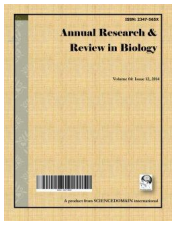


Annual Research & Review in Biology
11(1): 1-10, 2016, Article no.ARRB.28564
ISSN: 2347-565X, NLM ID: 101632869

SCIENCEDOMAIN *international*
www.sciencedomain.org

Histological Changes in Cuticle of the Red Swamp Crayfish, *Procambarus clarkii* During Molting Cycle

Mohammad A. Amer[1*], Awaad A. M. El-Sayed[1], Khalid A. Al-Damhougy[1] and Samir A. Zaahkouk[1]

[1]Faculty of Science, Al-Azhar University, Cairo, Egypt.

Authors' contributions

This work was carried out in collaboration between all authors. Author AAMES designed the study, wrote the protocol and interpreted the data. Author MAA performed the methods, anchored the field study, gathered the initial data and performed preliminary data. While authors KAAD and SAZ managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/ARRB/2016/28564
Editor(s):
(1) George Perry, Dean and Professor of Biology, University of Texas at San Antonio, USA.
Reviewers:
(1) Flavio de Almeida Alves Júnior, Universidade Federal de Pernambuco, Brazil.
(2) Venus Bantoto-Kinamot, Negros Oriental State University, Philippines.
Complete Peer review History: http://www.sciencedomain.org/review-history/16131

Original Research Article

Received 26th July 2016
Accepted 23rd August 2016
Published 9th September 2016

ABSTRACT

The present study was carried out to investigate histological changes in cuticle structure of the red swamp crayfish, *Procambarus clarkii* and number of layers of carapace cuticle during different molting stages. The crayfish samples were collected alive from the River Nile tributaries at Al-Kanater Al- Khairiya, Qalyoubiya Governorate during the period from spring 2010 to autumn 2011. These specimens were transported to the laboratory in the Faculty of Science, Al-Azhar University, Nasr City, Cairo. From the collected samples 10 individuals were selected according to molting stages morphologically, they were sexually mature and varied from 6.0 to 10.5 cm in standard length, 6.5 to 11.5 cm in total length and from 9.1 to 48.25 g in total body weight. These specimens were classified into five molting stages to study cuticle structure histologically. During intermolt stage, carapace integument is composed of four distinctive layers. These layers arranged from the outer surface to inner side as: epicuticle, exocuticle, endocuticle and membranous layer, respectively. During this stage, the epidermal layer composes of cuboidal epithelium. At the onset of premolt, a new endocuticle was arose above the membranous layer represents the new

**Corresponding author: E-mail: naser12_7@yahoo.com;*

carapace. It was characterized externally by a clear separation called an apolysis occurred between an old endocuticle and new one. During an ecdysis, both of pre-ecdysal cuticle and postecdysal one were distinct, pre-ecdysal cuticle comprises epi-, exo-, and endocuticle; while the postecdysal composed of epi- and endocuticle only (without exocuticle), in addition to membranous layer, the epidermis was composed of elongated columnar epithelial cells. All these results indicated that the cuticle structure changes obviously during the course of molting cycle in fresh water crayfish *P. clarkii*.

Keywords: Crayfish; epicuticle; endocuticle; exocuticle; Malpighian layer; epidermis.

1. INTRODUCTION

Molting is the main tool for increasing size in crustaceans [1,2,3,4]. During molting several internal (ex. Formation of gastroliths in cardiac stomach, mobilization of calcium and other reserves) and external changes (ex. morphological changes in carapace, apolysis, changes in uropod setae) are observed. Most of the freshwater and land crustaceans reabsorb biominerals, particularly calcium, from the old exoskeleton during premolting (stage D) and store it in special organs including hepatopancreas and tissues, or in specific paired discs called "gastroliths". The texture of exoskeleton becomes fragile, with faint or pale color during this stage. After molting, a rapid postmolt calcification of the exoskeleton is essential to all crustaceans, therefore, considerable amount of calcium were reabsorbed again from those storing organs or gastroliths, transferring the exoskeleton in gradual processes into flexible to hard structure with bright color [1,2].

The morphological changes in exoskeleton during molting cycle are necessary depending upon associated remarkable changes in cuticular structure, particularly the wall of carapace, accompanied with those events. The most pronounced changes are recorded in thickness, structure and number of different cuticular layers. Most of these changes were noticed through the preparatory stages (premolting stage D) and continue in an ecdysis (stage E), but reach stability during the long period, intermolting (stage C).

Therefore, this work aims to throw light on the histological changes in cuticle of carapace wall during molting stages of the crayfish, *Procambarus clarkii* (Girard, 1852) from the River Nile drainage canals, Egypt.

