Cleavage, blastula and gastrulation mechanism in embryonic development of giant fresh water prawn, *Macrobrachium rosenbergii* (De Man, 1879)

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Abstract
Sequential changes in the development of egg in the freshwater prawn, *Macrobrachium rosenbergii* was defined in the present study. In females, the ovaries are located dorsal to the stomach and hepatopancreas in the carapace cavity. When the female prawn is in ripe condition the deep orange or yellow colored ovaries are visible through the carapace extending from just behind the eyes to the first abdominal segment. An oviduct arises laterally from each ovary just anterior to the heart and extends ventro-laterally to the gonopore on the coxae of the third periopod. After mating, the eggs were deposited, or oviposited, on setae of the pleopods of the female. The fertilized eggs were opaque, greenish, round or oval in shape. The diameter of the egg was approximately 0.58 x 0.48 mm. As the development progresses, the yellow color changed into light orange, orange, brownish-orange and finally to dark brown or black in color about to hatch. The incubation periods varied from 12-18 days. Twelve stages were observed during embryonic development and tissue differentiation before the emergence of the first zoea larva in the *M. rosenbergii*. The process of embryonic development includes nuclear division, cleavage, blastula formation, segmentation, eye pigment development and larva formation. Embryonic development follows the normal blastula and gastrula stages, ending with the closing of the blastopore.

Keywords: *Macrobrachium rosenbergii*, embryonic development, Hatching, Blastula, Gastrula

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Introduction:
The fresh water shrimp; *Macrobrachium rosenbergii* belongs to the Phylum, *Arthropoda*; Class, *Crustacea*; Subclass, *Malacostraca*; Series, *Eumalacostraca*; Order, *Decapoda*; Suborder, *Natantia*; Section, *Caridea*; Family, *Palaemonida*; Genus, *Macrobrachium*; Species, *M. rosenbergii* De Man [1]. It can also be found in low salinity brackish water [2]. The body is divided into three main divisions: the head, thorax and abdomen. The head and thorax are joined to form a cephalothorax, which carries the mandibles, flagella, rostrum and the eyes containing a stalk and has five pairs of walking legs. The abdomen has six body segments with the last segment bearing a uropod or telson. The other five segments bear swimming apparatus known as swimmerets. A definite feature of *Macrobrachium* is that the second walking legs are modified to form the chelae. Most species are distinctively colored having either blue or brownish colors. The legs also have definitive features such as hairs or furs. Significant differences exist between the male and female. Mature males are considerably larger than females and the second walking leg is much thicker. The cephalothorax is also proportionally larger in the male than female while abdomen is narrower in the female. The genital pores of the male are between the bases of the fifth walking leg [2]. The female’s genital pores situates at the base of the third walking legs. The pleura of the abdomen are lower and broader in the female than in the male. The pleura of the female form a brood chamber in which the eggs are carried between laying and hatching. A ripe ovigerous female can easily be identified because the ovaries can be seen as large orange-colored mass occupying a large portion of the cephalothorax. Crustacean embryonic and larval systems offer a unique and valuable tool for furthering our understanding of both developmental processes and physiological regulatory mechanisms. Palaemonid females carry centrocelethial eggs in an external brood pouch during the development time [1]. This peculiarity of the palaemonids allows a systematic tracking of the embryonic development. Muller described the embryogenesis in *M. carcinus* and *M. acanthurus* [2, 3]. Since there is limited study on the embryonic development of *M. rosenbergii*, the present study was carried out to know the information on the embryonic development of *M. rosenbergii*.

