Review Article

# Embryonic nutrition and yolk utilization in the sand crab *Emerita asiatica*

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## Summary

This paper reviews information available on the composition of yolk and the pattern of yolk utilization during embryogenesis in an intertidal crab, *Emerita asiatica,* from the east coast of India. *Emerita* yolk primarily exists as a glycolipoprotein complex, conjugated with carotenoid pigment. In addition, free lipid globules and glycogen droplets are also stored in the ooplasm. Another feature of interest is the conjugation of several steroidal hormones such as ecdysteroids, estrogen and progesterone with the yolk molecules. The stage-specific release of a host of hydrolytic enzymes splits up the complex yolk molecules which not only serve as raw organic substrates for tissue build up, but also function as regulatory factors during embryogenesis. For example, the regulated release of active ecdysteroids from the conjugates at specific time during embryogenesis not only triggers embryonic cuticle formation but also accomplishes larval molting and egg hatching. In conclusion, the study on the *Emerita* yolk utilization provides information on energy release for embryogenesis as well as abbreviated larval development that occurs within the brooding egg in Crustacea.

**Key words:** Crustacea, *Emerita*, embryonic ecdysteroids, larval storage proteins, yolk utilization

## Introduction

Sexually reproducing animals are categorized into two groups, namely oviparous and viviparous, depending on the type of embryonic nutrition. In oviparous animals, embryonic development occurs entirely within the confines of the laid eggs, utilizing the stored yolk. Consequently, the macro- and micronutrients provided by the egg contents must guarantee the production of viable offspring. Hence, many of the oviparous animals accumulate enormous amounts of nutritive materials within the egg during oogenesis.

Decapod crustaceans lay a large number of yolky eggs. Crustacean vitellin, comprising the major yolk ingredient of the egg, is a high-density lipoprotein and is referred to as lipovitellin (Wallace et al., 1976). It differs from that of vertebrates in that it lacks protein phosphate, but has a higher content of lipids and carbohydrates. Although various aspects of yolk protein synthesis and deposition during egg maturation have been investigated in detail (Wilder et al., 2002), embryonic utilization of the crustacean yolk protein, including breakdown of the vitellin molecule, has not received adequate attention.

In crustaceans, embryonic development is generally abbreviated and the egg hatches into a primitive nauplius larva. However, in most marine decapods such as crabs, embryogenesis is epimorphic in that the naupliar stage is superseded by a period of extensive embryonic morphogenesis, widely known as a 'metanauplius' stage. Nevertheless, the developmental phenotype exhibited at hatching varies widely among crustacean species (Goudeau et al., 1990). Such abbreviated larval development, accompanied by embryonic molting within the egg, is evidenced by the occurrence of exuvial layers, subjacent to hatching envelop, at the time of hatching. For example, in the crab, *Carcinus maenas*, progression from the nauplius to metanauplius is recognized by an additional egg envelop. The post-metanaupliar embryonic development is associated with the formation of a loosely fitting cuticle, which is rapidly shed just after hatching (the prezoea), and a cuticle belonging to the first free-swimming zoea stage (Goudeau and Lachaise, 1980; Goudeau and Becker, 1982). To meet these extensive embryonic development as well as regulating the process of embryonic molting and hatching of the larvae, crustaceans have to accumulate both nutritive and

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morphogenesis-regulative hormonal molecules at the time of vitellogenesis. This paper reviews information on yolk protein biochemistry and the mechanism of yolk utilization in respect of embryonic nutrition in a sand crab *Emerita asiatica*. Special focus is provided on the regulation of embryonic molting as well as endogenous larval development by hormonal factors accumulated within the egg. *Emerita asiatica* is a typical sand-burrowing anomuran crab exhibiting both repetitive and continuous breeding in the east coast of peninsular India. It lays large numbers of yolky eggs which, after release from the oviduct, are attached onto the pleopodal hairs. The continuous availability of berried eggs in different stages of embryonic development makes this marine crab most suitable to study yolk utilization during embryogenesis (Subramoniam, 1979).

## *Emerita* yolk protein

The yolk materials in *Emerita* eggs are deposited mainly in the form of yolk proteins, called lipovitellins, which eventually form the source of nutrients for developing embryos. Crustacean lipovitellins are, in general, high-density lipoproteins with carbohydrate as the major covalently linked prosthetic group. As in many other decapod crustaceans, a special feature of *Emerita* lipovitellins is their conjugation to a variety of carotenoid pigments, imparting a bright colour to the eggs (Kour and Subramoniam, 1992). Astaxanthin is the most commonly identified carotenoid pigment in *Emerita* eggs in combination with the lipovitellin giving it a golden yellow color. Unlike the other carotenoid-protein complexes, in which the carotenoid components are directly linked to protein chains through amino groups, in the eggs of *Emerita*, they are dissolved non-stoichiometrically in the lipid prosthetic groups of the lipovitellins (Zagalsky et al., 1987). Additionally, carotenoids can also be esterified to the fatty acids of the lipovitellin molecules.

In *E. asiatica*, the yolk protein is mainly comprised of two lipovitellins, LvI and LvII, constituting as much as 90% of the total egg proteins. In SDS-PAGE analysis, LvI yielded two subunits with molecular weights of 109,000 and 105,000 Daltons; whereas, LvII resolves into six subunits with molecular weights of 65,000, 54,000, 50,000, 47,000, 44,000, and 42,000 Daltons (Tirumalai and Subramoniam, 1992). *Emerita* embryos also contain hemocyanin (Gunamalai, 2002), providing a source of protein and copper during yolk utilization. The carbohydrate component of the yolk exists in three forms, free carbohydrate and protein- and lipid-bound carbohydrates. The protein-bound carbohydrates are dominated by hexose, hexosamine and galactosamine. Further, LvII contains a higher amount of *N*-linked oligosaccharides than *O*-linked oligosaccharides. Sialic acid is specifically absent (Tirumalai and Subramoniam, 2000). In addition, three neutral glycolipids, monoglycosylceramide, diglycosylceramide and triglycosylceramide, are also present in the major yolk protein, LVII.

