Asian Fisheries Science **24** (2011):1-11

ISSN 0116-6514
E-ISSN: 2073-3720
https://doi.org/10.33997/j.afs.2011.24.1.001

Asian Fisheries Science
The Journal of the Asian Fisheries Society

A Simple Technique to Stage the Moult of *Penaeus monodon*

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Abstract

Growth in crustaceans and other arthropods is interrupted by cyclical moults, in which the old cuticle or exoskeleton is shed, and in the intervening growth phases the soft tissue swells and a new elastic cuticle gradually thickens and hardens. Marine prawns, *Penaeus monodon*, from a commercial farm in northern Queensland, Australia, were studied alive to assess their growth and moulting stages. The median part of the setae of inner uropods was examined by light microscopy. A finely graded classification of five stages and substages of postmoult, stage A consisting of substages A_1 and A_2, and stage B (1-16 hours); intermoult, stage C (1 day); premoult, stage D consisting of substages D_1, D_2 and D_3/D_4 (2-12 days) and ecdysis, stage E (about one minute) are described and illustrated. Ecdysis took place at night. Rapid and repetitive moult staging in individual prawns can be carried out without sacrificing the prawns.

Introduction

Crustaceans and other arthropods have outer cuticles that form the exoskeleton that are shed during repeated ecdysis or moults. In preparation for moulting, the inner soft tissue contracts and a thin new cuticle forms below the old one. At ecdysis, the old exoskeleton is shed, and the soft body grows in size by taking up water. The new cuticle gradually thickens and hardens, until at the next ecdysis the process is repeated. In small and young crustaceans, the moulting cycles are of short duration, but gradually increase with body size.

The moulting process can be staged either by recording the time intervals between moults, or by recording morphological changes in the soft tissues and the cuticle (Travis, 1957). The second method was first developed by Drach (Travis, 1955; Longmuir 1983) and later adapted by most researchers to determine the moult cycle of the crustaceans. The external changes of the exoskeleton and microscopical examination of setal development and epidermal withdrawal of the uropod and pleopod were commonly used to stage the moulting in penaeid prawns (Peebles, 1977; Longmuir, 1983; Smith and Dall, 1985; Robertson et al. 1987; Vijayan et al. 1997; Promwikorn et al. 2004). Smith and Dall (1985) also verified their findings with histological examination of the second abdominal segment. This physiological mechanism has been observed in *M. rosenbergii* (Peebles, 1977), *Penaeus merguiensis* (Longmuir, 1983), *P.*

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esculentus (Smith and Dall, 1985), *P. setiferus*, *P. stylirostris* (Robertson et al. 1987), *P. indicus* (Vijayan et al. 1997) and *P. monodon* (Promwikorn et al. 2004). Most of the authors agreed in dividing the moult cycles into 5 stages (A, B, C, D, and E) and various substages.

Since the growth rate depended on size and temperature, it was difficult to compare the moult cycle duration between species (Robertson et al. 1987). The moult cycle duration of the prawn is longer in prawn that are greater in size (Vijayan et al. 1997). Salinity and photoperiod also contributed to moult cycle variation. Premoult (D) occupied the longest period of the moult followed by intermoult (C) and postmoult (A and B) (Smith and Dall, 1985; Robertson et al. 1987; Vijayan et al. 1997). Ecdysis, the actual act of flicking out of the old exoskeleton, generally was less than 1 minute in penaeid prawns (Longmuir, 1983; Wassenberg and Hill, 1984; Smith and Dall, 1985; Robertson et al. 1987; Vijayan et al. 1997).

The stages of moult were an important factor in any stressful procedures such as handling, shipping, therapeutic treatment or harvesting (Robertson et al. 1987). These authors also claimed that moult staging of broodstock of penaeids was beneficial in predicting the cycles of larval production. Furthermore, because the rigidity of the prawns affected the market value, it was important to stage the moult before harvesting. All in all, determination of moult stages should be considered for pond management. This study presents a simple method to determine the moult stages of *P. monodon*.

Materials and Methods

Experimental animals

Prawns (*P. monodon*) were caught with a cast net from commercial farms in northern Queensland and transported to the Fish Laboratory of the School of Veterinary and Biomedical Sciences, James Cook University. Prawns were immediately acclimated and kept in two 1,000 L plastic bins with recirculating system and two aerators for each bin. At the start of each experimental run, the prawns were transferred and kept individually in glass aquaria (60 x 60 x 30 cm) with recirculation.

