ORIGINAL ARTICLE

Histological characterization of cuticular depositions throughout the molting cycle of the black tiger shrimp (*Penaeus monodon*)

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Abstract

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The aim of this study was to investigate changes of cuticular deposition throughout the molting cycle of the black tiger shrimp (*Penaeus monodon*). The molting stages were first determined using physical criteria. Then the deposition of collagen fibers, carbohydrate, lipid and calcium salt in the cuticle were examined histologically throughout the molting cycle. The results show that the mature cuticle of the intermolt stage-shrimp is composed of four sub-layers from exterior to interior including an epicuticle, an exocuticle, an endocuticle, and a membranous layer. The differences of cuticular depositions reflect the different functions of each cuticular sub-layer. We also investigated the changes of these depositions that were related to the molting cycle. We found that the dynamic process of cuticular degradation and regeneration occurred constantly throughout the molting cycle. During the premolt period, the new epicuticle and exocuticle (pre-ecdysial cuticle) were synthesized on the inside of the old ones. After exuviations (post-ecdysis), the syntheses of the endocuticle and membranous layer of the new cuticle progressed chronologically. When the synthesis of the membranous layer is complete, the shrimps then enter the intermolt stage. At the end of this paper, we also summarize the histological criteria to use for determining the stage of the molt in the black tiger shrimp.

Key words : cuticular sub-layers, molting stages, *Penaeus monodon*

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บทคัดย่อ

วราภรณ์ พรหมวิกร ปิยากร บุญยัง และ พรพิมล คีรีรัตน์

การเปลี่ยนแปลงสัณฐานทางเนื้อเยื่อวิทยาของเปลือกกุ้งกุลาดำตลอดวงจรการลอกคราบ

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ระยะของการลอกคราบในกุ้งกุลาดำถูกตรวจสอบโดยใช้เกณฑ์ทางกายภาพก่อนที่เส้นใยคอลลาเจนคาร์โบไฮเดรต ไขมัน และ เกลือแคลเซียม ในเปลือกจะถูกศึกษาด้วยวิธีการทางเนื้อเยื่อวิทยา ผลการศึกษาพบว่า กุ้งที่อยู่ในระยะระหว่างวงจรการลอกคราบจะมีเปลือกที่สมบูรณ์เต็มที่ซึ่งประกอบด้วย ชั้นย่อย ๆ 4 ชั้นเรียงจากด้านนอกเข้าด้านใน ได้แก่ epicuticle exocuticle endocuticle และ membranous layer โดยที่ในแต่ละชั้นมีการสะสมของเส้นใยคอลลาเจน คาร์โบไฮเดรต ไขมัน และ เกลือแคลเซียมที่แตกต่างกัน ซึ่งความแตกต่างนี้สะท้อนให้เห็นถึงหน้าที่ที่แตกต่างกันของแต่ละชั้นย่อย นอกจากนี้ยังพบว่าลำดับการย่อยสลายและเกิดใหม่ของเปลือกกุ้งกุลาดำเกิดขึ้นอย่างเป็นแบบแผนที่แน่นอนตลอดวงจรการลอกคราบ และในตอนท้ายได้สรุปลักษณะทางเนื้อเยื่อวิทยาของเปลือกที่สามารถใช้เป็นเกณฑ์บ่งชี้ระยะของการลอกคราบของกุ้งกุลาดำเพิ่มเติมจากลักษณะทางกายภาพที่เราเคยรายงานไว้ก่อนหน้านี้