Materials and Methods:

Study area: The present work was carried out in Kakati...
Aquatech (PVT) Ltd, Vijayawada, Andhra Pradesh, India. The berried females were collected from Bhimavaram and transported to hatchery site under oxygenated condition. Each berried female was kept in individual packet and all are stocked in 500 L brooders holding tanks and acclimatized to hatchery conditions (Fig. 1). Optimum temperature (28-30°C) and dissolved oxygen (5 ppm) was maintained in the brooders tank. The berried females were fed with crumbled sized rice and wheat twice in a day. Every day the excess feed, excreta and shed out eggs were siphoned out. The development of the eggs was closely observed every day. Daily color changes of the eggs during incubation period could be noted. Eggs were sampled aseptically by gently removing a bunch of eggs from the brood pouch using sterilized forceps in random locations and separated with the help of a needle and forceps without damaging the eggs. After each sampling, brooders were given a 1-min prophylactic fungus dip treatment in Treflan (1 ppm) before being returned to incubation tanks. All the developing embryos were examined with a light microscope to ensure that only viable embryos were sampled and the color change corresponding to the development and length of the incubation period was noted. The time course of embryonic development, as indicated by the appearance of specific morphological features recorded from spawning time onwards. This includes from fertilization to hatching of first zoea. The gradual changes in the embryonic development and increase in the size of the eggs were recorded to understand the different developmental stages.

**Fig 1: Brood stock holding**

Once the first stage zoea inside the egg was fully developed, the larva was ready to come out of the eggshell to start active life. The process of hatching was studied through hand lens and compound microscope with the developing embryo removed from the brood sac. This slow process was accompanied by continuous vibration at the mouth of the larva, and stretching of its rolled body, forcing the egg shell to elongate gradually. Vibration at the mouth became more and more vigorous followed by further stretching of the body. About an hour later the thoracic appendages started to vibrate vigorously but intermittently for about a few minutes with increasing length of pereiopods vibration. This became very vigorous and continuous. The body continued to stretch the rostrum and telson, which was held like a mask covering and protecting the eyes and head, which started pushing outward. Suddenly the eggshell break and the telson thrashed out followed by the head, and with a forceful flex and stretch of the body the hatched zoea larvae started swimming actively in the water column.

**Result:**

**Spermatophore transfer and fertilization:** During mating, the male placed its spermatophore on the thorax of a mature female, near the opening of the gonopores. As eggs were extruded from the oviduct, they passed across the spermatophore and were fertilized externally. Eggs were deposited, or oviposited, on setae of the pleopods of the female. The newly oviposited eggs were containing all the necessary material for synthetic processes associated with embryogenesis and morphogenesis and all the compounds required for oxidative metabolism and energy production. The eggs contain nutritive reserves in the form of proteinaceous yolk and lipid vesicles scattered throughout the cytoplasm. The fertilized eggs were opaque, greenish, round or oval in shape (Fig. 4).

**Fig 2: Colors of eggs**

**Fig 3: Developed eggs (ready to hatch)**

**Cleavages, blastula and gastrula formation:** At the time of spawning, the eggs of *M.rosenbergii* are elliptical and 0.58 x 0.48 mm in size and they are opaque and pale orange. At water temperature of 28.0 ± 0.4°C, within 5-6 minutes of fertilization the 1st polar body is discharged and the fertilization membrane expands. The 2nd polar body is then discharged. However, the eggs of *M.rosenbergii* next undergo nuclear division twice and attain a 4-celled stage. In this stage cleavage can becomes visible. Until then the cytoplasmic division cannot be clearly seen under the microscope. Beyond the 4-celled stage, nuclear division can be readily observed along with cytoplasmic division under the microscope. At a water temperature of 27-28°C, they attain the morula stage in about 20 hours after fertilization. Thereafter development passes through the blastula stage, the gastrula stage, and closing of the blastopore begins about 54 hours later, and then begins the determination of various rudiments and subsequent differentiation. After 80 hours, the embryo appears. After 170 hours heart beats are discernible and after 230 hours the compound eyes develop. After 320 hours the embryo assumes the shape of a Zoea, from this time the egg axis increases and after 430 hours has attained maximum length. Then the embryo is now 0.85 x 0.54 mm and ready to hatch. Usually, the eggs were homolecital type with yolk filled in completely and distributed uniformly. Even though various physical, chemical and biological parameters played a vital role, temperature and salinity plays key role in the duration of embryonic development and hatching. Twelve stages were observed during embryonic development and tissue differentiation before the emergence of the first zoea larva in the *M.rosenbergii*.