2. MATERIALS AND METHODS

The specimens of the freshwater crayfish, *Procambarus clarkii*, were collected alive for molting study from the River Nile tributaries at Al-Kanater Al- Khairiya, Qalyoubiya Governorate during the period from spring 2010 to autumn 2011. These specimens were transported to the laboratory in the Faculty of Science, Al-Azhar University, Nasr City, Cairo. All specimens were sexed and weighed to the nearest 0.1 gm using an electric balance with an accuracy of 0.01 g after blotting excess water with absorbent tissues. The total body length, standard length (length without telson and uropods), were measured with a Caliper Vernier with an accuracy of 0.01 mm. These specimens were divided into groups and kept for two weeks in tanks (50 L) containing fresh water aerated with air pumps at room ambient temperature, with an oxygen concentration ranged between 7 and 8 mg/L, and pH varied from 7.8 to 8.0. These animals were fed during this period with commercial pellets, frozen fish meat and squid flesh.

2.1 Determination of Molting Stages

Molting cycle for *P. clarkii* individuals were classified into five stages according to [1] and [2]. The classification of these stages are based on the morphological changes in carapace cases including: hardness, surface texture, rigidity, fragility and color patterns, in addition to color of abdominal muscles, presence of scratches, tubercles, mechanical damages, regenerated limbs, epiphytic and epizoic organisms on carapace, as well as the appearance of an ecdysal suture (apolysis) and processes of gastroliths formation.

2.2 Histological Studies

For histological studies several pieces (about one cm^2) from 10 individuals of crayfish *P. clarkii* carapace at different molting stages were taken

and immediately fixed in 10% formalin solution, then decalcified by mixture of 35 ml (20% sodium citrate): 65 ml (Formic acid). The decalcification process continued for 72 hrs. at room temperature, and was daily changed with fresh solution. The cuticular tissues of decalcified pieces of carapace were subsequently dehydrated in ascending concentrations of ethanol series started with 70 to 100%, and prepared for routine histological embedding in paraffin blocks. Paraffin sections of 0.5 µm thicknesses were routinely de-paraffinized and stained with Eosin and Hematoxylin stains. The stained sections were examined under light microscope (Leica DM – LB2) provided with a digital camera (Sanyo vcc-6580PE) for photography. For each section, several photographs were picked up, and saved for final examination and prepared according to different stages of molting.

3. RESULTS

The results of histological examination were treated and sorted according to molting stages, started with the intermolt, to follow up the beginning of changes, till the final formation of new carapace. All the collected individuals were sexually mature and varied from 6.0 to 10.5 cm in standard length, 6.5 to 11.5 cm in total length and from 9.1 to 48.25 g in total body weight. The histological structure of carapace wall is treated according to the following molting stages:

3.1 The Intermolt (Stage C)

During the intermolt stage, the carapace wall is well compact and full formed, consists of four distinctive noncellular layers with variable thickness lying above living cellular layer, called epidermal or Malpighian layer (Plate 1). The four noncellular layers compose mainly of lipoprotein, arranged from the outer to inner side as: epicuticle, exocuticle, endocuticle and membranous layer. The thickness of these layers varied from 124.4-172.6 µm with an average of 150.8 µm, and constitutes about 88.1% of the carapace wall. Exocuticle was the widest, ranged from 55.6 to 83.0 µm with an average of 69.5 µm, and represents 46.1% of the total wall thickness. The thickness declines gradually to 22.7 µm for eipcuticle (15.1% of cuticle), reaching to the minimum average of 16.7 µm for the membranous layer (11.1% of cuticle). The epidermal layer has deep blue color, and was composed of cuboidal epithelium, with large concentric nucleus. Its thickness was ranged from 13.9 to 27.8 µm and averaged 20.9 µm, representing about 12.2% from the total carapace wall.

Also, there are several vertical canals, observed throughout exo- and endocuticles which may be represent the tegumental glands. The fine structure of these canals may be observed more obvious with higher magnifications.