ภาควิชากายวิภาคศาสตร์ คณะวิทยาศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Crustacea molting cycles are controlled by neurosecretory hormones from the X- and Y-organs located in the eye stalks and near mouth region, respectively (Carlisle, 1957; Keller and Schmid, 1979; Soumoff and O'Connor, 1982; Skinner, 1985a&b; Lee *et al.,* 1998; Spaziani *et al.,* 1999; Watson *et al.,* 2001; Gilbert *et al.,* 2002; Nakatsuji and Sonobe, 2004). Ecdysteroid hormones are secreted from the Y-organ to stimulate "apolysis" and "ecdysis". Apolysis, corresponding to the D0 stage, is a process of separation of the old cuticle from the epidermis thus producing a space between the old cuticle and the epidermis underneath. This space is later replaced with newly synthesized cuticle to replace the old one. Ecdysis is the process at the brief moment of shedding the cuticle from the animal's body. Apolysis and ecdysis are followed by the process of cuticular re-building and increasing body size. These biological events are in a continuous cycle throughout the animal's life. Studies about the molting cycle have been investigated for seventy years (for example see Adams *et al.,* 1982; Skinner, 1985a&b; Lee *et al.,* 1998; Spaziani *et al.,* 1999; Musgrove, 2000; Watson *et al.,* 2001; Gilbert *et al.,* 2002; Gorokhova, 2002; Pratoomchat *et al.,* 2002a; b; Nakatsuji and Sonobe, 2004), however, the mechanisms that regulate the molting cycle are not yet fully understood, especially that of the molting cycle of the black tiger shrimp (*Penaeus monodon*), which is an important agricultural export product of Thailand. We are therefore attempting to understand the regulatory mechanism of ecdysis and the molting cycle in the black tiger shrimp. We hope that the results will be beneficial for promoting the growth the black tiger shrimp and other species of crustaceans. We have recently reported on the physical characteristics of the cuticle that can be used to determine the stage of the molt in the black tiger shrimp (Promwikorn *et al.,* 2004). In this paper we report further on the cuticular changes throughout the molting cycle as revealed by histological characterization of the collagen fibers, carbohydrate, lipid and calcium salt. We also summarize the histological criteria used as an alternative method for determining the stage of the molt in the black tiger shrimp.

Materials and Methods

Animals

Healthy black tiger shrimps (*Penaeus monodon*) were brought from commercial farms. During transportation to our laboratory in PSU, the shrimps were continuously oxygenated. The age of the shrimps was estimated at 90 days, and their body weight was between 10-20 g.

Shrimp culture

Natural sea water used in all experiments was stored in a tank for at least 2 weeks before use. Some days before the experiments, the sea water in the aquarium was continuously aerated. The salinity was adjusted to be similar to the sea water used in the farms (10-20 ppt. depending on the salinity of each farm). Once the shrimps had arrived, the molting stage of each shrimp was determined prior to its cultivation with continuous aeration. The shrimps were fed three times a day with commercial pellet. Natural day-light and atmospheric temperatures were used throughout the experiment.

Determination of molting stages

The molting stages were examined daily. Briefly, each shrimp was gently picked up by hand. They were then quickly examined with a light microscope. The physical criteria used for determining the stage of the molt were those used in our previous report (Promwikorn *et al.,* 2004). After 1–2 min examination, the shrimps were either placed back into the aquarium, or executed.

Histological study

The cuticular tissues at the carapace and trunk (first abdominal somite) of at least 10 shrimps at each molting stage were dissected and immediately fixed in Davidson's fixative to eliminate any calcium salt in the cuticle. The fixation stage was continued for 72 h at room temperature with a daily change of fresh fixative. The cuticular tissues were subsequently dehydrated in increasing concentrations of ethanol that ranged from 50 to 100%, and prepared for routine histological embedding in paraffin blocks. Paraffin sections of 0.5 µm thickness were routinely de-paraffinized and stained for collagen fibers with Masson's trichrome (Bancroft and Gamble modification, 2002a), and carbohydrate with periodic acid Schiff's reagent, PAS (Bancroft and Gamble, 2002b). The stained tissue sections were examined with a light microscope (Olympus BX 51) and photographed with a digital camera (Olympus DP11) connected to the microscope. For calcium salt staining, the cuticular tissues were fixed with 10% formalin instead of Davidson's fixative to preserve calcium salt in the tissue (Bancroft and Gamble, 2002c). For lipid staining, fresh cuticular tissues were sectioned with a cryostat (Leica CM1850) at -20 $^{\circ}$C, and stained with Oil Red O, before mounting in glycerine jelly (Sheehan and Hrapchak, 1980).