**Stage-1:** The eggs were homolecital type. Color of the egg
mass was yellow orange red.

Stage-2: Cleavage begins at this stage. It was holoblastic type. Cleavage began initially at the periphery and extended gradually towards the centre.

Stage-3: A short peripheral gap was formed at one side of the ovum, below the perivitelline membrane as a “yolk free part”. This was the beginning for the formation of primordial membranes by consuming yolk. It was observed that the yolk free part occupied nearly 1/6th of the total egg space, and the color of the egg changed by the addition of yellow pigment.

Stage-4: The yolk free part becomes increased and it occupied nearly 1/4th of the total egg. Faint brownish and horizontal membrane folding was observed in the yolk free part, which were faintly visible. The egg mass at this stage was yellowish.

Stage-5: Imagination was formed in the yolk containing part at this stage. The yolk free part at this stage occupied about 1/3rd of the total egg space. This was the gastrulation stage of development.

Stage-6: Organogenesis started at this stage. Single eye pigment differentiated at one side of the developing embryo inside the egg, the pigment appeared in half-moon shape.

Stage-7: Both the eye pigments were differentiated in this stage and these appeared in half-moon shape one on either side. Yolk free part increased due to decreased content of yolk. The egg mass at this stage became yellowish brown in color. Brown pigment bands started in this stage.

Stage-8: Pigment bands of developing embryo clearly differentiated. The bands were thin and light brownish black in color and were distributed all over the developing embryo and yolk containing parts. These bands were not containing parts. These bands were not continuous but distributed unevenly. Heart beat first appeared in this stage, but it was not rhythmic. The beat rate ranged between 70-120/min to 90-130/min. The eye pigments became bold and they appeared dark brownish in colour. Each eye pigment was curved in shape and the convex surface faced towards the periphery.

Stage-9: The pigments bands became thick and dark blackish in colour. Eye became more are less oval in shape. Yolk reduced to isolated patches. The egg mass at this stage became dark brown in color.

Stage-10: Heart beat became rhythmic and beat a rate of 120-130 / min. except two pigment bands, which run parallel behind the heart, all other bands disappeared. These two parallel bands were parts of the future abdomen. A network of pigment bands were found at the end of this abdomen. A net work of pigment bands were found at the end of these parallel bands. The heart was situated dorsally in the developing embryo in between the two eyes. Square type cells (cone cells) surrounded each at this stage. The eyes were very big at this stage. Egg mass gradually turned to pale black in color.

Stage-11: Un-segmented abdomen was differentiated at this stage. The little yolk over the egg remained attached
underneath the cephalothorax region. Contractile movements were observed in the embryo at various regions of the differentiating organs. Egg mass became brownish black color.

**Stage-12:** The developing embryo transferred itself to the first larval stage- ‘Zoea’, which was ready to hatch out. Abdomen became segmented and vigorous muscular movements of the abdomen and cephalothorax observed inside the egg. Little yolk was still left over the cephalothorax region. As the development progresses the yellow color changed into light orange, brownish-orange and finally to dark brown color about to hatch (Fig.2 & 3). At this stage, the developing larvae were observed under microscope. During this period, there was considerable increase in size of the egg in long axis. Fecundity was ranged between 10, 221 and 31,854 (60-90 mm total length of females). The incubation periods varied from 11-17 days. The process of embryonic development includes nuclear division, cleavages, blastula formation, gastrula formation, segmentation, ey pigment development and larva formation (Fig 5-12).

