

Carapace ultrastructure of the spider crabs *Schizophrys dahlak* (Majidae) and *Hyastenus hilgendorfi* (Epialtidae) from the Suez Canal, Egypt

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ABSTRACT

The microstructures of the carapaces of the spider crabs *Schizophrys dahlak* and *Hyastenus hilgendorfi* were examined under both scanning and transmission electron microscopes. Image analyses of the cross-sectioned carapaces showed that both species have a hierarchical microstructure with three well-defined layers. The two innermost layers (exocuticle and endocuticle) consist of stacked fibers arranged in a twisted plywood fashion producing a "Bouligand structure". They have a similar structure but different thicknesses and height of the stacks. The cuticles are inter-stitched with pore canals of varying width between the studied species, running transversely to the layered structure. This variation in the width of the pore canals in the two species seems to be proportional to the size of the carapace and its surface area.

INTRODUCTION

The functional properties of an organism's parts tend to have evolved optimal structural and material characteristics that provide adaptability and longevity over time (Wang *et al.*, 2015; Zhuang *et al.*, 2016). The hard exoskeletons of decapod crustaceans such as lobsters and crabs can be an adaptation to harsh environments by supporting the body, resisting impacts from external forces, protecting from erosion, predation, and disease, as well as preventing evaporation of water (Wanxi and Hong, 2000; Wang *et al.*, 2018). The exoskeleton of decapods consists mainly of chitin with a high degree of mineralization, typically calcium carbonate, which provides mechanical rigidity. The minerals are deposited within the chitin– protein matrix in the form of calcite or amorphous calcium carbonate (Lowenstam, 1981; Cameron, 1989).

The architecture of crab's exoskeleton seems to form to satisfy specific functional requirements (Alam, 2018). Underneath the exoskeleton there is the hypodermis: a layer of epithelial tissue that secretes exoskeletal materials (Travis, 1963). The cuticle, which forms the major part of the exoskeleton, is a layered, calcified structure deposited from the epidermal cells on the crab's soft body. The cuticle is composed of three fundamental

layers each with a different structure and function; the epicuticle, the exocuticle and the endocuticle. The structures of these layers differ somewhat from each other, and change over time through the mineralization process of the intermolt, premolt and production of the exuvium (Alam, 2018).

A remarkable feature of the crab's exoskeleton is its precise hierarchical organization at different structural levels (Chen *et al.*, 2008). At the molecular level, there are fibrils, 3 nm in diameter and 300 nm in length which are formed from long-chain polysaccharide chitins. These fibrils are wrapped with proteins and gathered into fibers of around 60 nm in diameter. These fibers assemble into bundles that arrange themselves parallel to each other, forming horizontal planes. The planes accumulate in a helicoid fashion, producing a twisted plywood structure. The layers, which are stacked and complete a 180° rotation, produce what is known as a "Bouligand structure". These repeat to form the exocuticle and endocuticle (Chen *et al.*, 2008). In a direction normal to the surface (the z-direction), there are pores which permeate the structure of the crab carapace from top to bottom in the shape of long canals. They are formed while the epithelial cells make a new integument prior to molting. Small bumps then protrude from the cell surfaces of the crustaceans' epithelium. Layers of chitin fibrils get woven around them and gradually, elliptical, helically twisted pore canals are created. Primarily, they serve as a transport system for the minerals used by the animals in order to quickly toughen the soft skeletal structure of the integument once the old one is shed (Alam, 2018).

The spider crabs *Schizophrys dahlak* Griffin and Tranter, 1986 (Family Majidae) and *Hyastenus hilgendorfi* de Man, 1887 (Family Epialtidae) inhabit the Suez Canal. Although these species are the most commonly caught brachyuran crabs along the canal (Osman *et al.*, 2015), they are inedible and have no commercial value or fishery potential and are constantly discarded by fishermen as a by-catch. Being relatively large compared to other local crustaceans and available at little or no cost, they are promising subjects for investigation. In this work, we examined the ultrastructure of the exoskeletal cuticle of these species using both scanning and transmission electron microscopes.