In agreement with the defining characteristic of marine eggs, *Emerita* eggs accumulate high levels of lipids, constituting the main source of metabolic energy during embryonic development. According to Tirumalai and Subramoniam (1992) the lipid content of major lipoprotein (LvII) is 30%. The purified LvII contains neutral lipids, glycolipids and phospholipids among which phospholipids constitute the dominant lipid class with phosphatidyl choline and phosphatidyl serine as the major species. In addition to the lipid bound to the yolk protein, free lipids are also distributed in the ooplasm of the eggs as droplets.

Yolk proteins of mole crabs also contain significant quantities of metal ions such as copper, iron, sodium, calcium and phosphorus. These ions constitute as much as 3.5% of the purified major yolk protein. Whereas calcium and copper are bound to lipid in LvII, phosphorus and sodium are both lipid- and protein-bound (Subramoniam and Gunamalai, 2003). The metalloprotein nature of *Emerita* lipovitellin signifies its metabolic role during embryogenesis. Vertebrate yolk protein is high in phosphorus content by virtue of phosphorylation of polyserine residues in the vitellogenin molecule, giving rise to a distinctive phosvitin subunit, consisting of polyserine domines (Wahli, 1988). Polyserine domines are lacking in crustacean vitellins, but the presence of a meager amount of protein-bound phosphorus in crustacean lipovitellin may result from the *O*-linked glycosylation of serine moieties prior to phosphorylation (Tirumalai and Subramoniam, 2000; Dhadialla and Raikhel, 1990).

Recent studies have indicated that *Emerita* yolk protein contains several conjugates of steroidal hormones (for review, see Subramoniam, 2000), suggesting a morphogenetic role for them during embryogenesis. Subramoniam et al. (1999) reported maternally-derived free and conjugated forms of ecdysteroids in the purified lipovitellin fraction. In addition, significant quantities of vertebrate type steroids, estrogen and progesterone, are conjugated to yolk proteins of *E. asiatica,* (Warrier et al., 2001).

Table 1. Levels of estradiol 17- Beta and progesterone in different embryonic stages of eggs of E. asiatica (Modified from Warrier et al. 2001)

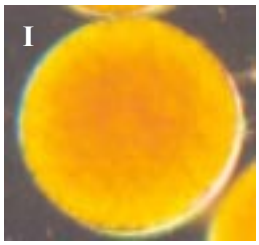
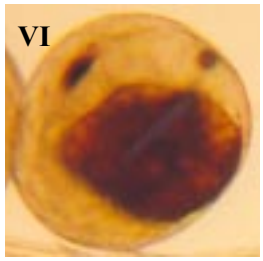
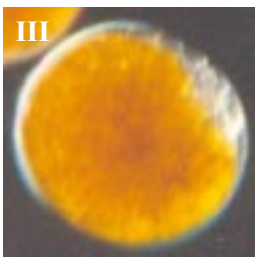
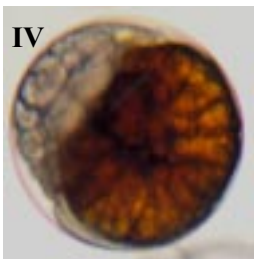
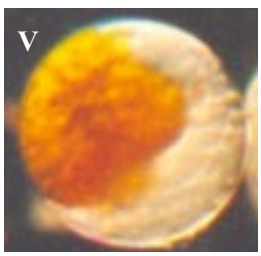
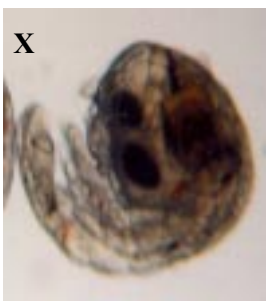
| Stage | Duration (days) at 28°c | Description | Stage | Duration (days) at 28°c | Description |
|---|---|---|---|---|---|
|  I | 5 | Freshly laid egg with dense yolk granules; egg mass bright orange in color |  VI | 2 | Two-thirds of the yolk is cleared; yolk is found in the vegetable pole; eyes are well developed |
| II | 5 | Cleavage has taken place and blastomeres are seen; egg mass bright organge in color. |  VII | 1 | Yolk is found a single cluster in center; append-ages are in developing stage |
|  III | 1 | A yolk free while streak (marked as arrow) makes its appearance at the animal pole |  VIII | 1 | Yolk is found as two clusters in the center well developed appendages and eyes pots; heart beat seen. |
|  IV | 1 | One quarter of the yolk cleared; embryo in the late gastrulation stage; | IX | 1 | Embryo is fully formed; egg mass white in color; no yolk globule seen; colorless yolk in the form of oil globules seen just below the eyes; about to hatch |
|  V | 2 | One third of the yolk is cleared: beginning of organogenesis |  X | 1 | Hatched out larvae with little yolk |

Table 2. Major organic constituents of eggs during different stages of development in *Emerita asiatica* after correction for water content (expressed as mg/10 mg of dry tissue, mean value only). (Data from Subromoniam, 1991)

| **Biochemical constituents** | **Embryonic stages** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **I** | **II** | **III** | **IV** | **V** | **VI** | **VII** | **VIII** | **IX** |
| Protein | 3.219 | 2.224 | 2.433 | 2.727 | 2.484 | 2.357 | 1.692 | 1.272 | 0.889 |
| Free carbohydrates | 0.293 | 0.489 | 0.718 | 0.969 | 1.161 | 1.714 | 2.000 | 3.090 | 4.000 |
| Glycogen | 0.012 | 0.012 | 0.031 | 0.051 | 0.036 | 0.093 | 0.089 | 0.089 | 0.115 |
| Protein bound sugars | 0.178 | 0.173 | 0.119 | 0.139 | 0.066 | 0.121 | 0.100 | 0.114 | 0.117 |
| Lipid | 4.609 | 3.837 | 4.795 | 5.606 | 5.806 | 2.286 | 2.000 | 2.318 | 2.722 |