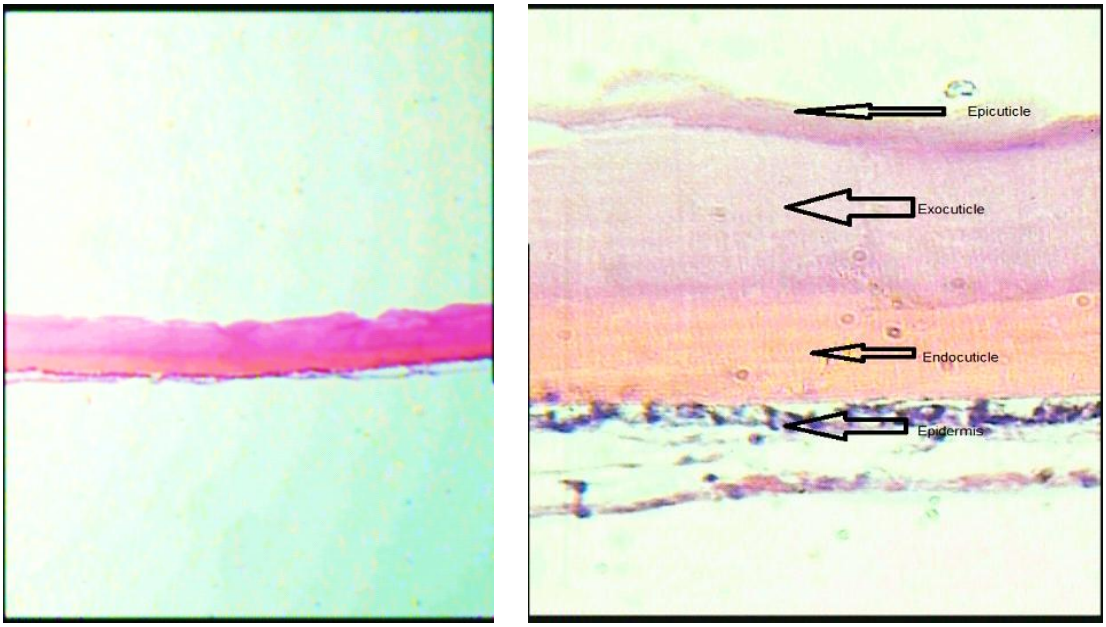
3.2 The Premolt (Stage D)

During this stage a clear separation called an apolysis was occurred between an old endocuticle and new one (endocuticle) above the membranous layer represents the new carapace (Plate 2). It reached 61.2 µm in its average. The cuticular layers of the old cuticle were morphologically differentiated by the appearance of striated myofibrils without membranous layer, due to the beginning formation of new cuticle underneath. These layers varied from 152.7 to 264.1 µm, and averaged 191.2 µm in thickness. Exocuticle was also the widest, and represented about 42.0% of all, and ranged between 69.5 to 83.4 µm with an average of 79.9 µm in thickness. It was observed that, the inner borders of both endocuticle and epicuticle have condensed or compact myofibrils, but showed loosely or slightly separated fibrils towards the outer borders.

On the other hand, the membranous layer is thin (6.9 µm) and tightly attached to the Malpighian layer. The cells of the last layer are cuboidal, with large concentric nucleus, have deep blue color. The thickness of this layer averaged 45.2 µm.

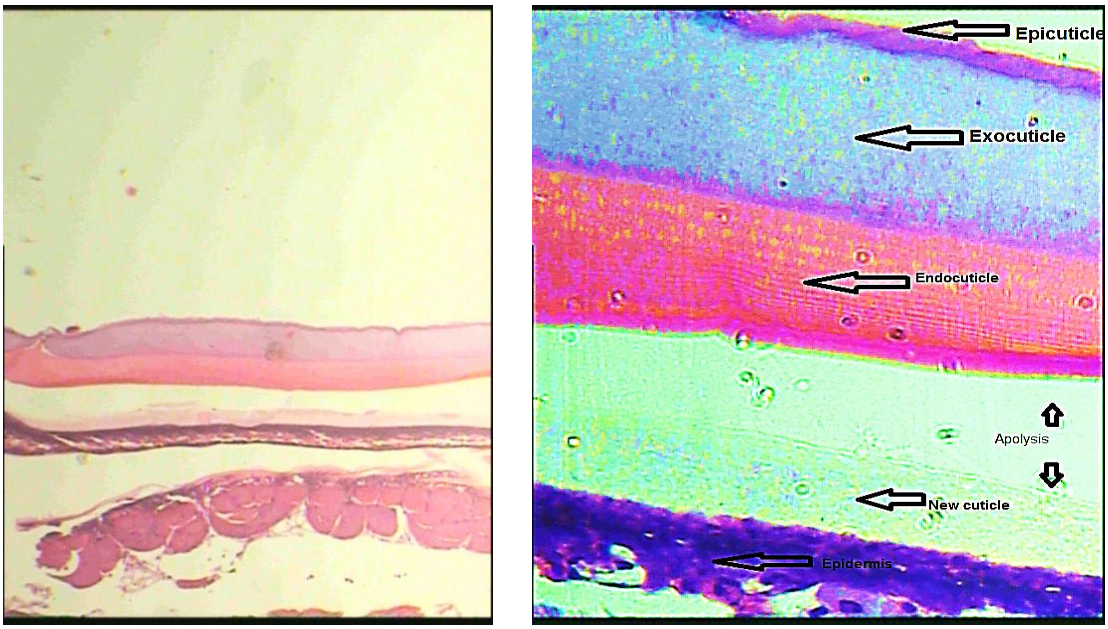
3.3 An Ecdysis (Stage E)