Results and Discussion

The crustacean cuticle is composed of 2 main layers from exterior to interior which are the epicuticle and procuticle, respectively. The procuticle is composed of three sub-layers including an exocuticle, an endocuticle and a membranous layer. The epicuticle and exocuticle are synthesized before ecdysis and form the so-called preecdysial layer. The endocuticle and the membranous layer are synthesized after ecdysis, and form the so-called post-ecdysial layer (Skinner, 1985b). The main components of the cuticle include both inorganic and organic materials in a 60:40 ratio. The inorganic materials found in the cuticle include calcium, chloride, copper, magnesium, manganese, phosphorus, potassium, and sulfur (Roer and Dillaman, 1984; Mangum, 1992; Compere *et al.,* 1993, Pierce, *et al.,* 2001; Pratoomchat *et al.,* 2002a; b; Wang *et al.,* 2003). The organic materials include lipid, glycoprotein, protein, glycosaminoglycans, mucopolysaccharides, carbohydrate, and chitin (Glynn, 1968; Vigh and Dendinger, 1982; Roer and Dillaman, 1984; Wheeler and Sikes, 1984; Marlowe *et al.,* 1994; Andersen, 1999; Roer *et al.,* 2001; Pratoomchat

et al., 2002b). Although these components are found in many species of crustaceans, mainly in crabs, the evidence of their location in the cuticle is limited. Using histochemical methods we have localised both organic and inorganic components including collagen fibers, carbohydrate, lipid and calcium salt in the mature cuticle of the black tiger shrimp. We have also investigated how these components are deposited in the cuticle with respect to the molting cycle.

1. Organic and inorganic contents are deposited differently in cuticular sub-layers.

Histochemical methods were used to locate the collagen fibers, carbohydrate, lipid and calcium salt in the cuticle of the black tiger shrimp. The collagen fibers, that give strength to the cuticle, were stained blue with Masson's trichrome (aniline blue, Ponceau S, and hematoxylin), carbohydrate was stained pink with periodic acid Schiff's (PAS) reagent, lipid was stained red-pink with oil red O (ORO), and calcium salt was stained orange-red with alizarin red S (ARS). It was shown that the mature cuticle of the intermolt shrimp consisted of four layers each with a distinctive texture and characteristic (Figure 1).

The outermost layer, an epicuticle, was a thin layer, which stained with PAS (deep magenta), trichrome (red), ORO (red-pink), and ARS (orange-red) methods. This indicates that the epicuticle is rich in carbohydrate, lipid, calcium salt and protein, but lacks collagen fibers. The red colour obtained after staining with Ponceau S in trichrome indicated that the epicuticle is rich in protein. Also the deep magenta colour obtained with PAS staining, gave a strong indication that glycoprotein is abundant in the epicuticle.

The procuticle could be divided into three distinctive layers from the exterior to the interior; an exocuticle, an endocuticle and a membranous layer. The exocuticle close to the epicuticle has typical alternating light and dark lamellae. It stained blue with trichrome, dark blue with PAS (counterstained with hematoxylin), and orange-red with ARS, but was not stained with ORO. This indicates that the exocuticle consists of bundles of collagen fibers arranged in a lamella-fashion with an intervening interlamella-matrix composed of carbohydrate and protein (the latter may be a precursor material for collagen fibers). Calcium salt is deposited throughout, but lipid is not detected in this layer.

The endocuticle lies inside the exocuticle. This layer stained light blue with trichrome, light pink with PAS and deep orange-red with ARS. Again the endocuticle did not stain with ORO. The characteristic of the outer part of the endocuticle is similar to that of the exocuticle, but has less content of collagen fiber, and carbohydrate. The inner part of the endocuticle has more delicate and compact lamellae of collagen fibers, carbohydrate and calcium salt, and closely resembles the innermost membranous layer. The membranous layer, found between the endocuticle and epidermis (sometimes referred as hypodermis, or epithelium), was clearly distinguishable from the endocuticle after staining by PAS. The membranous layer stained a darker blue with PAS, a stronger blue with trichrome, and orange with ARS when compared with the endocuticle, but did not stain with ORO. This indicates that the endocuticle and membranous layer are also rich in collagen fibers, protein, carbohydrate, and calcium salt, but lack lipid.

