

# Scanning Electron Microscopy of Vitellogenic Oocytes and Spawned Eggs of the Portunid Crab *Charybdis hellerii* (*Crustacea-Brachyura*) (Milne Edwards, 1867)

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## Abstract

**Background:** The portunid crab *Charybdis hellerii* is an economically critical aquatic species in the Mediterranean region. Several investigators have reported scanning electron microscopy (SEM) observations on some crustacean's eggs' morphology. Going through the previous studies, knowledge regarding the morphology of *C. hellerii*. vitellogenic oocytes and spawned egg membranes are not available. **Aims and Objectives:** In the present study, an attempt has been made to describe the morphology and the structure of the membranes of vitellogenic oocytes and the newly spawned eggs to provide necessary information for further studies on the reproductive and evolutionary biology of the crab *C. hellerii*. **Materials and Methods:** Samples of ripe pinkish orange ovaries of non-ovigerous females and the spawned incubated eggs of ovigerous females with orange and grey spawns were processed for scanning electron microscopy. The prepared samples were examined in a Zeiss DSM 940 scanning electron microscope. **Results:** The present SEM study revealed that, vitellogenic oocytes are highly packed with yolk inclusions, which appear to be embedded in a definite acellular matrix and surrounded by a distinct chorion, which is pierced by several pores. The follicle cells appear polygonal in shape and interconnected through thin lateral projections and strongly associated with vitellogenic oocytes. The brooded fertilized eggs are attached through a marked stalk (funiculus) and surrounded by three distinct envelopes, which showed specific ornamentations and variations in their surface topography. The outer envelope coarsely wrinkled, while the middle envelope showed finely wrinkled ornamentation, and the inner envelope appeared with its characteristic spongy, porous appearance. **Conclusions:** This study denotes a significant difference between mature vitellogenic oocytes inside the ripe ovary and the spawned ova. The differences have been shown in the structure and external ornamentation of their surrounding membranes. Unlike the vitellogenic oocytes, the spawned ova were surrounded by three distinct layers, which are differ in their surface architecture. Such membrane architecture is species specific characteristic and has been thought to be an adaptive feature for brooded fertilized eggs to survive from stressful environmental conditions.

**Keywords:** *Charybdis hellerii*, follicle cells, funiculus, vitellogenic oocytes, spawned eggs

## INTRODUCTION

The portunid crab *Charybdis hellerii* is extensively distributed in the Indo-Pacific region and often fished as a commercial catch and has been extending its distribution into the Atlantic Ocean.<sup>[1-4]</sup> The species was known to have reached the Mediterranean region.<sup>[5]</sup>

Scanning electron microscopy (SEM) observations on the morphology of some crustaceans eggs have been reported by several investigators.<sup>[6-24]</sup>

Subsequent studies on a number of crustacean eggs have revealed that these eggs are surrounded by an inner chorionic membrane and outer protective covering.<sup>[12,25-28]</sup>

Morphology and outer ornamentation of crustacean eggs are found to be varied in different species.<sup>[29-32]</sup> Ootaxonomy is based chiefly on the species specificity of chorionic

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architecture, which is constant within the representatives of a species.<sup>[33]</sup>

Going through the literatures, studies on the morphology of vitellogenic oocytes and spawned eggs of *C. hellerii* are not available; thus, the present study was conducted to describe the morphology and the structure of the outer membranes of the vitellogenic oocytes inside the ovary on the one hand and that of the newly spawned eggs which are hanged on the female pleopods on the other hand.