MATERIALS AND METHODS

Collection of samples:

Schizophrys dahlak and *Hyastenus hilgendorfi* were sampled from the Suez Canal at Timsah Lake. Crabs were taken from fishermen using trammel nets with an approximate mesh size of 33.5 mm that are dropped at sunset and collected at dawn. Fresh carapaces of both species in the intermolt stage with carapace width (CW) of ~44 mm and ~18 mm for *S. dahlak* and *H. hilgendorfi*, respectively were removed and fixed by immersing them immediately in a mixture of 4% formaldehyde and 1% glutaraldehyde (4F-1G) in phosphate buffer solution (pH 7.2) at 4°C for 24 hours. Both species are known to be heavily encrusted by epibiota. Only specimens with clean carapaces devoid of any epibionts were selected. A subsample of 2 males and 2 females were chosen to be examined using both scanning and transmission electron microscopes.

SEM:

For scanning electron microscopy, carapaces were post fixed in 2% osmium tetroxide (OsO_4) in phosphate buffer solution (pH 7.2) at 4°C for 2 hours. They were then washed in the buffer and dehydrated at 4°C through a graded series of ethanol and prepared as cross-sections. The prepared samples were then dried by means of a critical point method, mounted using carbon paste on an Al-stub and coated with gold up to a thickness of 400Å in a sputter-coating unit (JFC-1100 E). The coated samples were examined and SEM images were collected using scanning electron microscope (JOEL-JSM- 5300).

TEM:

For the transmission electron microscope, carapaces were de-calcified in 0.2 M EDTA at pH 7.6 for 2 weeks or until they were flexible. De-calcified carapaces were then post fixed in 2% OsO_4 in the same buffer at 4°C for 2 hours. The prepared samples were washed in the buffer and dehydrated at 4°C through a graded series of acetone then embedded in resin to polymerize them, cut into sections about 90Å in thickness. Sections were placed on a grid copper, stained by uranyl acetate for 5 min then lead citrate for 2 min. Stained sections were examined and TEM images were collected using transmission electron microscope (JEOL-JSM-1400 PLUS).

RESULTS AND DISCUSSION

Carapaces of the spider crabs *S. dahlak* and *H. hilgendorfi* were observed with SEM and TEM techniques using cross sections, with the representative images displayed in Figures 1-5. As evidently seen in Figures (1A and 2A), the carapaces have a total thickness of ~430 µm and ~450 µm for *S. dahlak* and *H. hilgendorfi*, respectively. **Elices (2000)** pointed out that a layered structure is characteristic of the exoskeletons of crustaceans. The SEM results showed that the cross-sections of the carapaces have three fundamental layers, each with its own structure. From outer to inner, these are the epicuticle, the exocuticle and the endocuticle (Figs. 1A and 2A) for *S. dahlak* and *H. hilgendorfi*, respectively. **Alam (2018)** reported that the structure of each layer differs somewhat from each other and this moreover changes throughout the mineralization process, which is typically divided into intermoult, premoult and exuviae. The key features and measurements of the structures from both species are summarized in Table (1) and will be discussed as follows.

The exoskeleton consists of three distinct regions in both species (Figs. 1A, 2A). In the outermost region, the epicuticle is much thinner than the other regions with a thickness of approximately (19-22 µm) (Fig. 1B) for *S. dahlak* and (15-18 µm) (Fig. 2B) for *H. hilgendorfi*. **Cheng et al. (2008)** reported that the epicuticle is a thin layer of wax lacking chitin, acting as a diffusion barrier on the outer surface of the carapace. Cross-sectional images of the epicuticle from the critical point dried samples (without decalcification) reveal a dense structure, where the microscopic features are hard to distinguish.