Our results indicate that collagen fibers, carbohydrate, calcium salt and lipid are the main components in the cuticle of the black tiger shrimp. However, they are deposited differently in each cuticular sub-layer. This presumably is a reflection of their functions. The lipid is detected only in the epicuticle where it could act as a water-shield or in body-temperature regulation. The collagen fibers, calcium salt, and carbohydrate are abundant in all sub-layers of the procuticle. The collagen fibers play a major role in cuticular architecture, and give strength to the cuticle. Calcium salt increases the hardness of the cuticle, possibly to protect the shrimp from external mechanical forces. In the epicuticle, the carbohydrate is in the form of a glycoprotein. The carbohydrate may be a

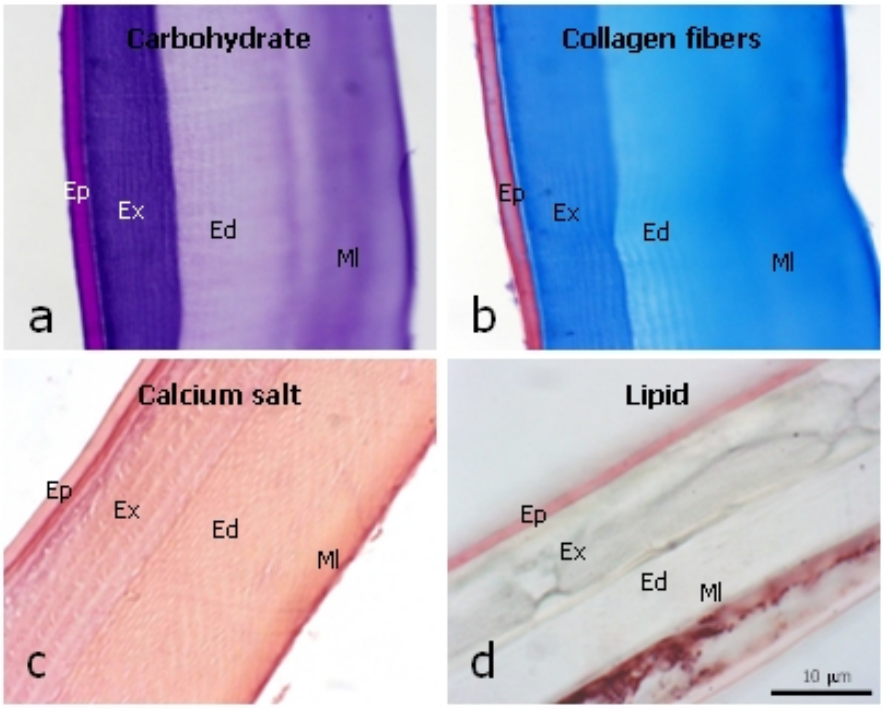

Figure 1. Localisation of carbohydrate (stained magenta, a), collagen fibers (stained blue, b), calcium salt (stained orange-red, c), and lipid (stained pink, d) in the mature cuticle of intermolt shrimp. Four cuticular sub-layers are shown; epicuticle (Ep), exocuticle (Ex), endocuticle (Ed), and membranous layer (Ml). Bar = 10 µm.

precursor for chitin, a polymer consisting of 80-90% N-acetylglucosamine and 10-20% glucosamine. The presence of protein and carbohydrate in the exocuticle and membranous layer may indicate the sites for chitin deposition. Our study provides additional information to previous studies on the location of collagen fibers, carbohydrate, calcium salt and lipid in the cuticular sub-layers of the black tiger shrimp.

2. The epicuticle and exocuticle are synthesized before ecdysis.

It would be of interest to learn how the degradation and regeneration of the cuticular sub-layers are related to the molting cycle. During the molting cycle, the cuticle changes dynamically in two aspects; i) the thickness of the cuticle, this depends on the number of cuticular sub-layers (Mykes, 1980; Stevenson, 1985), and ii) the deposition of the organic and inorganic components in the cuticle. We have shown that collagen fibers, carbohydrate, calcium salt and lipid are deposited in different cuticular sub-layers. We therefore examined in more detail of changes in the depositions of these components during the molting cycle. We found that during the early premolt period (D0-2), Figure 2, the membranous layer stained light blue with trichrome, light pink with PAS, and light orange-red with ARS, or often could not be clearly identified. These staining reactions were decreased if compared to the staining reactions found at the intermolt stage. This indicates that the membranous layer is being degraded during the premolt stages. This may allow the protein, carbohydrate, and calcium salt present in these two layers to be re-absorbed by