## MATERIALS AND METHODS

Adult ripe and ovigerous females of *C. hellerii* crab samples were collected from Alexandria Shores and brought alive to the laboratory. Ripe females were determined according to size and the color of the ovary as well as the ventral abdominal flap carapace color. The specimens used in this work included pieces of ripe pinkish-orange ovaries which were removed from nonovigerous females and the spawned incubated eggs which were taken from ovigerous females with orange and grey spawns. All specimens were fixed in 2% buffered glutaraldehyde, washed with in cacodylate buffer (0.05 M, pH 7.4) for 2 h at 4°C. After rinsing in cacodylate buffer, the tissues were post-fixed in 1% osmium tetroxide (OsO<sub>4</sub>) for 1 h at 4°C and rapidly washed in cacodylate buffer. The specimens were then transferred to an aqueous solution of 1% thiosemicarbazide, for 15 min, followed by an aqueous solution of 1% OsO<sub>4</sub> for 30 min at 4°C. After rinsing in distilled water, the specimens were dehydrated in graded ethanol at room temperature, critical point dried, and gold coated according to standard procedures.<sup>[34]</sup> The prepared parts were examined in a Zeiss DSM 940 scanning electron microscope, in the Electron Microscope Unit at the Alexandria University, Egypt. Photomicrographs were taken at various magnifications.

## RESULTS

The SEM study on *C. hellerii* vitellogenic oocytes and spawned eggs showed great differences between the vitellogenic oocytes inside the ovary and the spawned ova. These differences appeared in their structure and outer ornamentation of their surrounding membranes. Several pores pierced the outer surfaces of ovarian vitellogenic oocyte were observed [Figure 1a and b]. Follicle cells appeared closely associated with these oocytes [Figure 1c]. The magnified SEM photographs for the follicle cells showed that these cells are polygonal in shape and interconnected with each other by means of thin lateral projections [Figure 1d]. However, vitellogenic oocyte is highly occupied with yolk inclusions which appeared to be embedded in a clear matrix and surrounded by a distinct chorion [Figure 1e and f].

The brooded fertilized eggs of *C. hellerii* showed specific ornamentations [Figure 2a]. Many folds or wrinkles were observed on the surfaces of these eggs. Each spawned egg is surrounded by three different distinct outer, middle, and inner envelopes [Figure 2b and c]. The three envelopes showed a great variation in their surface topography. The outer envelope

possessed coarse, wrinkled ornamentation [Figure 2d], while the middle envelope showed a fine wrinkled one [Figure 2e] and the inner envelope appeared with its characteristic spongy porous appearance [Figure 2f]. The SEM study of brooded fertilized eggs showed that they were attached to each other through a marked highly twisted stalk (funiculus) [Figure 3a and b]. It was clearly observed that this funiculus was formed from only the outer envelope, as seen in Figure 3c. Brooding eggs, which are undergoing hatching process, are identified through the appearance of a characteristic emerging hatchling side [Figure 3d and e].