Beneath the epicuticle is the procuticle. The procuticle is divided into two parts, the exocuticle (outer) and the endocuticle (inner) (Figs. 1A, 2A). The exocuticle is stacked more densely than the endocuticle (Figs. 1C and 2C). These two regions have similar structures but have different thicknesses. The thickness of the exocuticle is approximately (54-60 μm) in *S. dahlak* (Fig. 1C) and (36- 40 μm) in *H. hilgendorfi* (Fig. 2C). The endocuticle is approximately (300-350 μm) and (340-380 μm) thick for *S. dahlak* (Fig. 1D) and *H. hilgendorfi* (Fig. 2D), respectively. The thickness of the endocuticle is approximately 5-6 times that of the exocuticle for *S. dahlak* and 8-9 times that for *H. hilgendorfi* (Figs. 1A and 2A). **Cheng *et al.* (2008)** reported that the thickness of the endocuticle in the American lobster *Homarus americanus* is approximately 3-4 times that of the exocuticle and 6-8 times that for the Atlantic blue crab *Callinectes sapidus*. **Chen *et al.* (2008)** stated that the procuticle is the main structural part, which is primarily designed to resist mechanical loads, in which the endocuticle makes up around 90% of the exoskeleton volume.

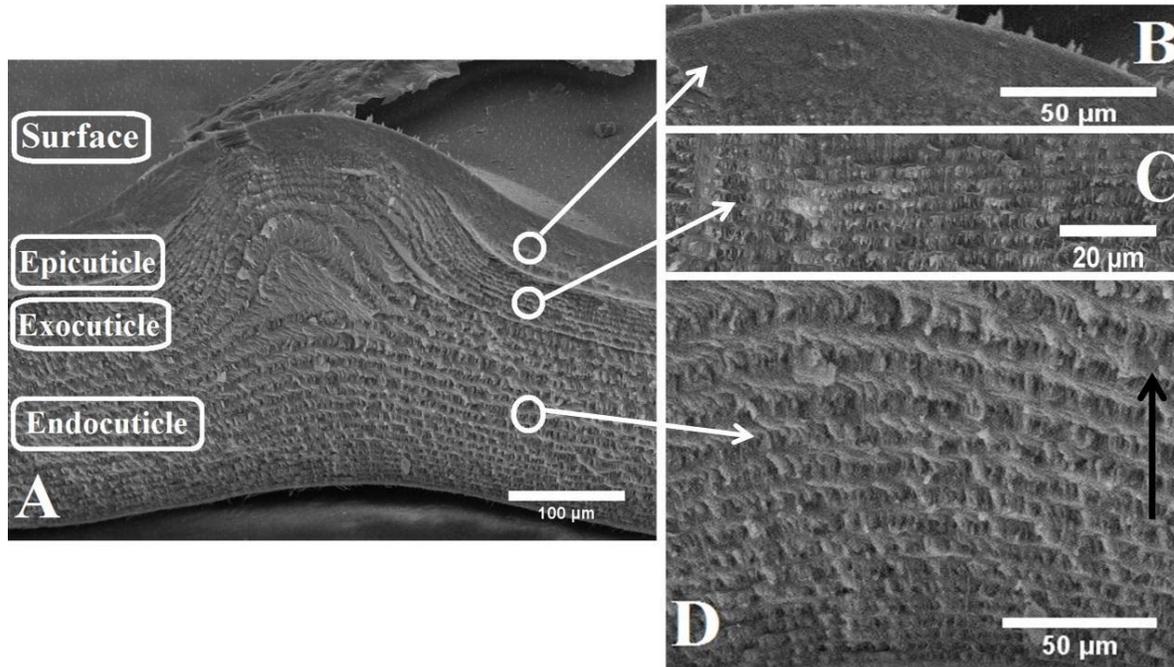


Fig. 1. Ultrastructure of *S. dahlak* carapace cuticle, imaging of the cross-fractured cuticle by SEM (A); overview of the cross section showed multilayered structure (B); epicuticle (19-22 μm thick) (C); exocuticle (54- 60 μm thick) (D); Endocuticle (300-350 μm thick). Black arrow points to the normal direction.