This stage is characterized by the presence of both pre-ecdysal or an old carapace (exuviae) and postecdysal or new carapace. The pre-ecdysal cuticle comprised epi-, exo-, and endocuticle. It is being widely separated from the new carapace beneath, and characterized by the presence of both of horizontal and vertical striation of myofibrils, which appear loose or coarse with clear interspace, may be attributed to the reabsorption of biominerals particularly calcium carbonates (Plate 3). The thickness of these layers decreased remarkably comparable with the previous stages. It varies between 111.1 and 159.9 µm, and averaged of 132.2 µm. The exocuticle was narrower than premolt stage, reached to 69.5 µm in thickness, followed by endocuticle (45.2 µm) and then the narrower epicuticle (17.4 µm). Note the vertical canals of tegumental gland in (Plate 3B), which extend through the exo- and endocuticle.



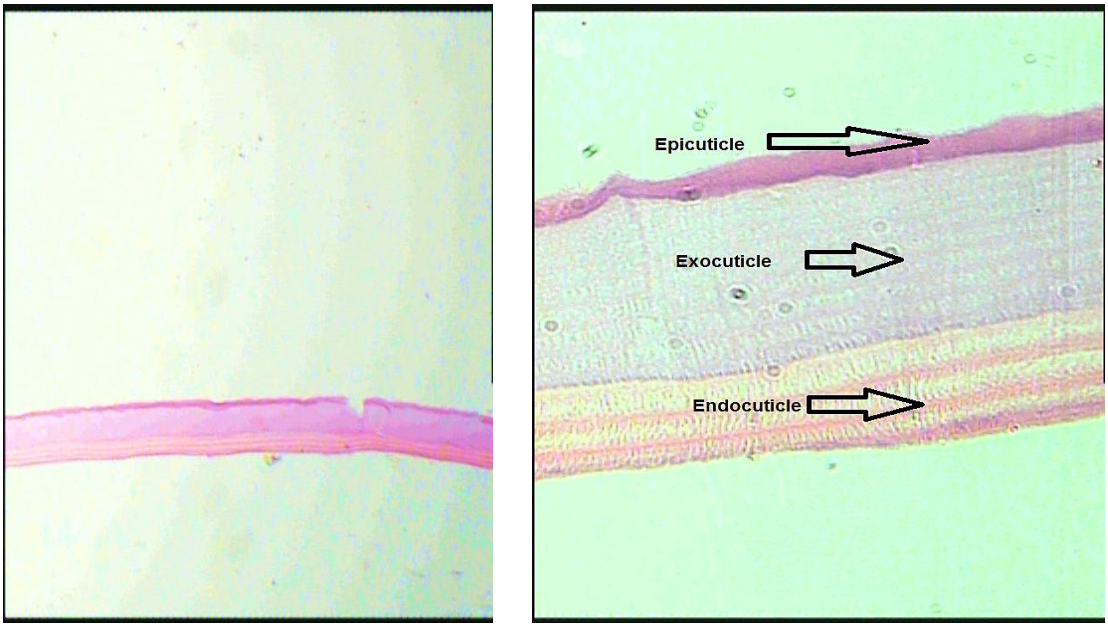
(A) (B)

Plate 1. Vertical section through the intermolt carapace stage showing layers of carapace wall, the arrows donate to each cuticular layer (Magnification: A, 10 X and B 20 X)



(A) (B)

Plate 2. Vertical section of carapace wall during premolt stage, the arrows donate to each cuticular layer with magnification of 5X (A), and 20X (B)



(A) (B)

Plate 3. Vertical section in the cuticle of the old carapace (exuviae) during an ecdysis with magnification of 5X (A), and 20 X (B)

On the other hand, the post cuticular layer of new carapace is composed of epicuticle and endocuticle only, in addition to membranous layer without exocuticle (Plate 4). The striation of myofibril appears fine and more compact. The thickness of these layers together averaged 109.3 µm, of which the endocuticle reached 95.9 µm in its average and represents 87.7% of the all cuticular wall.