Prior to use, the filter system and experimental aquaria were chlorinated with liquid chlorine (100 g/l) at 30 ppm overnight. The next day, the equipment was rinsed with fresh water overnight and once again the following day. During the experiment, salinity was maintained at 35 ‰ both in the bins and aquaria and temperature ranged from 28 – 30 ºC. The experimental animals were fed with commercial prawn pellets at 10% total body weight divided into 2 daily feedings. The waste was siphoned from the aquaria once every two days. Fifty-four prawns, with a mean body weight of 5.28 ± 2.38 g (1.94 – 12.92 g) and with a mean total length of 8.8 ± 1.8 cm (6.7 – 11.8 cm) were used for the first experiment. In the second, fifty prawns with a mean body weight of 15.17 ± 3.28 g (9.07 – 23.20 g) and with a mean total length of 12.2 ± 0.8 cm (10.4 – 14.0 cm) were used.

Moult staging

Moult was staged by microscopical examination of setal development and epidermal withdrawal in the inner uropod near the telson tip (Smith and Dall, 1985; Promwikorn et al. 2004). Prior to staging the moult, prawns were anaesthetised by placing them in iced water for a few minutes. Sampling was done soon after moulting, at 1 h, 2 h, 4 h, 8 h, 16h, 24 h, and 1 day until 12 days after moulting for the first experiment. For the second experiment sampling was carried out soon after moulting, the day of moulting, and 1 day to 13 days after moulting. Three animals were sampled at every time point for each experiment. Three prawns were kept alive as controls to observe the moulting period.

Results

The setal development (setogenesis) and retraction of epidermis from the setal bases (apolysis) of the inner uropod near the telson tip gave a clear indicator of the moult cycle of *P. monodon*. Based on these criteria, the moult cycle of *P. monodon* could be categorized into five stages: postmoult (stage A consist of substages A_1 and A_2, and stage B), intermoult (stage C), premoult (stage D consist of sub-stages D_0, D_1, D_2, D_3/D_4) and ecdysis (stage E).

Stage A

Stage A (early postmoult) could be divided into two substages, A_1 and A_2 (Figure 1, A_1, A_2). Substage A_1 began soon after the prawn had flicked clear of the old cuticle. The whole body and exoskeleton were very soft and slippery. Setae were also very delicate. The setae and setal bases were filled with cellular matrix. The setal cone could not be seen. The setal node was poorly developed. Substage A_1 lasted 1 hour after moulting in the first trial. In the second trial, the period of substage A_1 was not determined because sampling was not conducted on an hourly basis. Three prawns were observed for substage A_1 in the first experiment.

When the cellular matrix started to retract from the distal end of the setae, the prawn entered the substage A_2. Constriction of cellular matrix continued until only the distal half of setae was filled. Setal bases were still filled with cellular matrix. The setal node was more developed. The cuticle was still soft to touch but not slippery. However, hardening of the exoskeleton had begun especially in the carapace and the sixth abdominal segment. In the first experiment, stage A_2 lasted between 4 and 8 hours after moulting. The period of this substage in the second trial also was not determined. Three prawns were examined for substage A_2 in the first experiment.