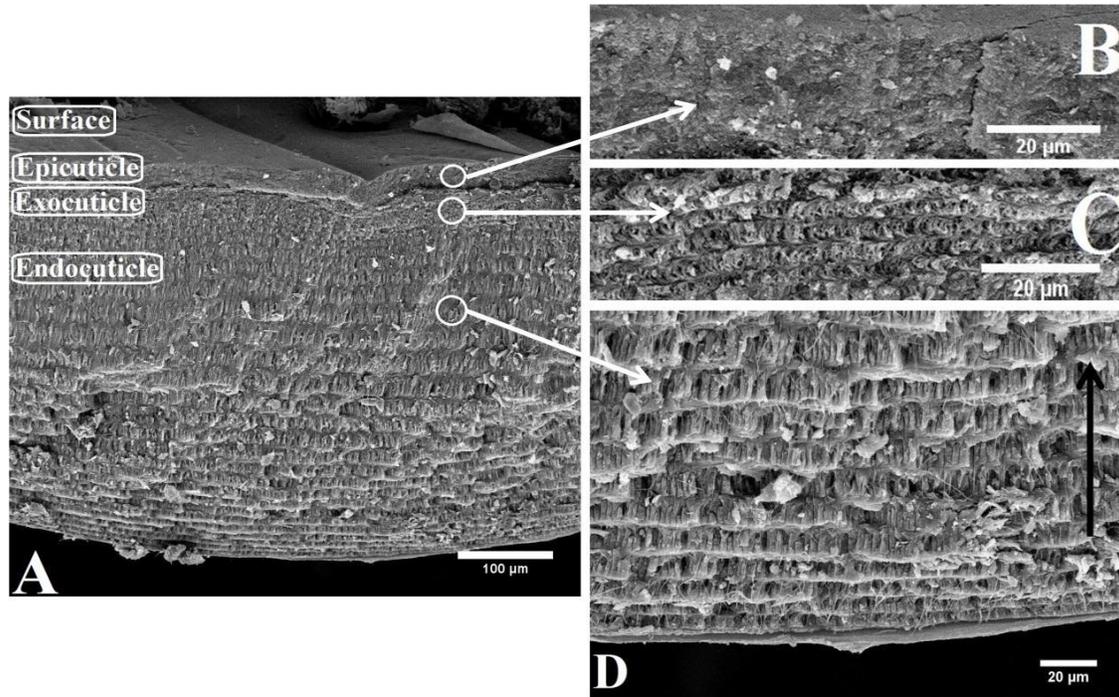


Fig. 2. Ultrastructure of *H. hilgendorfi* carapace cuticle, imaging of the cross-fractured cuticle by SEM (A); overview of the cross section showed multilayered structure (B); epicuticle (15-18 μm thick) (C); exocuticle (36- 40 μm thick) (D); Endocuticle (340-380 μm thick). Black arrow points to the normal direction.

Table 1: Structure comparison between *Schizophrys dahlak* and *Hyastenus hilgendorfi* carapaces, based on SEM image analysis.

Region	Property	<i>S. dahlak</i>	<i>H. hilgendorfi</i>
Epicuticle	Thickness	19-22 μm	15-18 μm
	Pattern	Fibers aligned perpendicular to the surface	Fibers aligned perpendicular to the surface
Exocuticle	Thickness	54-60 μm	36-40 μm
	Pattern	Bouligand structure	Bouligand structure
	180°-stack	3-5 μm	3-5 μm
Endocuticle	Thickness	300-350 μm	340-380 μm
	Pattern	Bouligand structure	Bouligand structure
	180°-stack	12-15 μm	21-27 μm
Pore canals	Diameter	0.35-0.65 μm	0.14-0.20 μm

The TEM images showed that the cuticle of our both species also have a layered structure, differentiated into three principal layers: epicuticle, exocuticle and endocuticle (Figs. 3A and 4A). Transverse pore canals are also dominant features, running perpendicular to these layers. The epicuticle has been reported as the outermost thin layer, which is composed predominantly of lipoproteins while the exocuticle is known to be a highly mineralized layer (Priester *et al.*, 2005).