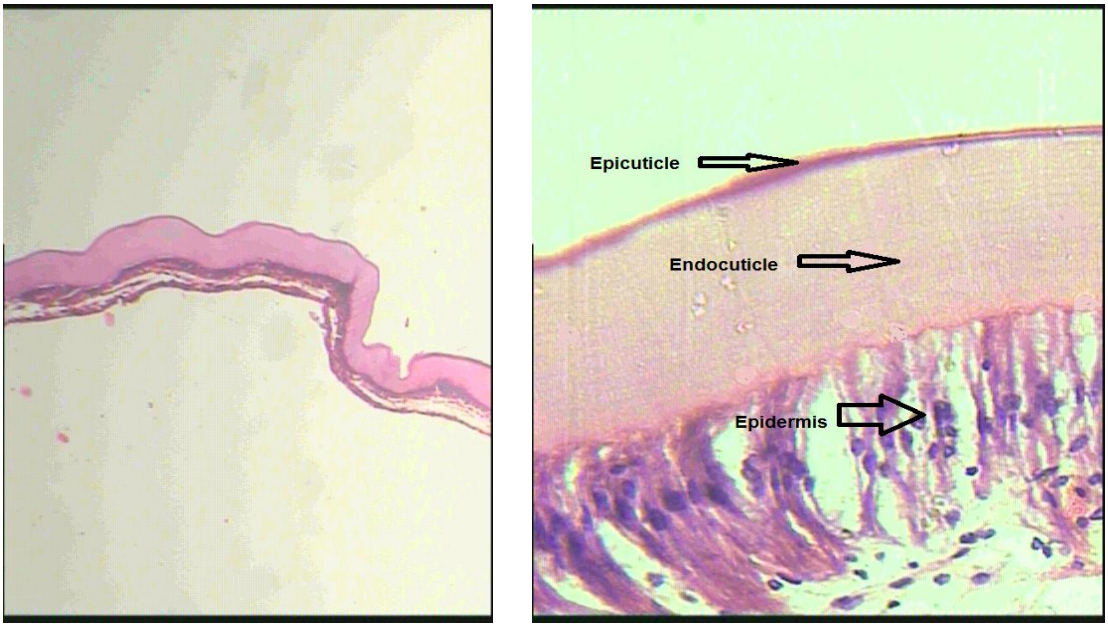
The epidermis of this stage was highly active, and characterized by the occurrence of elongated columnar epithelial cells, have semi-oval nucleus, located at either the mid- distance, or tend to be slightly found at the basal half of the cell. Due to the activity of these cells during this stage, the thickness of this layer beings nearly very close to that of endocuticle, with an average of 91.7 µm.

3.4 The Newly Molt (Stage A)

During this stage a complete formation of non-cellular cuticle including both of epicuticle, exocuticle, endocuticle and membranous layer was occurred (Plate 4). The thickness of these layers averaged 182.8 µm, of which the endocuticle was the widest, averaged 92.7 µm and represented 51.1% of the carapace wall. It followed by exocuticle with an average of 72.78 µm in thickness. The epidermis of this stage was still active, and has a columnar epithelial cells, have semi-oval nucleus, located at the mid-distance, or showing slightly tendency towards the upper side of the cell with thickness averaged 50.04 µm.

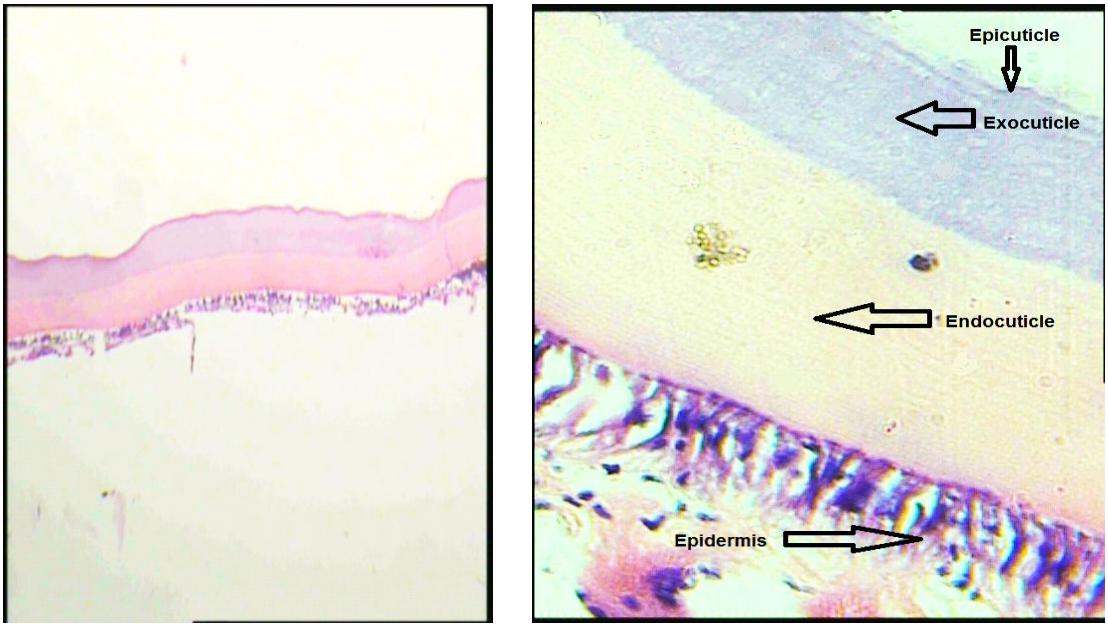
3.5 The Recently Molt (Stage B)