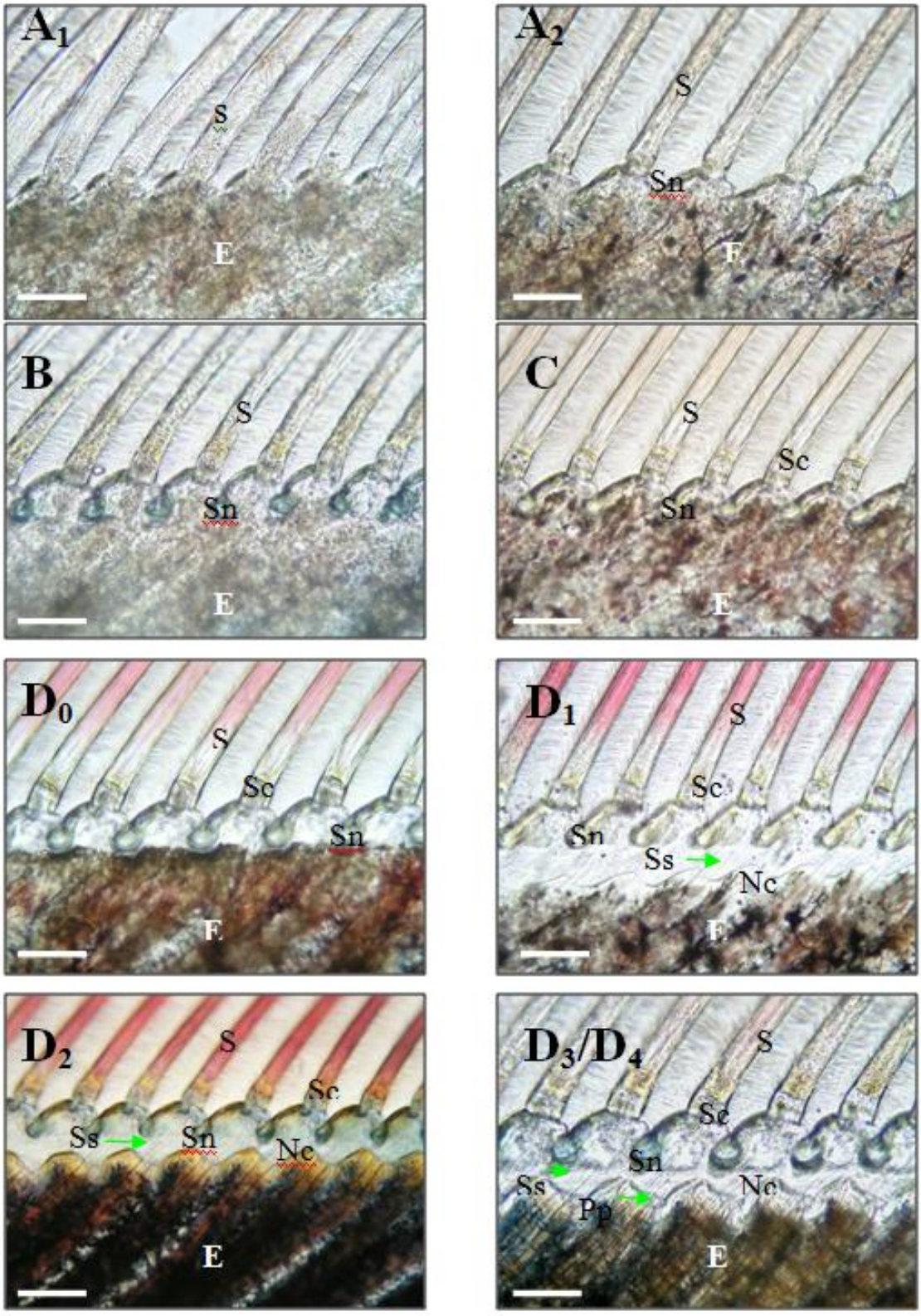

Figure 1. Moult staging of *P. monodon.* Setal development (setogenesis) and withdrawal of epidermis from the setal basis (apolysis) of the median part of inner uropod near the telson tip was examined to stage the moult and photographed under a light microscope (Olympus BH-2) connected to digital camera (Olympus Camedia C-5050 ZOOM). By this method, stage A (substages A_1 and A_2), stage B, stage C and stage D (substages D_0, D_1, D_2 and D_3/D_4) could be visualised. Scale bar = 100 µm. E, epidermis; S, setal shaft; Sc, setal cone; Sn, setal node; Ss, new setal shaft, Nc, new cuticle; Pp, pinpoints of light where new setal node will develop.

Stage B

Eight hours after moulting, stage B (late postmoult) started (Figure 1,B). This stage was marked by the retraction of the cellular matrix until the proximal half of the setae was emptied and was easily recognized by the clear space present in the base. The constriction of cellular matrix continued at the proximal end of the setae to form setal cones. Setal cones began to develop in some setae. Well developed setal nodes could be observed in most of the setae. Hardening of exoskeleton continued especially in the sixth abdominal segment and the carapace. This stage lasted around 16 – 24 h after moulting during the first trial. In the first experiment, 6 prawns were staged for stage B. In the second experiment, 3 prawns were observed in this stage.