Table 3. Carotenoid content in different egg development stages of *Emerita asiatica (mg/g wet weight).* (Data from Kour and Subramoniam, 1992)

| **Carotenoid Species** | **Stages** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **I** | **III** | **V** | **VII** | **VIII** | **IX** | **X** |
| a - carotene | 0.853±0.56 | 0.921±0.189 | 1.490±0.26 | 0.833±0.013 | 0.960±0.012 | 0.031±0.002 | - |
| b-carotene | 15.560±0.122 | 16.072±0.141 | 15.445±0.087 | 14.320±0.097 | 12.220±0.034 | 7.220±0.034 | 3.700±0.069 |
| Lutein | 2.080±0.067 | - | - | - | - | - | - |
| Echinenone | 0.846±0.031 | 1.960±0.036 | 3.540±0.036 | - | - | - | - |
| Isozeaxanthin | 4.373±0.068 | 1.500±0.019 | 3.540±0.039 | 3.380±0.048 | 0.031±0.010 | - | - |
| Zeaxanthin | 4.034±0.045 | - | 4.510±0.058 | 4.093±0.248 | - | 5.971±0.372 | - |
| Canthaxanthin | - | - | - | 2.972±0.323 | 5.806±0.528 | 4.613±0.264 | 2.606±0.264 |
| a- doradexanthin | - | - | - | - | - | - | 0.666±0.117 |
| Isocryptoxanthin | 6.712±0.198 | 5.100±0.197 | 3.910±0.153 | 3.630±0.161 | 2.540±0.236 | 2.136±0.142 | 2.104±0.173 |
| Free astaxanthin | 0.600±0.022 | 0.216±0.016 | 0.686±0.034 | 0.608±0.016 | 1.192±0.055 | 2.440±0.100 | 0.848±0.044 |
| Esterified astaxanthin | - | - | - | - | - | - | 4.280±0.018 |

## Embryonic development of *E. asiatica*

Different stages of embryogenesis in the sand crab could be observed after rearing the berried females in the laboratory. The table 1 summarizes the developmental stages in the embryonic development of *E. asiatica*, as observed in the laboratory-reared females. Yolk utilization, as revealed by the yolk clearance in the embryo, starts from stage III onwards and, organogenesis could be observed from stage IV onwards. Interestingly, the color of the egg mass changes gradually from bright yellow / orange to dirty brown and finally to white at the time of larval release. Obviously, this color change is due to metabolic conversions in the carotenoid pigments during embryogenesis.

## Biochemical changes during yolk utilization

The enormous amounts of organic and inorganic constituents accumulated in the lipovitellin as well as free lipid droplets and glycogen granules constitute the exclusive source of nutrition for embryogenesis and early larval development in *E. asiatica*. Biochemical analysis of the freshly-laid eggs showed that the major nutrients were lipid (4.6 mg / 10mg dry weight), followed by protein (3.2 mg / 10mg dry weight) and carbohydrates (0.48 mg / 10mg dry weight). Changes in these biochemical constituents of the embryo in different developmental stages are summarized on a dry weight basis in the table 2. It could be seen from the table that protein value steadily declined from stage I to IX, corresponding to increase in water content of the growing embryo. On the contrary, lipid content remained almost unaltered up to stage V; thereupon, the value fell precipitously, reaching the minimum of 27% (on dry weight basis) in stage IX. Compared to lipid and protein, the total carbohydrate was low initially, but different carbohydrate components exhibited an interesting pattern of fluctuation during embryonic development. For example, the total free carbohydrates, on a dry weight basis, were 2.9% in stage I, but increased to a value as high as 4% in stage IX. The free glycogen content also exhibited a similar increase during embryonic development. Conversely, the protein bound polysaccharides decreased from a high value of 0.178 mg / 10 mg of dry weight in stage I to 0.117 mg / 10 mg of dry weight in stage IX. During the entire embryonic development, there was steady increase in the water content from an initial value of 59% in stage I to 82% in stage IX (Subramoniam, 1991).

## Carotenoid metabolism during embryogenesis

Table 3 shows the variation in the occurrence of different carotenoids in the embryonic stages of *E. asiatica*. By far, the most abundant form of carotenoid deposited in the developing eggs is Beta-carotene, with its concentration varying between 15.4 mg. $g^{-1}$ wet weight and 16.1 mg. $g^{-1}$ wet weights in the early stages of embryogenesis. After maintaining almost the same level up to stage V, Beta - carotene started declining gradually to reach a low level of 3.7 mg $m^{-1}$ wet weight in the newly hatched out larvae. Beta- carotene also showed a declining trend during embryogenesis. Obviously, these two parent carotenoids of dietary origin undergo bioconversion into more oxidized forms such as hydroxyl and ketocarotenoids. The appearance of several of the intermediary compounds such as lutein, alpha-doradexanthin involved in the bioconversion of Beta -carotene into astaxanthin, has been recorded during *Emerita* embryo development (Kour and Subramoniam,1992). The stage-specific enzyme action that catalyzes the oxidative reaction of Beta -carotene metabolism during embryogenesis of *E. asiatica* provides a good model system to investigate the differential gene action during crustacean development. Apparently, astaxanthin is the final product of Beta-carotene metabolism. Esterification of astaxanthin towards the last stage of embryonic development is associated with the origin of chromatophores and the possible biosynthesis of visual pigments (Kour and Subramoniam,1992). The zoeal larvae of *E. asiatica* lead a long planktonic life before metamorphosing into megalopa which settles down on the beach (Israel et al., 2006). The presence of a large quantity of pigments in the larvae could subserve the functions of photoprotection and light shields as suggested for other crustaceans such as *Daphnia magna* (Herring, 1968).