From our electron micrographs, we can confirm that the exocuticle layer appears to have dense tissues in both species (Figs. 3B and 4B). Lamellae of helicoidally arranged fibers are distinctive in the exocuticle and endocuticle layers. In each layer, multiple layers of this lamellae are arranged parallel to each other. Each layer corresponds to a 180° rotation of the twisted plywood (Bouligand) structure (Fig. 5). This stacking sequence results in an apparent parabolic pattern in oblique sections of the cuticles for *S. dahlak* (Fig. 3C) and for *H. hilgendorfi* (Fig. 4C). Although both species had the same thickness (3-5 µm) of the 180° stack in the exocuticle layer, *H. hilgendorfi* had thicker 180° stack (21-27 µm) than that of *S. dahlak* (12-15 µm) in the endocuticle layer. Figures (3D and 4D) show a layered arrangement of lamellae in *S. dahlak* and *H. hilgendorfi*, respectively. **Dillaman *et al.* (2013)** explained that the exocuticle and endocuticle are composed of helicoidally arranged chitin-protein fibers, forming a lamellar appearance and contributing to cuticle stiffness and mechanical resistance. The procuticle, the main structural part, has well-marked plywood (Bouligand) structures (**Zhou *et al.*, 2016**). The Bouligand structure has been documented in several crustacean species and reported as a general hierarchical structural organization in their integuments (**Chen *et al.*, 2008**; **Cheng *et al.*, 2008**; **Zhou *et al.*, 2016**; **Wang *et al.*, 2018**).

In both species, in addition to the layered helicoidal structure, there are distinct through-the-thickness structural features: the pore canals (PC) which evenly distributed in both the exocuticle and the endocuticle and run perpendicular to the layers. In terms of diameter, *S. dahlak* has been observed to have wider pore canals (0.35-0.65 µm) (Fig. 3E) than those of *H. hilgendorfi* (0.14-0.20 µm) (Fig. 4E). **Giraud-Guille (1998)** believed that pore canals have at least two functions: enhancement of the mechanical strength of the cuticle and transporting of impregnating materials during the molting process. Many crustaceans have this porous structure in their fiber layers such as the green crab, *Carcinus maenas* (**Roer, 1980**) and the edible crab, *Cancer pagurus* (**Hegdahl *et al.*, 1978**).

Greenaway and Farrelly (1991) pointed to the existence of a direct access route between the exuvial space and the epidermal extensions within pore canals for premolt brachyurans. On the other hand, **Horst and Freman (1993)** reported that the function of pore canals in decapods is to provide access between the cuticle and the epidermal cytoplasm for materials that are reabsorbed from the old cuticle in the premolt stage and in calcium calcification during postmolt. Our two crab species differ greatly in terms of size where *S. dahlak* is probably 3 to 4 times larger than *H. hilgendorfi*. We therefore propose that the width of the pore canals could be proportional to the surface area of the carapace in both species thus facilitating the transport of materials required for the post molting process.

Both species under study are encrusted with an assortment of epibionts that helps conceal their outline (**Sallam *et al.*, 2007**; **Sallam and Wicksten, 2011**; **Madkour *et al.*, 2012**). This cover represents a load on the crab's integument. **Sallam and Wicksten (2011)** reported that males *H. hilgendorfi* carry a cover that is one and half their weight whereas berried females carries 3 times their weight. On that basis, we suspect that the ultrastructure of the integuments of these encrusted species differ either in terms of thickness of layers or in the degree of mineralization of the cuticle. Further investigations are recommended to focus on the impact of this covering on the ultrastructure of the integuments of these species in comparison to non-encrusted ones.