**Discussion**

Mating of *M. rosenbergii* is almost identical in all species of the genus. Immature, however the shrimps molt at night when cultured for a long time in the laboratory, they sometimes molt during the day. Thus a difference in time was observed. Usually in the case of prawns and shrimps, the females always moult before mating. This is because preparation for storage of the spermatophore (Paenaeides) or incubation (*Macrobrachium spp.*) is required. Hence this molt is known as the pre spawning molt. In most cases, mating lasts for a few hours from the time the female has undergone pre spawning molt; the scampi have a soft exoskeleton and are not able to creep properly. When male and female are reared separately indoors, unless the moulted female is allowed to mate with in 15-16 hours of the pre spawning molt, the success rate of indoor experiments have shown that the success rate is higher between 6-12 hours of mating and almost completed after 20-24 hours. After completion of the pre spawning molt, the female mates and receives the spermatophore. Within a few hours spawning begins. Just before spawning she briskly cleans the breeding dress on the thoracic part of the body and along the margins of the lateral plates of the abdomen, as well as the breeding setae in the coxae of the 1st to 4th abdominal appendages. Cleansing is done with the 1st pair of thoracic legs. Sometimes she folds the abdominal appendages forwards and continues to swim. Once spawning has started, the body is held slightly raised by means of the thoracic legs and the caudal part is bent downward. All the abdominal appendages are folded forward. Of these, the 1st pair is folded in such a manner that they cover the genital (spawning) apertures located in the proximal parts of the 3rd thoracic legs. The discharged eggs pass through the spermatophore adhering to the 1st abdominal legs and thorax and reach the incubation chamber. The incubation chamber or the brood pouch is divided into proximal parts of the 5th abdominal appendages nor is there any breeding dress along the margins on the lateral plates of the abdomen. Therefore incubation does not occur in the 5th compartment, which serves merely as a vestibular chamber. The eggs migrating continuously become attached to the brood pouch in a sequential manner from compartment No.4 to No.1. During this process the female shakes her body right and left, every few seconds to make the eggs spread in a uniform manner. During this period, the embryo’s investment coats (egg coats, egg envelopes) protect it from physical and chemical stresses and maintain the internal milieu. The outer investment coat, due to its immediate exposure to the aquatic environment, is of primary importance in this role. The outer coat has also been associated with the attachment of eggs to the maternal pleopods, selective permeability [4] and osmotic hatching [5], and it may serve as a substratum for aquatic microorganisms [6]. In the present study also, *M. rosenbergii* newly extruded eggs contain egg coats which helped the eggs to attach each other and to the pleopods. This helps to protect the eggs from physical and chemical damages. During oviposition, the female stood upright and the eggs moved into a chamber formed by the pleopods and the lateral epimeras (pleura) on the underside of her abdomen. Eggs were attached to each other and to pleopodal setae by a connecting or adhesive material that formed the outer investment coat. This occurred for both fertilized and unfertilized eggs. At sites of attachment, the adhesive material took the shape of a flattened strand or a twisted stalk [7]. The externally brooding caridean *P. macrodactylus* exhibited a mechanism of egg attachment that differs from accounts of macruran and brachyuran egg attachment. A substance produced and stored in the female pleopods appeared to be released at moult to coat the external surfaces of the pleopods. Extruding eggs, fertilized or unfertilized, were connected to the pleopodal setae and to each other by the adhesive material, which simultaneously formed the outer investment coat of the eggs. This mechanism is unlike that suggested for *Homarus* [4] in that cement glands or ducts were not observed in *P. macrodactylus*, and secretion of adhesive material occurred before, rather than during, oviposition. It also differs from that proposed for *Carcinus* [8] in that fertilization was not necessary for attachment and the outer layer was formed by material secreted from the female pleopods, not from individual eggs. Attachment in *Palaemonetes*, described by Burkenroad [9] and Jefferies [10], was probably the same as that described here for *P. macrodactylus*, but the adhesive material escaped detection because of its close conformity with the external surfaces of the pleopods. In the present study also similar mechanism was observed. The number of eggs produced by crustaceans varies widely [11]. According to Parson and Tucker [12], fecundity can vary seasonally, annually and between areas. In several crustaceans, there is a linear relationship between the number of eggs per brood and the size of the females. This has also been observed in *M. lamarrei* [13], the fresh-water crayfishes *Astacus leptodactylus* [14] and *P. (Austrocambarus) ilamasi* [15]. According to Manush et al. [16], fecundity of *M. rosenbergii* varies from 40,000 to 60,000 eggs (body weight 100 g). In *P. trituberculatus*, emphasized that oocyte number increased with