## Breakdown of lipovitellin

The majority of the yolk being represented as complex glycol-lipo-caroteno-protein, the yolk utilization in *Emerita* eggs entails mainly the breakdown of this complex molecule by enzymatic digestion. In the electrophoretic separation (native PAGE) of stage I eggs, the lipovitellin resolved into a thin proximal fraction, LvI, and a thick fraction, LvII, with a slightly higher mobility in the slow-moving region of the electropherogram. Both the fractions were of glycolipoproteinaceous nature, as revealed from their intense staining with Amido Black, Oil Red O, and PAS. During the progression of embryogenesis, there was natural cleaving of the lipovitellin fractions with the loss of stainability of the prosthetic groups such as lipids and carbohydrates. LvI cleaved apart into two fractions in stage VII, before totally disappearing in stage VIII / IX. Similarly, LvII also cleaved into three fast migrating fractions. From stage VII onwards, the staining intensities of LvII and its subunits declined and they

disappeared in stage VIII / IX, suggesting that the lipovitellin molecules would be dismantled in the midway of embryo development thereby loosing their lipid and carbohydrate prosthetic groups (Subramoniam, 1991). The breakdown products of vitellin subunits of *E. asiatica* during progressive embryogenesis are similar to the subunits of hemolymph vitellogenins separated on SDS-PAGE. An important consequence of lipovitellin breakdown was the release of ecdysteroid conjugates, which are then cleaved by esterase to form hormonally active ecdysteroids. It is of interest to note that the bound vertebrate steroids are also released during the mid-stage of embryonic development (Warrier et al., 2001).

## Enzyme activity during yolk utilization

At the commencement of embryonic development, the egg should possess a host of hydrolytic enzymes not only to dismantle the vitellin complex but also to release the constituent substrates in an utilizable form. Figures 1 and 2 show the stage-specific enzyme activity of esterases, proteases and glycosidases during embryogenesis in *E. asiatica*. Lipid being the major organic reserve of *E. asiatica*, non-specific esterase activity is intense during embryonic development (Subramoniam, 1991). However, its activity appears only from stage IV onwards, reaching the peak in stage V and declines thereafter. The zymogram pattern of esterase as revealed by slab gel electrophoresis showed five fractions (Fig. 3). However, no fraction could be observed in embryos up to stage III. In stage IV, the zymogram consisted of a major fraction (E1), a moderately staining E2 fraction and two other thin fractions (E3 and E4). In stages V and VI, E2 fraction decreased in intensity, but yet another fraction, E5, appeared in the fast moving zone in stage VI. The E5 persisted up to stage VII, but was absent in subsequent stages. In general, the E1 fraction did not change in intensity throughout development, but others decreased in intensity and disappeared in the last stages of development. In the freshwater prawn, *Macrobrachium borellii*, Heras et al. (2000) also found high esterase activity during mid-embryonic stage (V). This enzyme activity was correlated with the highest activities of lipid synthesis or hydrolysis at the time when the embryos were under active organogenesis in *M. borellii* (Gonzalez-Baro et al., 2000). Furthermore, all the esterase enzyme fractions of *E. asiatica* are isozymes of carboxylesterase, as they were inhibited by silver nitrate and malathione and unaffected by pCMP, EDTA and eserine sulphate (Subramoniam, 1991). An interesting observation is that the thick fraction, E1, appeared to overlap the main lipovitellin in its relative mobility. Similar observation was reported in the crab *Scylla serrata* in which E1 fraction of the ripe ovary showed similarity in its relative mobility with lipovitellin (Ezhilarasi and Subramoniam, 1985). Interestingly, this esterase fraction was found to be synthesized in the hepatopancreas and then released into the hemolymph. Obviously, this esterase could bind to vitellogenin and then get incorporated into the oocyte along with the yolk precursor protein. E1 may be kept in an inactive condition in the ovary and then activated during embryogenesis. In a parallel instance, a cathepsin-B-like thiol protease is secreted by the fat body as a latent proenzyme and then accumulated by developing oocytes,

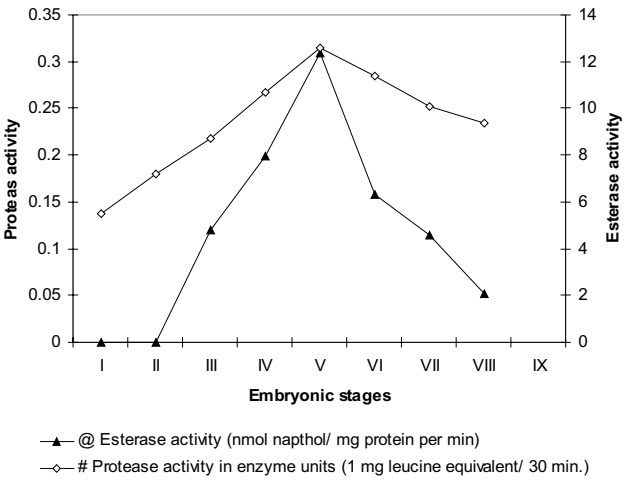



**Fig. 1.** Fluctuation of enzyme activity during embryonic development in the crab Emerita asiatica. Protease and esterase activity. (Based on data from Subramoniam, 2000)

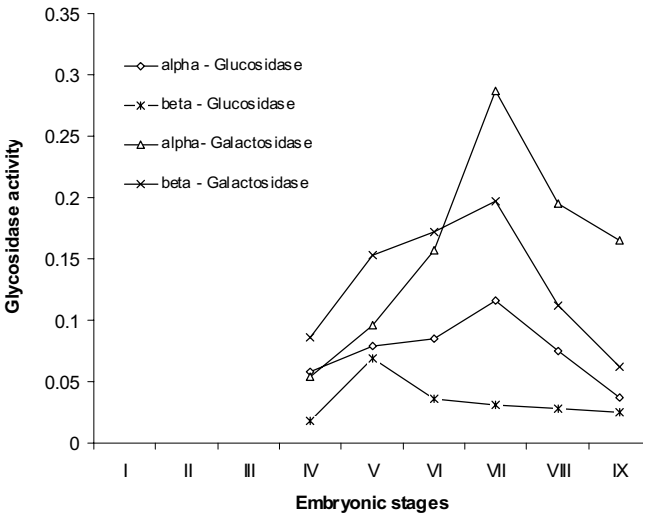



**Fig. 2.** Fluctuation of enzyme activity during embryonic development in the crab Emerita asiatica. Glycosidase activity (iM p-nitrophenol released/10mg embryo). (Based on data from Subramoniam, 2000).

where it is stored in yolk bodies of the mosquito *Aedes aegypti* (Cho et al., 1999). This inactive protease undergoes further processing at the onset of embryogenesis to an active state, and is involved in embryonic degradation of vitellin. Suggestively, there is a programmed activation process to release the protease for degradation of specific yolk protein during embryogenesis to meet the metabolic demands in organogenesis.