Stage C

Stage C (Figure 1,C) started 24 h or day 1 after moulting. The cellular matrix was absent from the setal lumen. The secretion and hardening of the exoskeleton reached a maximum at this stage and it was marked by the complete development of setal cones. The setal bases were filled by the epidermis as well as around the setal nodes. Stage C ended when the epidermis retracted from the setal bases. In each experiment, 3 prawns were observed at this stage.

Stage D

When the separation of cuticle at the setal bases began due to the retraction of epidermis and the development of new setae, the prawn entered the premoult, stage D. Most researchers agreed in dividing the premoult stage into 5 substages from D_0 to D_4. Stage D_0 was marked by the withdrawal of epidermis (apolysis) from the setal bases of uropod (Figure 1, D_0). The retraction of epidermis from the setal bases continued until it formed a straight line at the bases of setae. This was the end of the substage D_0. This substage was observed between 2 and 3 d after moulting in the first trial or between 1 and 4 d after moulting in the second trial. There were 4 and 7 prawns staged at stage D_0 in the first and second experiment, respectively.

In stage D_1, further withdrawal of epidermis from the setal bases was recognised by a clear zone between the setal bases and epidermis (Figure 1, D_1). The clear zone marked the formation of new cuticle. Setal invagination appeared like a tube for the first time and setal tips could be observed. Retraction of epidermis from the setal bases continued and formed a wavy pattern. Sometimes pigment was observed to form a straight line under the wavy epidermis. New setal shafts of the developing setae appeared as translucent fibre from the wavy edge of the epidermis. At the end of stage D_1, the scalloping of the epidermal edge became uniform and reached a maximum. This stage extended for a period of 3 to 8 d after moulting in the first experiment and around 3 to 13 d in the second experiment. In the first trial, 13 prawns were observed at this stage. In the second experiment, there were 19 prawns determined at this stage.

At the next premoult, stage D_2, setal nodes started to deform (Figure 1, D_2). Later, the setal shaft changed its colour to red-orange. This was the most obvious feature that could be

observed in stage D_2. This stage could be observed from 7 to 12 d after moulting in the first experiment and 6 to 12 d after moulting in the second experiment. There were 11 and 9 prawns staged at D_2 in the first and second trial, respectively. The present study did not differentiate between moult stage D_3 and D_4 due to the need for sacrificing the animals at every sampling time for another experiment. Therefore, these two stages were expressed as a single stage observed before ecdysis. Stage D_3/D_4 (Figure 1, D_3/D_4) was characterized by the appearance of light pinpoint in the new setal nodes when examined under the light microscope. This stage was observed 10 – 12 d after moulting in the first experiment and day 13 after moulting in the second experiment. This stage lasted for a few hours before moulting. Five and two prawns respectively were determined at this stage in the first and second experiment.

Stage E

Stage E was the last observed and called ecdysis. The prawns flicked out of the old exoskeleton and extended the setae of the new exoskeleton. The exuviation process began in the carapace followed by the abdomen while the animal was lying on it side on the bottom of the aquarium. This extraction process took less than 1 minute. Three prawns were observed undergoing moulting in the first experiment.

Staging the moult of the prawns was sometimes difficult. It was commonly found that setogenesis was completed before the withdrawal of epidermis in the setal bases (apolysis). However, in some prawns the epidermis retracted before the setal cones in the setae were formed (Figure 2). In staging the moult, if this occurred in the median part of the inner uropod of the prawn, then the withdrawal of epidermis was used to determine the moult stage rather than the development of setae.

Observations on the cycle of the moult from 3 control prawns in each experiment revealed that one moult cycle took around 8 to 12 d in the first experiment or between 8 and 22 d in the second experiment. It was also found the premoult occupied the longest period of the moult (2 – 12 d after moulting), followed by intermoult (1 d after moulting) and postmoult (1 – 16 h after moulting). Some mortality occurred during the experiment. In the first experiment there were 5 mortalities, 4 prawns jumped out of the tanks and 1 prawn got stuck in corner filter. In the second experiment 5 mortalities occurred, 3 prawns jumped out, 1 prawn died during moulting, and another 1 died for no known reason.