During this stage the layers of cuticle are clearly differentiated as in the previous stage, in addition to the occurrence epidermal layer (Plate 5). The thickness of all cuticular layers averaged 189.0, distributed as 12.2, 83.4 and 79.5 µm for epi- exo- and endocuticle, respectively, showing a decrease in endocuticle thickness, and slightly increase in exocuticle, which may be very close in their thickness. It was also observed that the color of the two layers took the same pattern as in the newly molt stage, with blue and faint red color for exo- and endocuticle, respectively. On the other hand, the epidermal layer became narrower (36.1 µm) comparable with the newly molt. It has cuboidal cells tightly attached to endocuticle and subcutaneous muscle below.



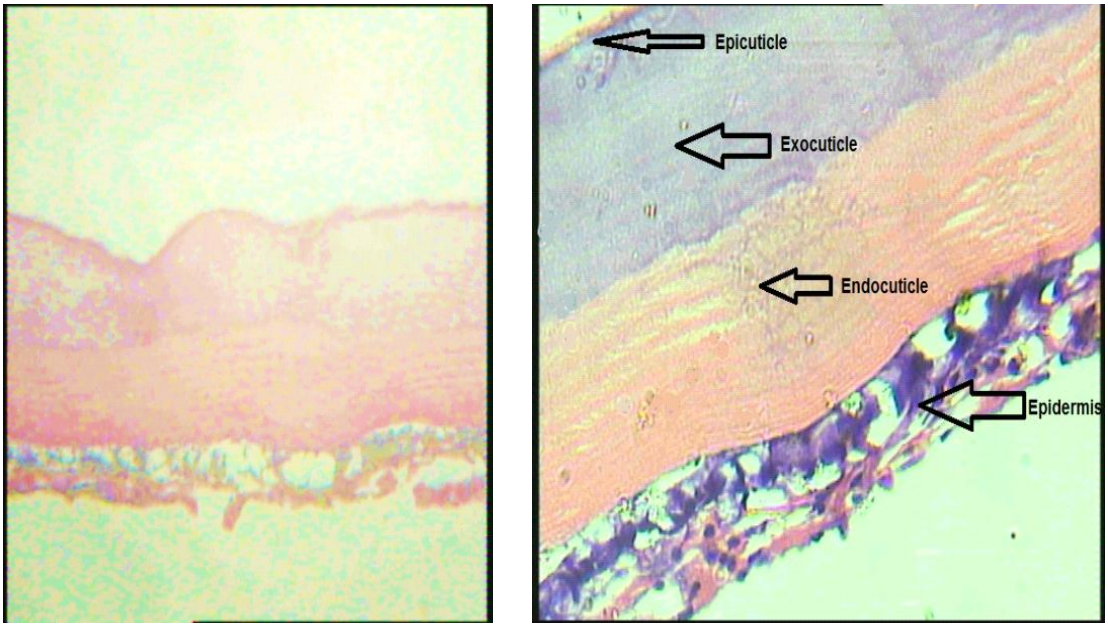
(A) (B)

Plate 4. Vertical section of a new carapace during an ecdysis, with magnification of 5X (A) and 20X (B). Note the elongate columnar epithelial cells of epidermis (B), and disappearance of exocuticle (A) as well as the wrinkled of the new cuticle (A)



(A) (B)

Plate 5. Vertical section in soft new carapace during newly molt stage showing four layers of carapace wall (with magnification power of 5X (A) and 20 x (B)



(A) (B)

Plate 6. Vertical section in new rigid carapace during recently molt stage showing all 4 layers with 10 X (A) and 20 x (B) magnification power

4. DISCUSSION

Changes in integument reflect, more than other tissue, the periodic regulated growth that taking place in the crustaceans during molting [3]. During molting cycle, the cuticle changes dynamically in two main aspects: (1) the thickness of the cuticle, which depends on the number of cuticular sub-layers; and (2) the deposition of the organic and inorganic components in the cuticle certain stages [5]. Therefore, the main components of the cuticle include both inorganic and organic materials in a ratio of 60:40 [4,6]. The inorganic materials found in the cuticle include calcium, chloride, copper, magnesium, manganese, phosphorus, potassium, and sulfur [3,6,5,7,8,9,10]. While as the organic materials include lipids, glycoproteins, proteins, glycosaminoglycans, mucopolysaccharides, carbohydrates, and chitin [3,5,9,11,12,13, 14,15,16].