Conversely, all other isozymes (E2-E5) of *E. asiatica* might be synthesized by the embryonic cells, with stage-specific appearance or disappearance, indicating changes in cellular differentiation and metabolic pathways. Another enzyme active at the time of lipovitellin degradation (stages V and VI) in *E. asiatica* is phospholipase C (Ramachandran, 1992). Esterase activity, in addition to releasing the free fatty acids from conjugated lipids, may also release carotenoids that are esterified to long chain fatty acids of lipovitellin. Further more, esterase activity during embryogenesis of *E. asiatica* resulted in the release of vitellin-bound ecdysteroids into their free forms, such as 20-hydroxyecdysone and ecdysone (Subramoniam et al.,1999).

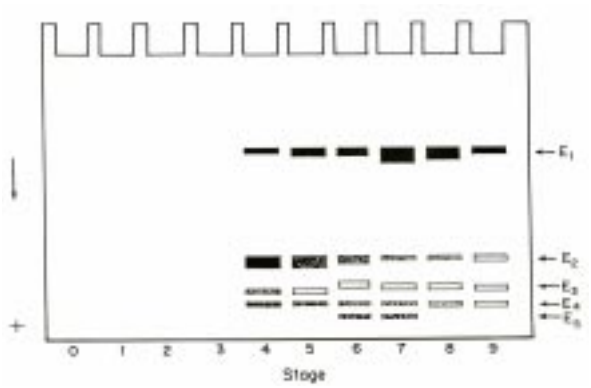



**Fig. 3.** Zymogram of esterases from the ovary (=O) and different embryonic developmental stages (1-9) in Emerita asiatica. E1-E5 represent different isozymes.
(Redrawn from Subramoniam, 1991).

Contrary to esterase activity, protease activity during embryonic development in *E. asiatica* commenced quite early and increased during mid-stage with only a slight reduction in activity registered during the end stages, suggesting protein degradation throughout embryogenesis. Interestingly, protease activity also peaked during stage V of embryonic development, concomitant with maximum yolk degradation and active organogenesis (Fig. 1). Protease activity also corresponded to the protein substrate utilization which was continuous throughout embryogenesis. Biochemical characterization of the protease indicated that it is of serine-protease nature in *E. asiatica*.

In the Crustacea, the yolk precursor protein, after its endocytotic uptake into the oocytes, undergoes proteolytic cleavage and is deposited as yolk granules (Warrier and Subramoniam, 2003; Okuno et al., 2002). Yolk granules, which are subcellular structures, specific of developmental systems, play a major role in embryo development. Embryonic utilization of yolk protein contained in the yolk granules has been well studied in Artemia salina. In this brine shrimp, the yolk granules found in the oocytes and early embryonic cells undergo membrane rupture when the dormant dry cysts are hydrated and development resumes. This allows the hydrolytic enzymes to degrade the lipovitellin components of the yolk into simpler units. The proteases that metabolize yolk into amino acids and usable energy include a neutral cytosolic protease and another protease of lysosomal location (Ezquieta and Vallejo, 1985). The cytosolic proteases were localized inside the yolk granules as trypsin-like proteinase, associated to lipovitellin. These storage yolk granule-proteases are kept in an inactive condition until hydration, when the release of these enzymes into the cytosol occurs. This protease undergoes further processing in order to reduce its molecular weight and becomes activated to digest the vitellin molecules. The programmed events of deinhibition of proteases, and their activation due to molecular processing coincide with the cellular differentiation that restarts after hydration of the *Artemia* cysts. In the insects, yolk granules contain several proteases (cysteine, aspartyl and serine) that degrade the corresponding proteins. They invariably exist as inactive proproteases and the mechanism controlling protease activation involves yolk granule acidification, a process common to vertebrate embryogenesis also (Liu et al., 1996).

In accordance with the relatively high carbohydrate content of the crustacean lipovitellin, *Emerita* eggs also store different carbohydrases. Gunamalai (1993) investigated the existence of two forms of alpha –galactosidases and three forms of Beta-galactosidases as well as alpha - and Beta-glucosidases in the developing embryos of *E. asiatica* and found their activity peaking in stage VII / VIII of embryonic development (Fig. 2). These glycosidases may be required to release the bound glucose and galactose from the glycolipid and oligosaccharide components of the lipovitellins and to hydrolyse stored glycogen during embryogenesis of *E. asiatica* (Tirumalai and Subramoniam, 2000). A consequent accumulation of free sugars such as glucose

towards the final stages of embryogenesis may be required for the synthesis of chitin precursor molecules for the embryonic cuticle synthesis. Interestingly, the activity of all the three enzymes is correlated with breakdown of the complex yolk proteins into simpler utilizable subunits and the onset of organogenesis.

## Ecdysteroids and embryonic molting

An important consequence of lipovitellin degradation during embryogenesis of *E. asiatica* is the release of maternally derived conjugated hormones such as ecdysteroids and vertebrate-type steroids such as estradiol and progesterone. Among them, ecdysteroids assume important functions as morphogenetic hormones partaking in the control of embryogenesis and early development. Subramoniam et al. (1999) reported the occurrence of a complex mixture of free and conjugated ecdysteroids in the developing embryos of *E. asiatica* (Fig. 4). These hormone complexes exhibited multiphasic fluctuation during the course of embryonic development. The first rise in free active ecdysteroids was noticed in stage III, when the blastoderm extends. The next increase in free ecdysteroids was seen in stage VI, when the embryonic eyes are fully developed and the first limb buds become apparent. The sharp rise of total free ecdysteroids towards the pre-hatching period may be correlated with the deposition of embryonic cuticle of the proto- and prezoea larvae. Ecdysteroid fluctuations within the embryos of *E. asiatica* were common to both free and conjugated forms, reflecting interconversions between them. However, the concentration of free ecdysteroids always predominated over the conjugated ones in all the developmental stages.