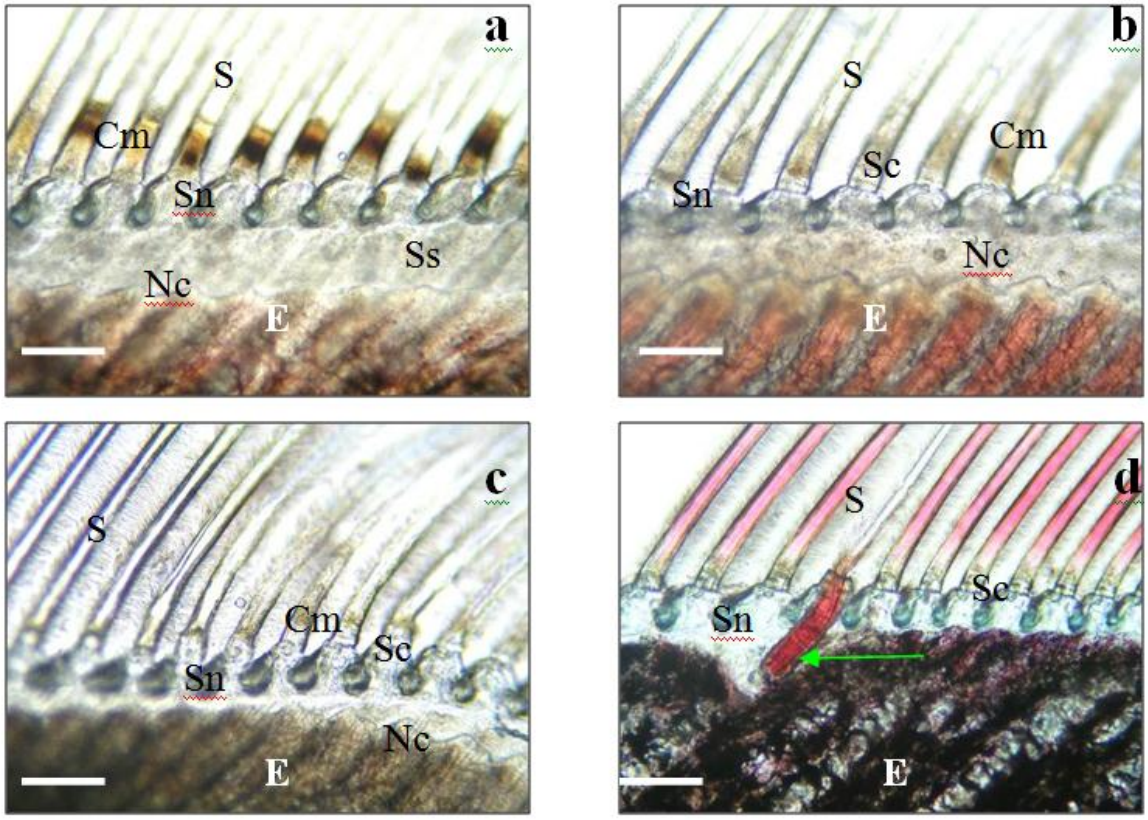

Figure 2. Abnormal setal development in the median part of the inner uropod near the telson tip of *P. monodon*. (a) Further retraction of epidermis from the setal bases suggested stage D_1 while the constriction of cellular matrix in the proximal end of setae where the setal cones would be formed pointed to stage B; (b) late stage D_2 was marked by the colour change of the setal shaft to red orange while setal cones have not yet formed in the setae (stage B); (c) apolysis in stage D_2 with some setae already having setal cones (stage B), some setae were not symmetrical (swollen like) in deformity of uropod; (d) one setum (arrow) just grew like the final stage before moulting (stage D_4) while the epidermis had already retracted from the setal bases (stage D_1). Scale bar = 200 µm. E, epidermis; S, setal shaft; Ss, new setal shaft, Sc, setal cone; Sn, setal node; Nc, new cuticle; Cm, cellular matrix.

Discussion

In the present study, the method used by Promwikorn et al. (2004) was applied in combination with criteria developed by Smith and Dall (1985) without the histological examination of the abdominal segment of the prawn. Setal development and epidermal withdrawal of the inner uropod near the telson tip gave clear characteristics to determine the stage of the moult in *P. monodon*. This method was simple, rapid and repeated moult staging could be performed on the experimental prawns because sacrificing the animal was not necessary to assess the stage of moult. However in this experiment, repetitive moult staging for individual prawn was not conducted because the animal was sacrificed after staging the moult for other experimental reasons.