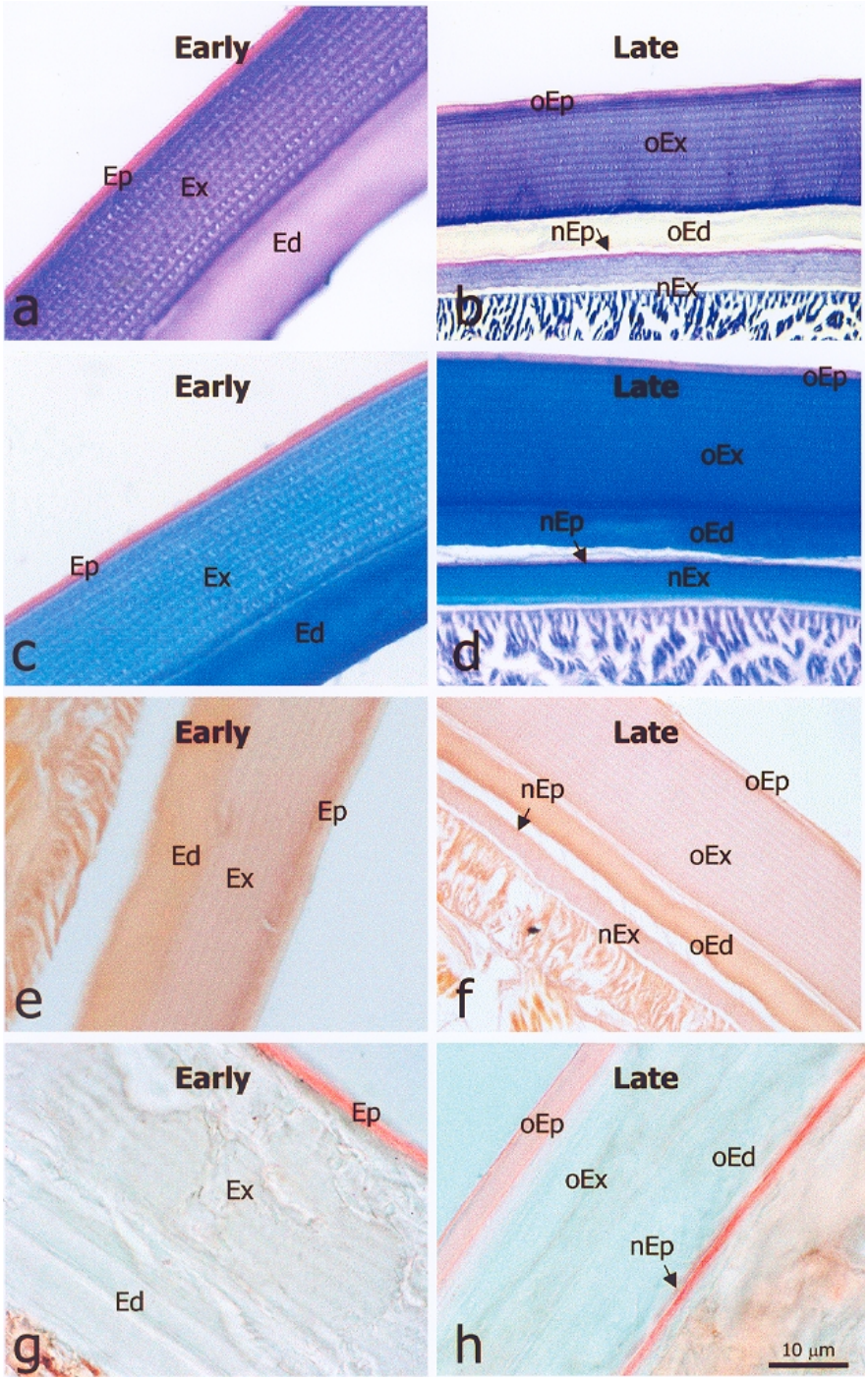

Figure 2. Localisation of carbohydrate (a,b), collagen fibers (c,d), calcium salt (e,f), and lipid (g,h) in the cuticle during the early premolt period (left column) and late premolt period (right column). New epicuticle (nEp) and exocuticle (nEx) is synthesized on the inside of the old exocuticle (oEx), and thickening in a later stage. Bar = 10 µm.