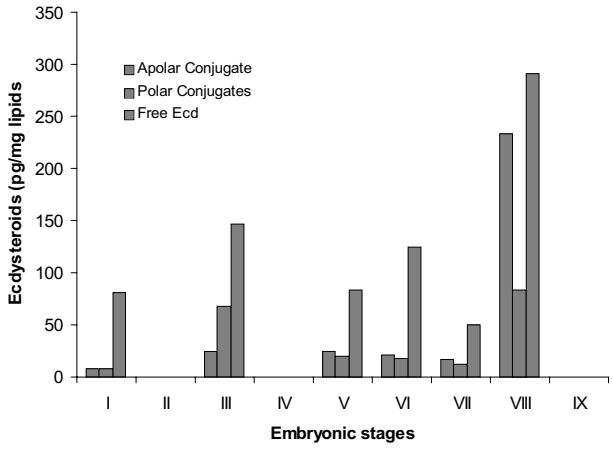



Fig. 4. Separate determination of titers of conjugated and free ecdysteroids during embryonic development of *E. asiatica.* (From Subramoniam et al., 1999)

The consistent rise in free ecdysteroids throughout embryogenesis with a peak at pre-hatching stages may also suggest fresh synthesis of these hormones by the newly formed embryonic Y organ. In fact, in a penaeid shrimp, *Sicyonia ingentis*, the bulk of embryonic ecdysteroids is derived from the embryonic Y organ (Chang et al., 1992). In this penaeoidean shrimp, the freshly spawned eggs contain very little ecdysteroids, suggesting meager maternal accumulation of these hormones. Nevertheless, the secretion of larval envelops as well as the pre-hatching molt occur when the embryonic ecdysteroids reach the peak level. In another work on the freshwater shrimp *Palaemon serratus*, Spindler et al. (1987) correlated the stages in embryonic development, including the formation of Y organ, with the level of embryonic free ecdysteroids. Le Roux (1983) also showed the appearance of Y-organ during the period just prior to eye development in the same shrimp. Thus, the Y organ in *Emerita* embryos as well could be functional during stage VI, when the eyes are fully formed. Hence, the highest amount of ecdysteroid accumulation in the pre-hatching stage could be due to combined contribution from maternally derived as well as *de novo* synthesized ecdysteroids by the developing Y- organ.

Morphogenetic role of edysteroids during larval development and embryogenesis is well understood in insects. In *Blaberus craniifer,* an orthopteran insect, in which the entire embryonic development takes place inside the incubating pouch, three ecdysteroid peaks have been observed during embryo development. The first peak coincides with the end of metamerization, the second peak with the secretion of the first cuticle and the third with the second cuticle which is the larval cuticle when the egg hatches, and the last peak also corresponds to the hatching period (Bulliere et al., 1979). Goudeau et al. (1990) have elegantly detailed the secretion of as many as six embryonic envelops in the European lobster before hatching and correlated with ecdysteroid peaks appearing in each layer formation. Such a correlation between cuticulogenesis and ecdysteroid peaks has been observed in many decapod crustaceans including *E. asiatica*. However, in other crustaceans, in addition to 20-hydroxy ecdysone (20E), ponasterone A (PoA) is also reported to be involved in embryonic molting. Whereas in the penaeid shrimp *Sycionia ingentis,* and the anomuran crab *E. asiatica* 20E is the principal hormone that controls both adult and embryonic molting, in the shore crab *Carcinus maenas,* PoA is the chief ecdysteroid to control embryonic molting (Lachaise and Hoffmann, 1982). Yet, in the European lobster *Homarus gammarus*, both 20E and PoA appear to exert a sequential but independent control on embryonic

envelop secretion; 20E predominating at the time of secretion of envelop 6 and PoA is the major ecdysteroid to control secretion of envelop 3 (Goudeau et al., 1990).

Another feature of interest is the mode of accumulation of ovarian ecdysteroids in *E. asiatica*. Radioimmunoassay of 20E of ovary and hemolymph during different molt stages revealed gradual sequestration of ecdysteroids during the entire intermolt period (Gunamalai et al., 2004). This is followed by a drastic decline during the premolt stage, corresponding to a steep rise in the hemolymph ecdysteroids level. This decline may be due to the release of ecdysteroids from the ovary back into the hemolymph, causing its rise in the circulating ecdysteroids. Alternatively, the reduction in the ovarian ecdysteroids may be caused by their esterification with long- chain fatty acids of the lipovitellin, giving rise to the storage forms of conjugated ecdysteroids. Understandably, excessive ecdysteroids released into the hemolymph may be stored inside the ovary to function as morphogenetic hormone during embryogenesis. Yet another function attributed to ovarian ecdysteroids both in insects and crustaceans is their non-genomic action on the induction of resumption of meiosis during oocyte maturation (Lanot and Cledon, 1989).

Apart from being used as recyclable conjugates, catabolism of ecdysteroids would result in other products such as 20, 26-dihydroxyecdysone (McCarthy and Skinner, 1979) and ecdysonic acids (Lachaise and Lafont, 1984), both forming the inactive end products for elimination through storage excretion.