Promwikorn et al. (2004) did not mention which part of the uropod that they used to stage the moult. The consistency, with which the area of the appendages is examined, is an important part in determining the moult cycle. For example, in the inner uropod of penaeid prawns, setal development and retraction of the epidermis from the setal bases in the proximal area developed slowly, while in the distal area, it progressed rapidly. The median part of the

inner uropod could be utilized to average these two extremes. Similar changes of setogenesesis have been found in the pleopod of *P. merguiensis* (Longmuir 1983).

Postmoult (stage A and B) could be defined by observing the distribution of cellular matrix within the setae. Stage A_1 started soon after the prawn shed the old cuticle. The new cuticle was very soft and slippery, as was the setae. The protoplasmic matrix filled the setae and setal bases. When the cellular matrix began to retract from the distal end of the setae, the prawns commenced stage A_2. Even though they were still soft to touch, the hardening of the exoskeleton began, particularly in the carapace and sixth abdominal segment, and the slippery feel of the cuticle disappeared. Setal cones still could not be observed. According to Smith and Dall (1985) in *P. esculentus*, when the secretion of the endocuticle and the retraction of the cellular matrix began, the exoskeleton lost its slippery feel.

Stage B was marked by the formation of setal cones. Formation of setal cones was indicated by the constriction of cellular matrix at the base of setae. Longmuir (1983) suggested that cellular matrix formed the setal cones. The cellular matrix might also contribute to the invagination of the setae, in muscular activity at moulting, and supported the skeleton of the setae until they hardened. In this stage, the hardening of the exoskeleton continued until it reached the necessary rigidity. In *P. esculentus*, the changing of skeletal hardness from a membranous to a parchment-like consistency occurred in stage B (Smith and Dall, 1985), while in *P. indicus* this happened in stage A (Vijayan et al. 1997). This discrepancy was due to the differences in defining "parchment like" as a measure of exoskeleton rigidity (Smith and Dall, 1985).

The appearances of cones in most of the setae and the absence of cellular matrix within the setae were the most striking features that could be observed in intermoult stage (stage C). The exoskeleton achieved maximum rigidity in this stage. Most authors agreed in defining the postmoult and intermoult stage in penaeid prawns (Peebles, 1977; Longmuir, 1983; Smith and Dall, 1985; Robertson et al. 1987; Vijayan et al. 1997; Promwikorn et al. 2004). However in the later moult stages, there was inconsistency in dividing stage D into substages due to the methods that they used.

The retraction of epidermis from the setal bases signalled the end of the intermoult stage and the beginning of the premoult stage (stage D_0). This could be seen clearly from the transparent edge of the appendages below the setal bases. The withdrawal of pigment and epidermis from the setal bases occurred at the same time. Similar patterns were found in *P. esculentus* (Smith and Dall, 1985). A straight line below the setal bases as a result of epidermal withdrawal marked the end of stage D_0.

Further separation of epidermis from the setal bases was marked by the appearance of a clear zone between the epidermis and the setal bases and defined the stage D_1. The clear zone implied the formation of the new cuticle (Promwikorn et al. 2004). Stage D_1 continued to develop until it achieved a uniform and maximum wavy edge of epidermis. This stage was the longest time in *P. monodon* and could be observed from 3 to 13 d after moulting.

In *P. esculentus*, *P. merguiensis*, and *P. indicus*, stage D_1 was further substaged to $D_{1'}$, $D_{1''}$, and $D_{1'''}$ according to the developmental stage of the new setae (Longmuir, 1983; Smith and Dall 1985; Vijayan et al. 1997). In previous moult staging of *P. monodon* when the epidermal line changed from straight to wavy, the moult stage was categorized as stage D_2 (Promwikorn et al. 2004), whilst in other penaeid prawns, it was classified into $D_{1'}$ (Longmuir, 1983; Smith and Dall, 1985; Vijayan et al. 1997). In this study it was termed D_1. It seemed that Promwikorn et al. (2004) used the development of scalloping epidermal tissue to define stages D_1, D_2, and D_3. Other authors (Longmuir, 1983; Smith and Dall, 1985; Vijayan et al. 1997) applied the terminology $D_{1'}$, $D_{1''}$, and $D_{1'''}$, while the present study simplified these stages into one stage, D_1.