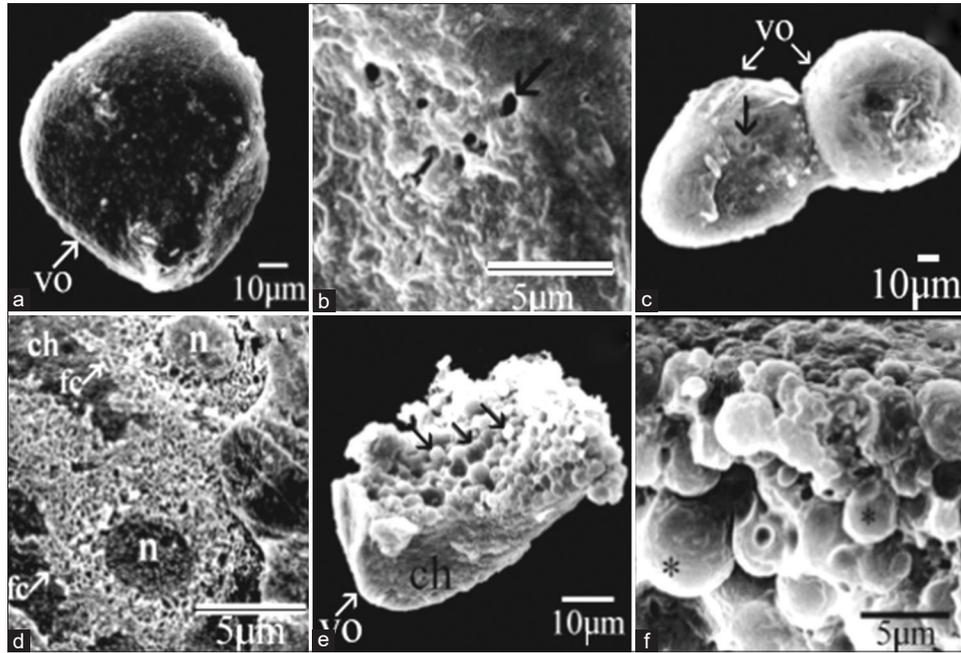
## DISCUSSION

In the present study, the vitellogenic ovarian oocytes of *C. hellerii* are highly condensed with yolk and are surrounded externally by a comparatively thick chorion. This was also coinciding with the observations made by Mazzei *et al.*<sup>[35]</sup> who mentioned that before fertilization, the egg of *Armadillidium vulgare* was surrounded by only one envelope (chorion).

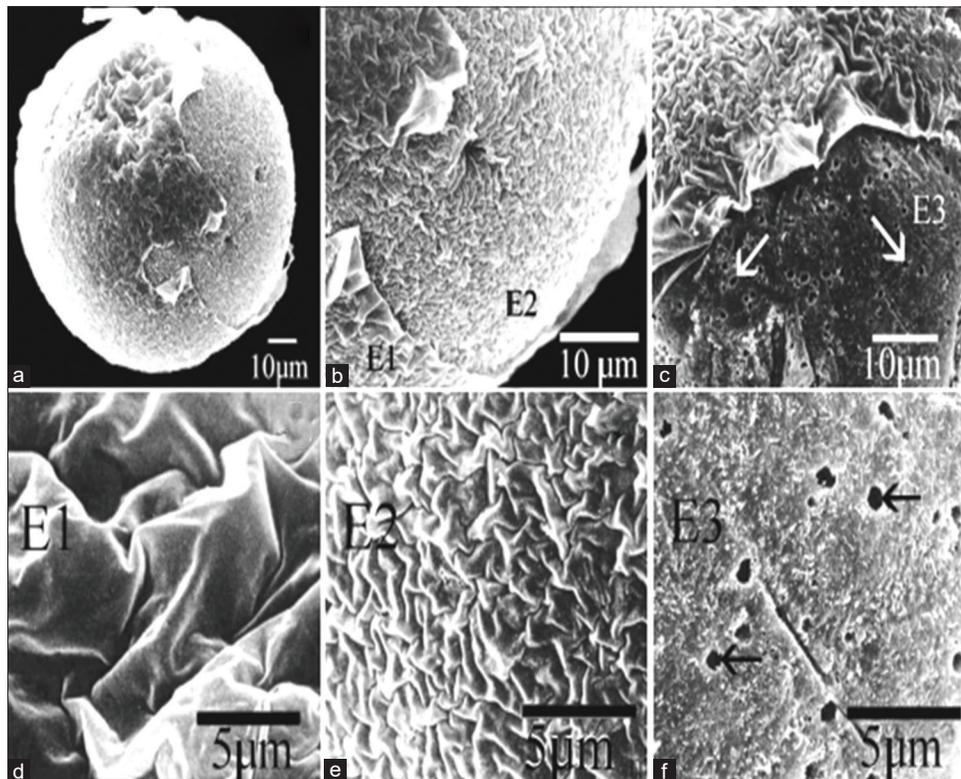
The spongy appearance of chorionic surface of *C. hellerii* vitellogenic oocytes was owing to the presence of numerous pores. In *Portunus pelagicus*, El-Sherief<sup>[12]</sup> observed fine microvilli projected into and through the chorionic pores. However, these fine microvilli were not observed in the present SEM investigation. In *Homarus americanus*, Talbot<sup>[36]</sup> observed numerous channels in the chorion of its oocytes. In the same lobster, Schade and Shivers<sup>[37]</sup> mentioned that the most apparent fine structural feature of vitellogenic ovarian oocytes is the presence of a large number of coated pits. They assume that these pits are involved in the pinocytotic activity of the oocytes. The transmission electron microscopy-based findings of Molleberg *et al.*<sup>[38]</sup> in *Mithracidae* species from three different genera such as *Mithrax hispidus*, *Mithrax tortugae*, *Mithraculus forceps*, and *Omalacantha bicornuta* confirm the presence of coated vesicles on the oolemma and many cytoplasmic endocytic vesicles. Such vesicles are responsible for the extracellular uptake of different compounds, a characteristic of the exogenous vitellogenesis. Similarly, the cortical cytoplasm of previtellogenic and vitellogenic oocytes of *Astacus leptodactylus* were characterized by the presence of coated vesicles.<sup>[39]</sup> The authors suggested that before the onset of vitellogenesis, follicle cells can deliver other substances into oocytes by receptor-mediated endocytosis.<sup>[39]</sup>