## Neuropeptide regulation of embryonic molting

Molting in adult crustaceans is negatively controlled by the eyestalk neuropeptide molt-inhibiting hormone (MIH), which represses ecdysteroid synthesis by Y-organ (Chang, 1989). In addition, crustacean hyperglycemic hormone (CHH) has also been shown to influence ecdysteroid synthesis as well as induce water uptake during molting (Chung et al., 1999). These authors further indicated that crustacean cardioactive peptide (CCAP) secreted by the pericardium is also involved in the control of stereotyped ecdysis behavior. In a recent study on embryonic molting in *Carcinus maenas*, Chung and Webster (2004) studied the expression pattern of MIH, CHH and CCAP during embryonic molting and found them similar to that of adult molt cycle. These authors also observed the expression of CHH in extra-eyestalk sources (abdominal peripheral neurons) before hatching, an event analogous to eclosion in insects. Similarly, there is a gradual increase in X-organ derived CHH levels in late embryos, culminating with a dramatic increase prior to eclosion, and an impressive decline within an hour of this event, which corresponds with shedding of the prezoeal cuticle. Evidently, this prehatching surge of CHHs is related to larval eclosion and water uptake necessary for egg shell rupture and hatching. In addition, two crustacean pigmentary effector hormones, red pigment-concentrating hormone and pigment dispersing hormone, with neuromodulating functions, have also been identified in the eye stalk neurons during later part of the embryo development in *C. maenas* (Chung and Webster, 2004).

## Vertebrate steroids

Recent studies have revealed the occurrence of vertebrate steroid hormones such as estradiol 17 Beta ($E_2$) and progesterone (PG) in several decapod crustaceans, including *E. asiatica*, and their fluctuation in the hemolymph during ovarian cycle has been correlated with control of vitellogenesis (Subramoniam, 2000; Gunamalai et al., 2006). Other studies have also indicated their accumulation in the ovary during vitellogenesis (Wilder et al., 2002). Warrier et al. (2001) found that these hormones were conjugated to lipovitellin in the oocytes of *E. asiatica* and *Scylla serrata*. It was also shown that the profiles of both $E_2$ and progesterone followed a pattern in the *Emerita* eggs, from stage I to IX of embryonic development, with a peak in stage V (Fig. 5). Stage V of *E. asiatica* embryogenesis represents breakdown of complex yolk protein which would release not only the constituent

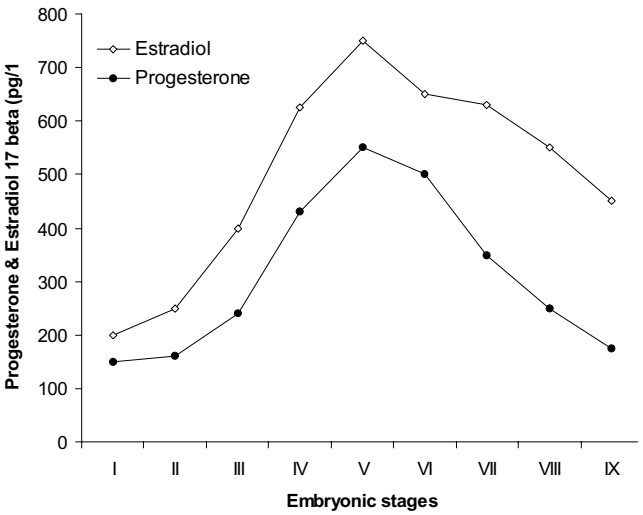



Fig. 5. Levels of estradiol 17-b and progesterone in different embryonic stages of eggs of E. asiatica (Modified from Warrier et al., 2001)

prosthetic groups but also the conjugated hormones. Thus, the upsurge of the two steroid hormones is due to release of the protein-bound (by protease action) and conjugated (by esterase action) steroids into the general pool of free steroids. The subsequent decline in the hormone levels would suggest their role in the organogenesis, as morphogenesis controlling factors.

## Vitellin-like storage protein and larval development

Most crustacean species are not only oviparous but release different kinds of larvae. These larval forms, in general, lead planktotrophic life before metamorphosing into juveniles. *E. asiatica* releases planktotrophic zoea with a long pelagic life. The zoea larva, after several molts, metamorphoses into the crab-like megalopa, which settles onto the sandy beach (Israel et al., 2006). However, the zoea larva contains colorless yolk globules at the time of release. These lipid reserves contribute to buoyancy of the pelagic larvae on their release and provide instant nutrition for larval growth. Remnants of egg yolk vitellin have been reported in other crustaceans such as *Artemia salina* (de Chaffoy de Courcelles and Kondo, 1980). Ituarte *et al*. (2005) observed retention of yellowish transparent yolk droplets in the hatched out zoea larvae of the palaemonid shrimp, *Palaemonetes argentinus*, and their utilization for molting into the second stage zoea, when reared in complete absence of food.

In addition to the maternally derived egg yolk protein, a special class of yolk protein has also been reported to be produced by the cypris larva of Balanus amphitrite (Shimitzu et al., 1996). In this barnacle, remnants of egg yolk protein are found in the released larvae, but they disappear completely in stage I nauplius. Barnacle life cycle consists of 6 feeding naupliar stages, followed by metamorphosis to a non-feeding cypris stage. Interestingly, nauplius larvae start synthesizing a larval storage protein which accumulates maximally in the non-feeding cypris stage. This protein is termed as Cypris Major Protein (CMP). The CMP is related to the heavy chain of barnacle yolk protein both structurally and immunologically. Further, this protein is comparable to the storage protein of insect larvae inasmuch as both the proteins are used as sources of energy and organic substrates in the transformation of larva into adult. The CMP's similarity with yolk protein is inferred from the synthesis of both yolk protein and CMP under induction by estradiol -17 Beta (Billinghurst et al., 2000). Evidently, larval storage protein of crustaceans could have evolved from vitellogenin to subserve yet another nutritive function during larval development.

Another glycoprotein called Settlement Inducing Protein Complex (SIPC), found in juveniles and cyprid larvae of the barnacle *Balanus amphitrite,* has also been found to be expressed in the ovary and egg mass, with immunological and peptide sequence similarity with cirripede yolk proteins (Dreanno et al., 2006). Evidently, cirripede larval storage protein and the SIPC might have shared a common ancestor with yolk protein. Alternatively, crustacean yolk protein genes would have undergone duplication to give rise to different proteins necessary for larval metamorphosis and the gregarious larval settlement in these sessile barnacles.