The most noticeable feature that could be observed in stage D_2 was the colour change of the new setal shaft to red-orange at the end. In this stage the setal cones began to deform. In a previous study, this sign commenced the stage D_4, the last stage before ecdysis (Promwikorn et al. 2004). When the light pinpoint appeared from the new setal nodes, the prawns entered the stage D_3/D_4. This stage could not be separated into two different moult stages due to the requirement for the experimental animals being sacrificed at every sampling time for another experiment. Maximum reabsorption of components of the old exoskeleton such as nutrients and minerals occurred at this stage and are reincorporated into the new exoskeleton (Longmuir, 1983; Smith and Dall, 1985; Withers, 1992). As a result, the exoskeleton of the prawns became soft.

The stage D_3/D_4 was the last stage that could be seen in *P. monodon* before ecdysis. Even though the previous study of Promwikorn et al. (2004) defined the moult stages until D_4, this stage was defined as D_2 in the present study. In *P. merguiensis* the moult stages also lasted to stage D_3 - D_4 (Longmuir, 1983), in *P. indicus* (Vijayan et al. 1997), *P. setiferus* and *P. stylirostris* (Robertson et al. 1987) it lasted to early stage $D_{2\text{-}3}$, while in *P. esculentus*, it could be defined until stage D_4 (Smith and Dall, 1985). The inconsistency in defining stage D in penaeid prawns might be due to the different methods and species used.

The last stage observed was ecdysis (moulting). The prawns shed the old exoskeleton and extended the new setae of the new exoskeleton. At ecdysis, the plastic exoskeleton was stretched by water uptake and an expanded haemocoel (Withers, 1992). The exuviae of *P. monodon* was removed in two parts, firstly the carapace and secondly the rest of exuviae (appendages and abdomen). Similar observations were seen in *P. esculentus* (Wassenberg and Hill, 1984), *P. merguiensis* (Longmuir, 1983), and *P. indicus* (Vijayan et al. 1997).

The exuviation process took less than 1 minute in *P. monodon* similar to *P. esculentus* (Smith and Dall, 1985), *P. setiferus* and *P. stylirostris* (Robertson et al. 1987). The ecdysis process of *P. indicus* lasted between 30 - 50 seconds, in *P. merguiensis* (Longmuir 1983) it was about 40 seconds, while Wassenberg and Hill (1984) found it much shorter, approximately 18.1seconds in *P. esculentus*. This discrepancy was suggested as being due to the differences in defining ecdysis (Wassenberg and Hill, 1984; Vijayan et al. 1997). It was commonly found that this exuviation process occurred at night. In addition, the duration of moulting period between the two experiments was different. It was found that duration of moulting period in the second

experiment was longer than the first trial. The differences may be caused by the different body size in the two experimental runs.

It seemed that the prawns began to stop feeding at the end of stage D_2 and started to feed at stage B - C. According to Charmantier-Daures and Vernet (2004) animals did not feed from the end of stage D_2 until stage B and used the stored reserves in the hepatopancreas to supply the energy required for ecdysis. When they began to feed, prawns also ate the shed exuviae to recycle nutrients and minerals (Withers, 1992).

Since stage A, B, (postmoult) and C (intermoult) were determined according to the development of setae (setogenesis) and stage D (premoult) was based on the epidermal retraction from the setal bases (apolysis), a problem arose when these two criteria did not develop synchronously. It was generally found in normal prawns that apolysis occurred after setogenesis was completed. However in several prawns, the withdrawal of epidermis from the setal bases had already occurred while the cellular matrix had just retracted at the proximal end of setae (Figure 2). As a result, it was difficult to differentiate between stage B, C, and D. To overcome this problem, the retraction of epidermis from the setal bases was used to stage the moult. This problem might indicate that setogenesis developed slowly and also suggested slow growth of the animals. Another appropriate method should be considered in staging the moult of abnormal prawns.

Acknowledgements

The authors would like to thank to Mr. Laurie Reilly for helping to take the photographs and Mr. Norman Taylor for editing the manuscript. Rusaini is an awardee for the Australian Development Scholarship (ADS) Program from the Australian Agency for International Development (AusAID).

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Received 23/08/2006; Accepted 27/9/2010 (MS 06-60)