In the present investigation, the follicle cells were found to be associated with vitellogenic oocytes and connected with each other by means of thin lateral projections. These follicle cells resemble to those described in other decapods species such as the lobster *H. americanus*,<sup>[36,37]</sup> and marbled crayfish.<sup>[40]</sup> The investment of follicle cells around oocytes termed as: "folliculogenesis" is a prerequisite for heterosynthesis as reported in various crustaceans like *Fenneropenaeus indicus*<sup>[41]</sup> and *P. pelagicus*.<sup>[42]</sup>

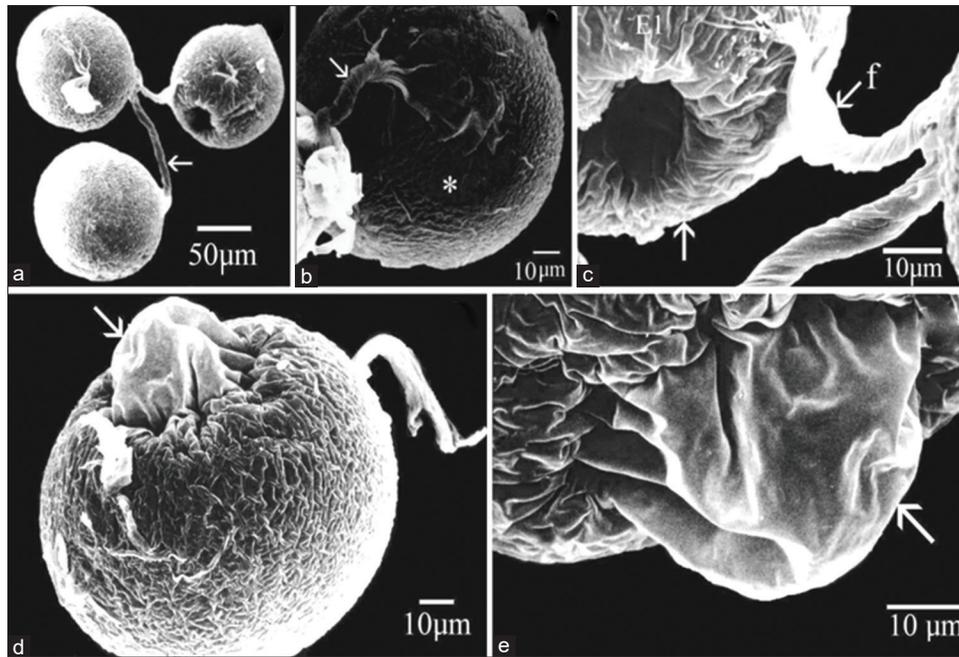
In most decapod crustaceans, fertilized eggs were extruded from the gonopore and attached to the ovigerous hairs within