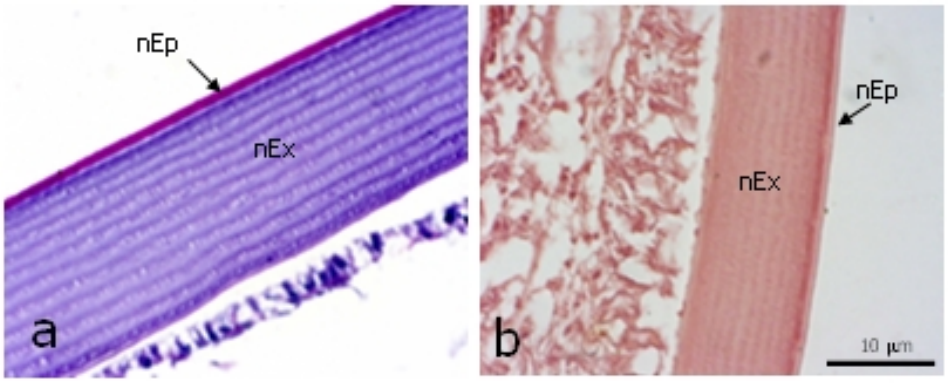

Figure 3. The cuticle of the black tiger shrimp immediately after ecdysis. Only the new epicutilce (nEp) and exocuticle (nEx) are present. The cuticle was stained with PAS for carbohydrate (a), and alizarin red S for the calcium salt (b). Bar = 10 µm.

the underlying epidermis. In the late premolt period (D3-4 stage), newly secreted epicuticle (nEp) and exocuticle (nEx) were sequentialy produced inside the old cuticle. These data indicate that during the premolt period the endocuticle and membranous layer of the old cuticle are re-absorbed, prior to the formation of the new epicuticle and exocuticle and the shedding of the exuviae.

3. The endocuticle and membranous layers are synthesised after ecdysis.

Immediately after ecdysis, during A1 - 2 stages, only an epicuticle and an exocuticle were observed in the intact cuticle (Figure 3). Ten hours after ecdysis, a small layer of endocuticle was seen at the inner border of the exocuticle. This layer stained blue with trichrome, pink with PAS, and light orange-red with ARS, but did not stain with ORO. Two days after ecdysis, in the B2 stage, the thickness of the newly synthesized endocuticle increased. The stain for the calcium salt was now clearly visible (Figure 4f). Three to four days after ecdysis, all layers of the cuticle including the membranous layer were visible (see Figure 1). These findings indicate that the syntheses of the new epicuticle and exocuticle have finished before ecdysis. However, another two layers are synthesised after ecdysis. The synthesis of the endocuticle starts immediately after ecdysis, followed by the synthesis of the membranous layer. It is noted that the late post molt stage C1-2 is difficult to distinguish from late B stage by these methods. When all layers of the cuticle have been produced in the cuticle, the shrimp is then designated as being in the intermolt stage (C3-4). At this time the thickness of the post-ecdysial layer (endocuticle and membranous layer), approximately 24 mm, was approximately twice that of the pre-ecdysial layer (epicuticle and exocuticle). Determination of the intermolt stage by physical examination is difficult, and can easily be misdiagnosed. Using the histological characteristics of the cuticle is a better way to determine the intermolt stage of the shrimp. We would like to emphasise here that observation of the membranous layer in the cuticle could indicate the level of maturity of the cuticle, hence the existence of the intermolt stage.

Taken together the results indicate that the biological processes of degradation and regeneration of the cuticle occur throughout the molting cycle. These processes include the syntheses and degradations of collagen fibers, protein, carbohydrate, calcium salt and lipid. Synthesis of collagen fibers, and carbohydrate occur constantly throughout the molting cycle, while glycoprotein and