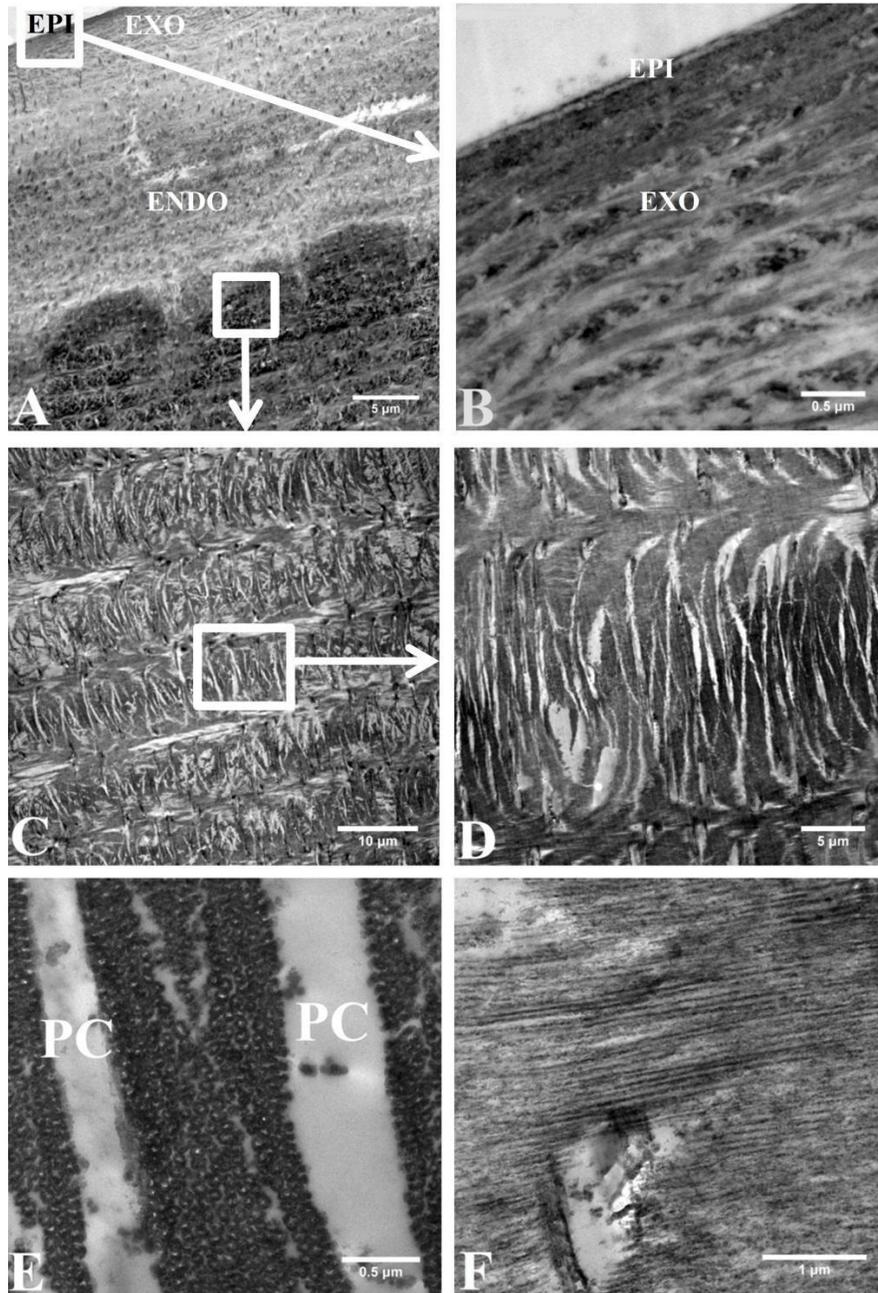


Fig. 3. Decalcified an ultrathin section in *S. dahlak* carapace cuticle, imaging by TEM (A); Ultrastructure of the carapace cuticle showing the three layers epicuticle (EPI), exocuticle (EXO) and endocuticle (ENDO) (B); The epicuticle layer (EPI) and higher stacking density of exocuticle layer (EXO) (C); Parabolic pattern of chitin-protein matrix can be seen in the endocuticle (D); Fibrils sectioned longitudinally in narrow lamellar bands and crosswise in broad lamellar bands (E); Pore canals (PC) (F); Longitudinally sectioned fibrils.