## Conclusions

During the recent past, significant progress has been made in the understanding of cellular and biochemical aspects of yolk accumulation in crustacean oocytes. However, most of the studies on crustacean yolk utilization are made with regard to energy transformation during embryogenesis and the ecophysiology of the organism (Pandian, 1970). Understandably, yolk utilization is the central event of embryogenesis inasmuch as stored yolk components supply both energy as well as organic building blocks for embryonic growth. To meet such a demand, oviparous animals have contrived a mechanism to store all organic and inorganic nutrients necessary for the independent embryonic development in a single protein molecule called vitellin. Although this yolk protein evolved among animal species primarily to serve as a source of nutrients, it has undergone considerable changes in its amino acid sequence to perform diverse roles. As an example, the evolution of polyserine segment (phosvitin) in vitellin molecule of vertebrates is related to bone formation in vertebrate embryos, thus paralleling the important evolution of internal skeleton in all true vertebrates (Byrne et al., 1989).

The main feature in the evolution of crustacean yolk protein is its ability to bind lipids and steroidal hormones. Recent structural and immunological studies on crustacean vitellogenin (the precursor molecule of vitellin) have unraveled homology with lipid binding proteins of disparate functions, with only limited sequence homology with yolk proteins of other animal groups (Subramoniam, 2002). Warrier and Subramoniam (2003) found a strong immunological relationship between the crab vitellogenin / vitellin and the mammalian atherogenic lipoproteins as revealed from their immunoreactivity with antibodies of human LDL, VLDL, and apoB. This structural trait of crustacean vitellogenin is consistent with the fact that the yolk protein contains as much as 30%

lipids and, hence, lipid transport to the growing oocytes via vitellogenin is considerable. Furthermore, the crab vitellogenin receptor also showed considerable cross-reactivity with mammalian LDL receptor, suggesting a co-evolution along the lines of ligand and receptor (Warrier and Subramoniam, 2002). The biological similarities of crustacean vitellins and atherogenic lipoproteins such as LDL, VLDL, and apoB would suggest the invertebrate origins of the vertebrate lipid transporting system. An additional feature in the evolutionary relationship of crustacean yolk protein is evidenced in the occurrence of yolk protein-like larval storage proteins and settlement inducing protein complex in cirripedes. Understandably, the conservative yolk protein gene of crustaceans has given rise to other genes coding for larval nutritive proteins.

Biochemical analysis of the yolk components revealed extensive reshuffling of substrates, especially during the early stages of embryonic development, suggesting changes in the metabolic pathways involving interconversion of stored substrates. The initial high content of lipid in *Emerita* egg is characteristic of lecithotrophic eggs. However, protein utilization during embryogenesis takes precedence over lipid in *E. asiatica*. This condition is similar to bony fishes, in which protein is preferentially used during the entire course of embryogenesis (Lasker, 1962). On the contrary, the eggs of the freshwater crab *Paratelphusa hydrodromous* expend enormous reserves of lipid continuously during embryogenesis with a concomitant increase in the protein level (Pillai and Subramoniam, 1985). Conceivably, embryonic development of *E. asiatica* represents a condition intermediate between cleidoic and non-cleidoic developmental extremes.

Analysis of enzyme activity during embryogenesis is also in agreement with the pattern of substrate utilization. Particularly, esterase activity during the progression of embryonic development is related to the timing of the release of hormones bound to lipovitellin molecules. Embryonic ecdysteroids, both free and conjugated, play pivotal role in controlling morphogenetic events such as secretion of several embryonic cuticles and their subsequent molting. In this respect, crustacean embryogenesis draws parallel with that of insects, which utilizes maternally derived ecdysteroids to control embryonic molting and possibly eclosion. In contrast to insects, crustaceans complete several larval molts before egg hatching, with more than one ecdysteroid species, partaking in the control of different embryonic molting stages (see above).

Conspicuous increase in the titer of free ecdysteroids during eye-forming embryonic stage of *E. asiatica* and other decapods may imply a role in neurogenesis, as suggested for the insects (Kirschenbaum et al., 1995). In insects, ecdysteroids also play important role in the remodeling of muscles during larval metamorphosis (Hegstrom et al., 1998). Enormous increase in active ecdysteorids during the prehatching stage of several decapod crustaceans could also mean a role in muscle remodification inasmuch as several larval stages are condensed and completed before hatching. A recent finding that ecdysteroid receptors are expressed in the developing embryos of the crab *Metapograpsus messor* (G. Anil Kumar, personal communication), also supports the developmental role of ecdysteroids during crustacean embryogenesis. Interestingly, the functional ecdysteroid receptor dimerizes with another nuclear receptor ultraspiracle (usp) in insects, whereas in crustacean ovary and embryos, EcR dimerizes with retinoid X receptor (Chung et al., 1998; Durica et al. 2002).

Unlike insects, there is a continual synthesis and secretion of ecdysteroids from the X-organ in the adult crustaceans and the ovary sequesters them for use during embryogenesis. Interestingly, crustacean ovary constitutes an important site of ecdysteroid metabolism by converting them to active 20E, using 20-hydroxylase enzyme, as reported in the crab *Cancer antennarius* (Spaziani et al., 1999). In addition, the ovulated eggs also contain a host of enzymes to convert the active ecdysteroids into inactive polar and apolar conjugates.

In contrast to insect embryogenesis, embryonic molting in crustaceans also involves the molt inhibitory neuropeptides, as in adult molt. Unlike the maternally derived ecdysteroids, these neuropeptides could be synthesized *de novo* by the embryonic eyestalk neurosecretory centers. Understandably, the epimeric embryogenesis found in several of the decapod crustaceans calls for additional regulatory molecules to coordinate both inhibitory and stimulatory factors to achieve successful embryonic molt cycles. An interesting speculation would be a role for vertebrate steroids that accumulate in large quantities in the embryos of decapod crustaceans.

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