increasing female’s body size and predicted estimates ranged between 0.8 and 4.5 million for the carapace width of 130-240 mm. Depending on the size of _M. idea_, it carries about 40-160 developing eggs [18]. In present study, _M. rosenbergii_ fecundity was ranged between 21,880 to 62,078 (65 and 90 mm total lengths). During the development in _M. rosenbergii_ the color of the eggs changed through yellow, orange, brownish-orange and dark brown in color. At the time of development, the color of the egg changed through brown to gray as the yolk is used up and the outline of the embryo becomes visible. The eyes and pigment spots appear first followed by the outlines of the abdomen and cephalothorax [19]. The color change was caused by absorption of the yellow yolk and development of dark pigment in the eyes [20, 21]. Extruded eggs of _Macrobrachium_ species are of two colors either green, like in _M. acanthurus_ [22] and _M. amazonicum_ [23] or orange in colour as in _M. heterochius_, _M. ohione_ [24], _M. rosenbergii_ [25] and _M. carcinus_ [26]. In _Macrobrachium_ spp, eggs with embryos turn either grey or dark brown prior to exclusion [27]. Whereas in _M. gangeticum_, the colour of eggs is green yellow and become grey corresponding to embryonic development [28]. In _M. lamarrei_ and _M. lamarrei lamarrei_, the eggs were green in colour [29, 30]. [31-33] classified the eggs into four different developmental stages. However, Ajith Kumar [34] classified eggs into 4 stages based on the color of the eggs in _M. rosenbergii_ and _M. idella idella_. Many workers have divided the crustacean egg stages based on the appearance of distinctive morphological features such as eye, heart beat and appendages formation. However, such morphological characters only begin to appear mid-way during embryonic development. [35] emphasized the importance of a detailed classification of early development of decapod crustaceans to understand the changes in the metabolic pathways involving inter conversion of already stored substrates within the closed system of egg development. The quantity and distribution of yolk in the eggs of different crustacean species is closely related to cleavage and embryonic development patterns [36, 37]. Holoblastic or total cleavage usually occurs in eggs containing a small amount of yolk (oligolecthal eggs), in which the establishment of morphological characteristics occurs relatively fast, resulting in the development of the typical free nauplius larvae with three pairs of appendages [7, 38]. This pattern is observed in most branchiopods and maxillopods, and in penaeids of the malacostraca [38]. Crustaceans with yolky eggs (centrolecithal eggs) present meroblastic or partial cleavage. The large amount of yolk triggers a delayed embryonic development that results in further structuring of the embryonized-nauplius (also called egg-nauplius), with the formation of paired appendages, growth of the caudal papilla and organization of appendages in the post-naupliar region [39,40]. This pattern is observed in most malacostracans in which the hatching form is the _zoea_ [36]. The embryonic development in _M. rosenbergii_ followed the general pattern of embryogenesis described for other species that have centrolecithal eggs, as _P. varians_ [41], _M. carcinus_ [42], _P. pugio_ [43] and _M. acanthanus_ [44]. The formation of the _zoea_ structures follows the organization of the basic body plan observed in the development of oligolecitic species eggs [45] where first larval phases correspond to the embryonized post-zoea in the meroblastic pattern [46]. The cleavage process observed in stage II indicates that development follows a holoblastic pattern, since cleavage furrows can be seen in the surface of the whole egg, individualizing the blastomeres. However, these cleavage furrows are shallow, and they do not reach the central yolk mass the subsequent organization of the germinal disc seen in stage III, followed by the organization of the embryonized _zoea_ and post-_zoea_, are typical of the meroblastic developmental pattern [47]. The recognition of both holoblastic and meroblastic developmental traits during the cleavage stage is common in most decapods species, due to the particular quantity and distribution of the yolk in the eggs [36]. The development of the _M. rosenbergii_ shows that the initial morphogenesis is quite intense until organization of the embryonized _zoea_. The _zoea_ could be visualized due to the large size of the egg, superficial position of the embryo and color contrast between embryonic cells and yolk mass. In the present study, the egg size of the _M. rosenbergii_ increased mainly in long axis during the embryonic development. These changes in egg size were also reported to most of the malacostracan species, as the brachyuran _Eriocheir japonicus_ [48] and the prawn, _M. offersi_ [49]. According to Odinetz-Collart and Rabelo [50] and Narciso and Morats [51], in crustaceans the egg diameter tends to increase until hatching. Churchill [52] specified that egg diameter was not related in any way to female size and also the egg diameters increased at a relatively steady pace throughout ontogeny. Under constant environmental conditions, the variability in egg size and biomass has been attributed to variation in female size or age [53, 54] and genetic factors [55]. The growth of egg size is associated, among other factors, to increase of water content in eggs, as the embryo develops [48]. The eggs of aquatic invertebrates range widely in size. Even within a single taxonomic group such as decapods or amphipods, egg size can vary enormously between species, and also within species. For example, echinoderm eggs vary in diameter from 50 to 1500 μm [56]. In general, of course, species with smaller eggs have higher fecundities than those with larger eggs, and the selective advantages of the different egg size have been discussed extensively in the literature of marine invertebrate reproduction [57-59]. The number of eggs containing embryos during development depends on the size of the mother shrimp, as is known for _M. lamarrei_ [13], _M. amazonicum_ [60], _M. idae_ [64] and _M. ohione_ [24]. Associated with these variations in egg size are differences in the time taken for the embryo to develop and hatch. This can vary from a few days in some tropical species to at least 18 months in some polar isopods [60-66]. In crustacean with yolky eggs, different developmental times are observed from spawning to hatching, such as 40 days for _Cherax destructor_ [69] and 180 days for _H. americanus_ [39]. The incubation of developing embryos by most female decapod crustacean may be one of the reasons for the success of this group. This ensures greater survival
against predators and other adverse environmental conditions. Their different incubation times are related to the endogenous factors of development and can also be influenced by exogenous factors like water temperature, as described by Celada et al. [69-71]. In P. sanguinolentus embryonic development last for 8-11 days [70]. In M. rosenbergii embryonic development last for about 18.5 days [65]. The incubation time is 12-15 days for M. malcolmsonii and comparatively less duration of 12-13 days for in M. gangeticum. Whereas in giant freshwater prawn M. rosenbergii, longer period for incubation and embryonic development was reported at 18-25 days [29]. However, in the present study the water temperature was controlled, suggesting that the endogenous factors, like egg size and the amount of yolk were decided to the variation of the development time. The embryonic developments of M. idella rosenbergii last for about 13-14 days. A number of species belonging to the genus Macrobrachium are known to migrate from fresh water to brackish water for breeding purposes [72]. Populations of M. idae inhabiting the rice fields along the west coast of south India are known to migrate into the backwaters during the breeding season. During the embryonic development, the eggs of M. idae increase their salt (ash) content from 4 to about 7% (dry body weight) by absorbing salts from the surrounding water. The gravid females of M. rosenbergii that were gradually exposed to salinities of 8 ppt during the last part of incubation had a higher number of larvae released. In the present study, the M. rosenbergii brooders were maintained at 6-8 ppt during incubation period [73]. The species of M. olfersti, P. pandaliformis and P. argentinus have similar sized eggs and have presented similar development times. On the other hand, the longest length of development was observed in M. potiuna, whose voluminous eggs allowed for a more prolonged embryogenesis, which results on a development of more complex structures [2, 63]. According to Jalihal et al. [74], the species of Macrobrachium, which have larger eggs, tend to show a smaller fecundity, fewer larval stages, and a reduction of the larval period. These features have also been observed in other palaemonids, such as M. nattereri [69], M. theringi [70], M. borelli [71] and M. jelkii [75-77]. A similarity of the length of the embryonic periods shows that a specific amount of time to the organization of embryonic features is necessary. The prezoea and zoea periods are faster due the organization of less complex embryonic structures. The post-zoea period is longer because the structures have to be finalized and to acquire functionality before hatching. In M. potiuna, the post-naupliar stage is even longer, because this species hatches as more complex larvae [42, 63]. In the present study also, prezoea and zoeal periods are faster than the post-zoeal period.

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