**Figure 1:** Vitellogenic oocyte of *Charybdis hellerii* taken from ripe ovary. (a) The outer surface of the vitellogenic oocyte; (b) magnified part of previous vitellogenic oocyte surface, showing the choric surface of vitellogenic oocyte and choric pores (arrow); (c) the outer surface of vitellogenic oocyte and the associated follicle cells (arrow); (d) enlarged micrograph showing the connections between the adjacent follicle cells; (e) a part of vitellogenic oocyte showing ooplasm packed with various yolk inclusions (arrows) and the surface of chorion; (f) magnified part of ooplasm of vitellogenic oocyte showing various sizes of yolk inclusions (asterisks) embedded in ooplasm. ch: Chorion, fc: Follicle cells, n: Nucleus, vo: Vitellogenic oocytes



**Figure 2:** Spawned egg of *Charybdis hellerii*. (a) outer surface ornamentation of spawned egg; (b) the outer envelope (E1) and mid envelope (E2) of spawned egg; (c) the inner envelope (E3) of spawned egg, which is characterized by its spongy porous appearance white arrow (d) magnified part of outer envelope (E1) of spawned egg; (e) magnified part of mid envelope (E2) of spawned egg; (f) magnified part of inner envelope (E3) of spawned egg showing numerous pores on its surface (arrows)



**Figure 3:** Brooding eggs of *Charybdis hellerii*. (a) Connection of three brooding eggs through funiculus (arrow). (b) Brooding egg (asterisk), showing highly twisted funiculus (arrow); (c) magnified part of brooding egg, which originated from outer membrane (E1); (d) The brooded egg at the beginning of hatching process. Note: emerging hatchling (arrow); (e) magnified part of the previous brooded egg showing emerging hatchling region (arrow). f: Funiculus

the incubation chamber of the female. The attachment is through a stalk called the funiculus.<sup>[7,19,24,25,43-45]</sup> In the present study, the funiculus function is not only to attach the incubated eggs to the abdominal appendages but also to bind the brooded fertilized eggs to each other. Egg-to-egg adherence phenomenon was observed in other decapod crustaceans such as lobsters in the genus *Homarus*<sup>[25,47]</sup> and shrimps in genera *Palaemonetes*<sup>[48]</sup> and *Palaemon macrodactylus*.<sup>[7]</sup> In *Sesarma haematocheir*, however, egg-to-egg attachment was rarely observed as mentioned by Saigusa *et al.*<sup>[19]</sup>

The origin of a funiculus has been discussed in a number of studies. In some decapods, the funiculus formation has no contribution from the egg layer. In the shore crab, *Carcinus maenas* and in the estuarine crab, *S. haematocheir*, the funiculus is formed within the abdominal cavity with the aid of long setae.<sup>[7,19]</sup> In the narrow-clawed crayfish *A. leptodactylus* the funiculus is simply formed by deposition of the substance excreted by pleopodal glands.<sup>[22]</sup> Similarly, In *Aegla platensis*, the funiculus is formed by addition of an adhesive substance to pleopodal setae.<sup>[46]</sup> In *Austropotamobius pallipes*, the funiculus has a dual origin long setae and egg outer layer.<sup>[49]</sup> In the present investigation, it was clearly observed that the funiculus is derived from the outermost envelope of the egg. This result coincides with an observation made by Yonge,<sup>[25]</sup> in *Homarus vulgaris*. Conversely, in the newly laid egg of *C. maenas*, the highly stretched funiculus consists of two superimposed vitelline envelopes.<sup>[7]</sup>