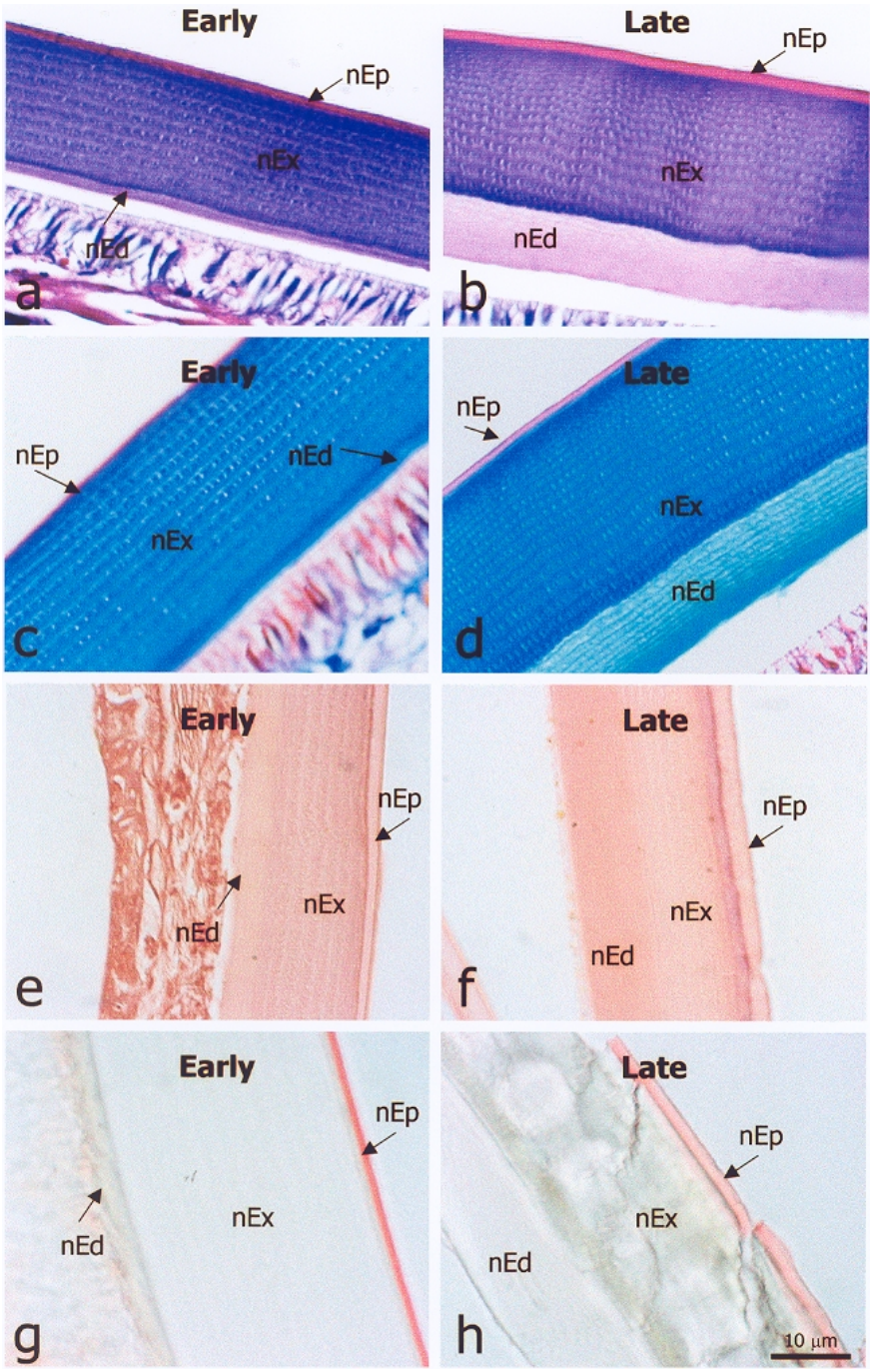

Figure 4. Localisation of carbohydrate (a,b), collagen fibers (c,d), calcium salt (e,f), and lipid (g,h) in the cuticle during the early postmolt period (left column) and late postmolt period (right column). New epicuticle (nEp) and exocuticle (nEx) are now mature. The new endocuticle (nEd) is being synthesized in early postmolt period. It is noted that the stain for calcium salt in the endocuticle became more intense in the late postmolt stage (compare nEd in 4e and 4f). Bar = 10 µm.

lipid syntheses occur only at the end of the premolt stage. Deposition of calcium salt increases from the late premolt until the intermolt stage. This information may be useful for managing the feeding regimes to ensure that the shrimp's demand for a particular nutrient at a particular stage in the molting cycle is met. This could support maximum shrimp growth and health after each round of the molting cycle. We are continuing our investigations to uncover potential links between cuticular changes and other organs. This knowledge will help us to understand the processes that regulate the molting process in the black tiger shrimp.

Conclusions

1. The mature cuticle of the black tiger shrimp is composed of four sub-layers from the exterior to the interior: an epicuticle, an exocuticle, an endocuticle, and a membranous layer. Each sub-layer contains different organic and in-organic components. Carbohydrate and calcium salt is found in all sub-layers. Collagen fibers deposit in most sub-layers, except the epicuticle. Lipid deposits only in the epicuticle.