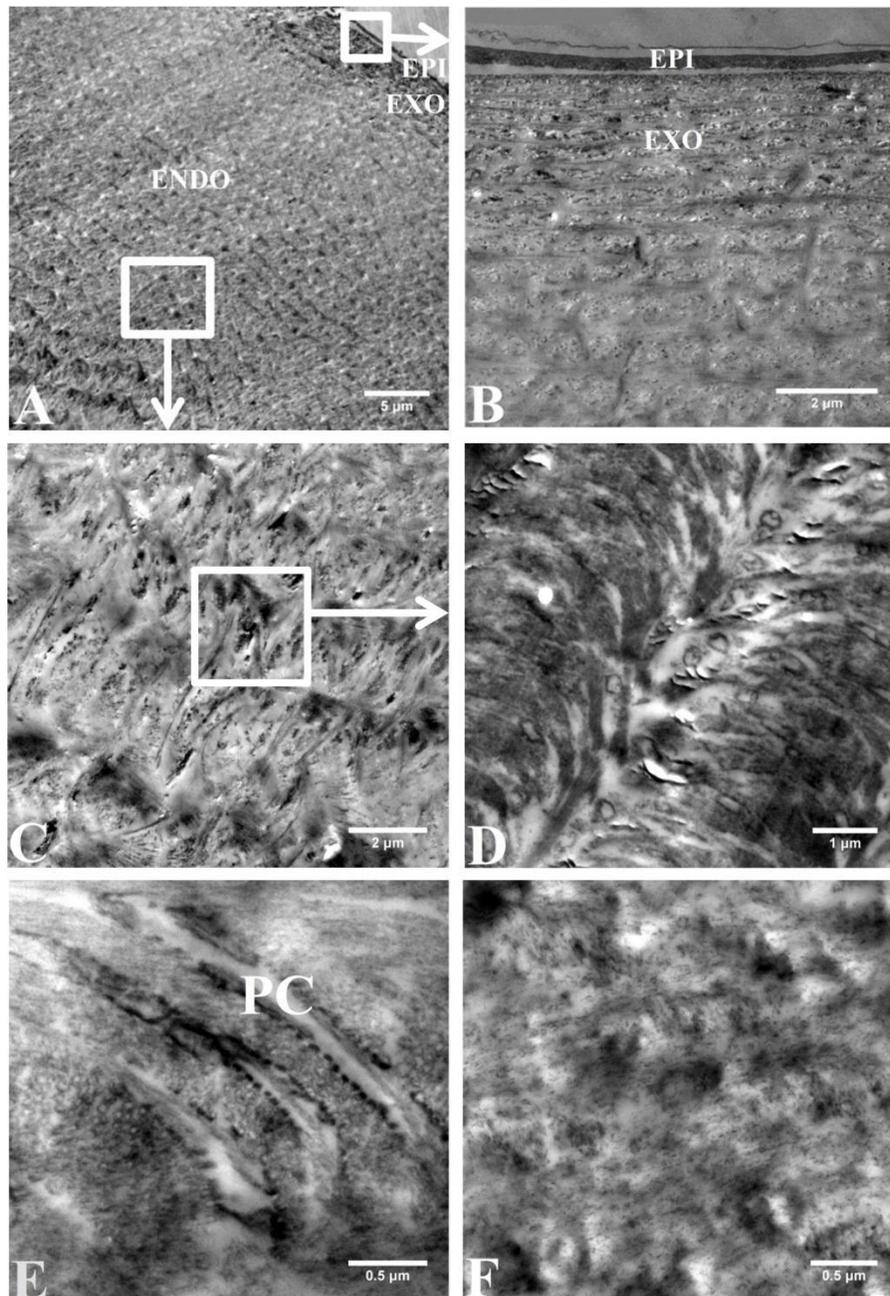


Fig. 4. A decalcified an ultrathin section in *H. hilgendorfi* carapace cuticle, imaging by TEM (A); Ultrastructure of the carapace cuticle showing the three layers epicuticle (EPI), exocuticle (EXO) and endocuticle (ENDO) (B); The epicuticle layer (EPI) and higher stacking density of exocuticle layer (EXO) (C); Parabolic pattern of chitin-protein matrix can be seen in the endocuticle (D); Fibrils sectioned longitudinally in narrow lamellar bands and crosswise in broad lamellar bands (E); Pore canals (PC) (F); Longitudinally sectioned fibrils.

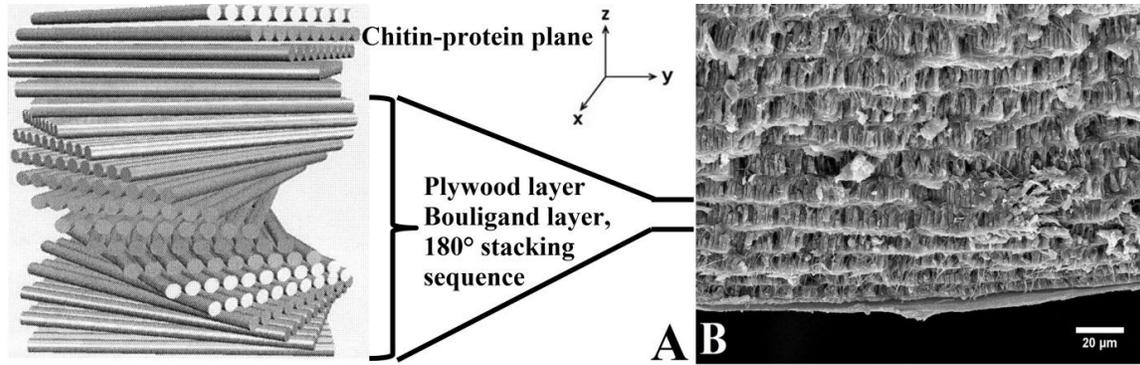


Fig. 5: (A); Simplified schematic presentation of the Bouligand structure (B); Each layer corresponds to periodic 180° rotation of stacking sequence of the endocuticle in *H. hilgendorfi*.

CONCLUSION

In this work, we have investigated carapaces of the two common spider crabs in the Suez Canal: *Schizophrys dahlak* and *Hyastenus hilgendorfi*. The investigation was based on extensive SEM and TEM analyses in order to establish their ultrastructure.

The principal conclusions are as follows:

- The carapaces of both species are multilayered, with an epicuticle and a procuticle which is divided into the exocuticle (outer) and the endocuticle (inner). The thickness of the endocuticle is approximately 5-6 times that of the exocuticle for *S. dahlak* and 8-9 times that for *H. hilgendorfi*.
- Cross sections of the procuticle of the carapace reveal a Bouligand structure. Each layer of the fibrous layer consists of several regularly arranged fiber bundles, where many pore canals between neighboring bundles penetrate this plywood layer.
- The Bouligand layers in the exocuticle have a dense structure (3-5 μm each layer) in both species. The endocuticle has a more open structure (12-15 μm each layer) in *S. dahlak* and (21-27 μm each layer) in *H. hilgendorfi*. *S. dahlak* has wider pore canals (0.35-0.65 μm) than those of *H. hilgendorfi* (0.14-0.20 μm).

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