SEM study revealed that the spawned eggs of *C. hellerii* are enfolded by three distinct envelopes outer, middle, and inner

envelopes. The egg envelopes are elaborated by follicle cells and laid down in a well-defined sequence.<sup>[50]</sup> These envelopes are extracellular structures that surround the egg cell and the embryo after fertilization. Their basic function is to protect the embryo from potentially harmful aspects of the external environment.<sup>[22]</sup> A survey on the morphology and the structure of crustaceans' egg envelopes showed that they are greatly varies among the different species. The eggs of the copepod *Calanus sinicus* had revealed a complex five-layered structure, which seemed to be originated from the egg. The second to the fifth layers were newly formed after spawning, while the first layer, which might be a vitelline envelope, separated from the cell membrane just after spawning.<sup>[16]</sup> Hinsch and Cone<sup>[51]</sup> reported that the mature oocyte of *Libinia emarginata* has an egg membrane with two distinct layers, as observed by electron microscope. In the swimming crab, *Portunus trituberculatus*, two membranes formed after fertilization and the outer membrane, which played an important role in attachment to the pleopods.<sup>[52]</sup> Minagawa *et al.*<sup>[53]</sup> mentioned the presence of the two-layer egg membrane at the prematuration stage in the crab *Ranina ranina*. In the portunid crab *Portunus sanguinolentus*, the newly spawned eggs were spherical and surrounded by two transparent membranes, an inner and outer membrane.<sup>[54]</sup> Saigusa *et al.*<sup>[19]</sup> determined three envelopes enfolding the egg of the estuarine crab *S. haematocheir* by the use of transmission electron microscope. He added that the outermost layer consists of two further sublayers. In the crayfish *A. leptodactylus*, the egg case is made of three layers; the external, the middle, and the inner layers. All layers are composed of an electron-dense material; the most distinct ultrastructural feature is the presence of polyhedral grains, which

are interspersed with multiple aeropylar areas and channels in the middle layer. In addition, the second (middle) layer consists of two sublayers of equal thickness but with variable grain sizes and aeropyle structures. The third and innermost layer is made of tightly packed grains, smaller than those in the middle layer. It includes a few, small aeropylar channels.<sup>[22]</sup> In *Oratosquilla massavensis*, spawned eggs were covered by the characteristic chorion with three different regions: the marginal, central, and frontal regions. The most conspicuous characteristics are the appearance of irregularly shaped projections and dark spots. In the central region, the author suggested that these spots appeared to be a secretion released from conspicuous openings between the projections.<sup>[24]</sup>

In the present investigation, the spawned eggs of *C. hellerii* differ from those before spawning in their outer ornamentation. The important difference is the presence of wrinkles in the outer and middle envelopes. These wrinkles or folds certainly may increase the surface area of the attaching membrane and may assist this outer covering as a protective and large coat for the time when the egg grows up during embryogenesis. Different egg ornamentation was also observed in other crustacean species such as anostracans of the genus *Chirocephalus*,<sup>[29]</sup> anostracans of the genus *Branchinecta*,<sup>[31,32,55,56]</sup> in *P. pelagicus*,<sup>[12]</sup> *A. leptodactylus*,<sup>[22]</sup> and *O. massavensis*.<sup>[24]</sup>

From the present investigation, it was corroborated that hatching process of brooding eggs was identified through the appearance of a characteristic emerging hatchling region. This in agreement with Vogt *et al.*<sup>[40]</sup> who found similar structure during the hatching process of marbled crayfish brooding eggs.

In summary, the vitellogenic oocytes of *C. hellerii* are surrounded by one layer “the chorion,” while the newly spawned eggs, which are hanged on the female pleopods, are enfolded by three distinct layers. It seemed that the nature of ornamentation of *C. hellerii* spawned egg membranes may have a protective role in keeping them as safe as possible and add more protection against stressful conditions in the aquatic environment. The information regarding the egg morphology of *C. hellerii*, is needed to indicate phylogenetic relationships among the crab taxa.

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### Conflicts of interest

There are no conflicts of interest.

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