2. These cuticular sub-layers change chronologically throughout the molting cycle. Before ecdysis, the endocuticle and membranous layer of the old cuticle are degraded while the epicuticle and exocuticle are synthesized on the inside of the old cuticle. After the old cuticle is shed the new endocuticle and membranous layer are sequentially synthesized together with the depositions of carbohydrate, collagen fibers, calcium salt, and lipid. In the intermolt stage, syntheses of all the cuticular sub-layers are completed. The ratio of the thickness of the pre-ecdysial and post-ecdysial layers should now be approximately 1:2. (see Figure 5 for the summary).

3. The histological properties used to determine the stage of the molt are given below

i) D0-2 (early premolt), lightly stained membranous layer
ii) D3-4 (late premolt stage), the existence of new epicuticle and exocuticle on the inside of the old cucticle
iii) A stage (early postmolt stage), the existence of an epicuticle and an exocuticle
iv) B stage (late post molt) the existence of the developing endocuticle and
v) C3-4 stage (intermolt stage), the existence of a membranous layer in the cuticle. The ratio of the thickness the pre-ecdysial : post-ecdysial cuticle is approximately 1:2.

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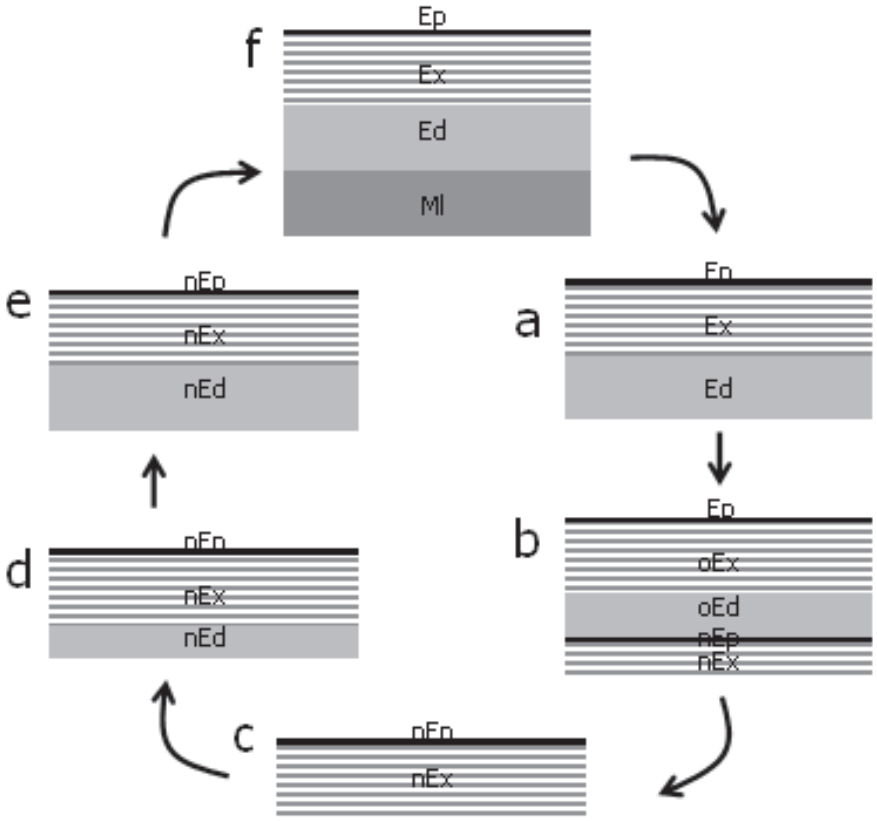

Figure 5. Summary of cuticular-degradation and -regeneration throughout the molting cycle. Early premolt (a), the endocuticle and membranous layer are being degenerated from the old cuticle. The new epicuticle and exocuticle are next synthesized (b). Immediately after ecdysis, both new epicuticle and exocuticle are present, while the old cuticle was shed (c). Early postmolt, the endocuticle is newly synthesized (d). End of postmolt, the newly secreted endocuticle becomes thicker (e). When the synthesis of the membranous layer is begun, the shrimp is said to be in the intermolt stage (f). Ed = endocuticle, Ep = epicuticle, Ex = exocuticle, Ml = membranous layer, nEd = new endocuticle, nEp = new epicuticle, nEx = exocuticle, oEd = old endocuticle, and oEx = old exocuticle.

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