The results of the present study indicated that the wall of carapace during the intermolt stage is relatively thick, rigid and forms a well completed cuticle. The cuticle consists of four distinctive layers above epidermal (Malpighian) layer. These layers are noncellular, composed mainly of lipoproteins, arranged from the outer to the inner surface as: epicuticle, exocuticle, endocuticle and membranous layer. This structure is in well agreement with previously mentioned by [1,2,3,4,17,18]. The exocuticle was the widest, because it contains calcium carbonate and the thickness of epicuticle was the narrower, may be due to it lacks calcium carbonate. While as the epidermal layer composes of cuboidal epithelium, with large deep blue color concentric nucleus. Their small size may reflect inactivity during this stage which is in agreement with [18,19,20]. These results also showed that, during this stage both the reinforcement of the cuticle and the condensation of the epidermis tissues continue to a maximum, accompanied by a gradual reduction of lacunar spaces and conspicuous tissue growth.

By the onset of the premolt, remarkable changes were observed in carapace wall denoting to increasing internal activity during this stage. This stage was characterized by a clear apolysis occurred between the old endocuticle and a new

cuticle beneath, which appears gradually above the membranous layer. The cuticular layers of the old cuticle were morphologically differentiated by the appearance of striated myofibrils and disappearance of membranous layer. The exocuticle was very close to the epicuticle and has typical alternating light and dark lamellae. It has blue color with hematoxylin, may be due to the presence of organic components especially, lipids and protein, which constitute the major components of the epicuticle. Exocuticle was also the widest, and represented about 42.0% % of all cuticular thickness. The inner borders of both endocuticle and epicuticle have condensed or compact fibrils, but showed loosely or slightly separated fibrils towards the outer borders due to beginning of reabsorption of minerals from the old cuticle. When morphogenesis has been completed, a very thin new cuticle is secreted on the surface of new epidermal structures, while a gap (an apolysis) was appeared between the old and the new cuticle, increased with the advances of the stage. At the same time, an endocuticle layer had been observed in the new cuticle which is similar with that mentioned by [21]. These results are also in agreement with that mentioned by [1,17,21,22]. On contrast, [19] and [23] stated that, the formation of an exocuticle was firstly begun at the same stage, which may be called for the previous endocuticle. However, the membranous layer was thin (6.9 µm) and tightly attached to the Malpighian layer. The cells of the last layer were also cuboidal, with large deep blue color concentric nucleus. The thickness of this layer averaged 45.2 µm, indicating to their high activity, which agrees with those mentioned by [17,19].

On the other hand, during ecdysis the molting process takes normally a few minutes [1,2,21]. The presence of pre-ecdysal or an old carapace (exuviae) comprised epi-, exo-, and endocuticle only and postecdysal or new carapace were found. The separation between each may be attributed to the reabsorption of biominerals particularly calcium carbonates. The thickness of these layers decreased remarkably comparable with the previous stage, but the vertical canals of tegumental gland were extending through the exo- and endocuticle. In crayfish, it is initiated by flexing movements, followed by a dorsal rupture of the cuticle between the cephalothorax and the first abdominal segment. Subsequently, the crayfish sheds also the anterior parts of its exuviae or old carapace and a new carapace appears, so that a new molting cycle begins. These findings are in agreement with those mentioned by [1,18,24].

However, during newly molt and immediately after an ecdysis, the cuticle was thin and wrinkled, and the body is completely soft ascertained by probing with delicate forceps [21]. Microscopic examination revealed that a spongy epidermal tissue structure with numerous large and irregularly shaped lacunar spaces was found. The epidermis of this stage was highly active, and characterized by the occurrence of an elongated columnar epithelial cells, have semi-oval nucleus, located at either the mid- distance, or tend to be slightly found at the basal half of the cell. Whereas the cuticle in recently molt, becomes more rigid, and the epidermal tissues begin to concentrate along the inner surface of the cuticle. It was obvious that, these results are in full agreement with [18].

5. CONCLUSSION

The histological examination of cuticle in *P. clarkii* during molting cycle showed changes in cuticle structure and number of layers, during intermolt stage, carapace integument composed of four distinctive layers, they arranged from the outer surface to inner side as: epicuticle, exocuticle, endocuticle and membranous layer, respectively. These layers changes in thickness, number and shape of epithelial layer during molting stages. The most obvious event arises in premolt stage (apolysis) a separation between old cuticle and new one. All these changes could be used as criteria for molt staging in this species beside morphological characteristics in purpose of agriculture.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Warner GF. The biology of crabs. Elek Science, London; 1977.

2. Lowery RS. Growth, molting and reproduction. In: Holdich, D.M., Lowery, R.S. (Eds.), Freshwater Crayfish, Biology, Management and Exploitation. Croom Helm Press, London. 1988;83–113.
3. Yamaoka LH, Scheer BT. Chapter: 11: Chemistry of growth and development in crustaceans. In: Chemical Zoology; Academic Prees, London; V(A). 1970;321-338.
4. Promwikorn W, Kirirat P, Thaweethamsewee P. Index of molt staging in the black tiger shrimp (*Penaeus monodon*). Songklanakarin J. Sci. Technol. 2004;26(5):765-772.
5. Mangum CP. Physiological aspects of molting in the blue crab, *Callinectes sapidus.* Amer. Zool. 1992;32:459-469.
6. Roer R, Dillaman R. The structure and calcification of the crustacean cuticle. Amer. Zool. 1984;24:893-909.
7. Compere P, Morgan JA, Goffinet G. Ultrastructure location of calcium and magnesium during mineralization of the cuticle of the shore crab, as determined by the K-pyroantimonate method and X-ray microanalysis. Cell Tissue Res. 1993;274: 567-577.
8. Pierce DC, Butler KD, Roer RD. Effects of exogenous *N*-acetylhexosaminidase on the structure and mineralization of the post-ecdysial exoskeleton of the blue crab, *Callinectes sapidus*. Comp. Biochem. Physiol. 2001;(128)B:691-700.
9. Pratoomchat B, Sawangwong P, Pakkong P, Machado J. Organic and inorganic compound variations in haemolymph, epidermal tissue and cuticle over the molt cycle in *Scylla serrata* (Decapoda). Comp. Biochem. Physiol. 2002a;(131)A: 243-255.
10. Wang W, Wang A, Wang D, Wang L, Liu Y, Sun R. Calcium, phosphorus and adenylate levels and Na+-K+-ATPase activities of prawn, *Macrobrachium nipponense*, during the moult cycle. Comp. Biochem. Physiol. 2003;(134)A:297-305.
11. Glynn JP. Studies on the ionic, protein and phosphate changes associated with the moult cycle of *Homarus vulgaris*. Comp. Biochem. Physiol. 1968;26:937-946.
12. Vigh DA, Dendinger JE. Temporal relationships of postmolt deposition of calcium, magnesium, chitin and protein in the cuticle of the Atlantic blue crab, *Callinectes sapidus* Rathbun. Comp. Biochem. Physiol.1982;(72)A:365- 369.
13. Heller G. Compt. Rend. Soc. Biol. 1984;121-414.
14. Marlowe RL, Dillaman RM, Roer RD. Lectin binding by crustacean cuticle: The cuticle of *Callinectes sapidus* throughout the molt cycle, and the intermolt cuticle of *Procambarus clarkii* and *Ocypode quadrata*. J. Crustacean Biol. 1994;14: 231-246.
15. Anderson SO. Exoskeletal proteins from the crab, *Cancer pagurus*, Comp. Biochem. Physiol. 1999;(123) A:203-211.
16. Roer RD, Halbrook KE, Shafer TH. Glycosidase activity in the post-ecdysial cuticle of the blue crab, *Callinectes sapidus*. Comp. Biochem. Physiol. 2001; (128)B:683-690.
17. Highnam KC, Hill L. The comparative endocrinology of the invertebrates. (English Language Book Society) First Edition. 1969;298.
18. Nagabhushanam R, Kulkarni GK. Endocrine regulation of reproduction in the marine female, *Parapenaeopsis hardwickii* (Miers) (Cruastacean, Decapods, Penacidae) Proc. Symp Coastal Aquaculture. 1983;1:40-47.
19. Skinner DM. The structure and metabolism of a crustacean integumentary tissue during a molt cycle. Biology Bulletin Marine Biology Lab. Woods Hole. 1962;123: 635-647.
20. Skinner DM. The structure and metabolism of a crustacean integumentary tissue during a moult cycle, New York, Department of Physiology and Biophysics, New York University School of Medicine, Doctor of Philosophy in the Subject of Biology; 1963.
21. Hayd LA, Anger K, Valenti WC. The moulting cycle of larval Amazon River prawn *Macrobrachium amazonicum* reared in the laboratory, São Paulo, Brazil. Nauplius. 2008;16(2):55-63.
22. Jenkin PM. Apolysis in arthopoda in molting cycle. Nature. 1966;221:871.
23. Promwikorn W, Boonyoung P, Kirirat P. Histological characterization of cuticular depositions throughout the molting cycle of the black tiger shrimp (*Penaeus monodon*), Thailand. Songklanakarin J. Sci. Technol. 2005;27(3):499-509
24. Wilder MN, Huong DTT, Jasmani S, Jayasankar V, Kaneko T,

Aida K, Hatta T, Nemoto S, Wigginton A. Hemolymph osmolality, ion concentrations and calcium in the structural organization of the cuticle of the giant freshwater prawn *Macrobrachium rosenbergii*: Changes with the molt cycle, Aquaculture. 2009;292: 104–110.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/16131