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Morphological and histological studies on the embryonic development of the freshwater prawn, *Macrobrachium rosenbergii* (Crustacea, Decapoda)

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Abstract The present work was carried out to describe the embryonic changes during development of the freshwater prawn *Macrobrachium rosenbergii* based on some morphological and histological features. In addition the biochemical composition of eggs was investigated during the embryonic development of the studied species. Results revealed that eggs of *M. rosenbergii* completed their development in 20 days at 28.5 ± 0.45 °C. The present investigation showed that primordial germ cells (PGCs) were detected early in the examined embryos. In 6.5 days old embryo, a cluster of PGCs occupied the dorso-medial region behind the yolky portion. In addition, the biochemical data indicated that the protein content was significantly increased, while lipid and carbohydrate contents decreased during the embryonic development. The lowest water content was found in the bright orange eggs and reached its highest level in the deep brown eggs. It was noted that the increase in the water content was correlated with the increase in the egg diameters. It was also concluded that, variations in the biochemical compositions of eggs reflected changes in their morphogenesis during the embryonic development.

Introduction

One of the reasons for the reproductive success of Crustacea is related to the fact that many of them carry their eggs until hatching resulting in a high survival rate for the eggs (Gieseand Pearse, 1974; Charniaux- Cotton et al., 1992). In Palamonids, the eggs are large and laid in bulk (Nazari et al., 2000), making it possible to follow the embryonic development through the transparent chorion (Sandeman and Sandeman, 1991; Odinetz-Collart and Rabelo, 1996). Several staging methods have employed to characterize the meroblastic developmental pattern and to recognize progressive morphological changes during embryonic development (Perkins, 1972; Helluy and Beltz, 1991; Nazari et al., 2000; Muller et al., 2003). The embryogenesis of yolky eggs enables all morphological changes to be followed daily by the examination of the gross morphology and histological sections. However, some methodologies also permit the evaluation of

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development by measurements of some externally observed embryonic structures such as the eyes (Beltz et al., 1992), relating the quantitative variables to the morphological descriptions. Yolky eggs of Palamonid prawns exhibit diverse incubation periods, as described for *Palaemonetes argentines*, 10 days (Nazari et al., 2000); *Palaemon pandaliformis*, 13 days (Muller et al., 1999); *Macrobrachium olfersi*, 14 days (Muller et al., 2003); *M. rosenbergii*, 16 days (Clarke et al., 1990); *M. potiuna*, 21 days (Muller et al., 2004). If compared with other decapods, the incubation period of Palamonids can be considered short, since in the lobster, *Homarus americanus*, it is 180 days (Helluy and Beltz, 1991); in the crab, *Erimacrus isenbckii*, 360 days (Nagao et al., 1999) and in the crayfish, *Virilastacus araucanius*, 120 days (Rudolph and Rojas, 2003).

M. rosenbergii (De Man, 1879) is a decapods crustacean of the family Palaemonidae, and it is distributed throughout the tropical and subtropical regions of the world. This species has been transferred from its natural location to many parts of the world initially for research purposes and ultimately for commercial production (Brown, 1991; Jay Chandran and Joseph, 1992). In Egypt, *M. rosenbergii* was imported from Hawi to Seuz Canal University, in 1988. It showed a great economic interest because the wide brackish water surfaces are available, the climate situation is very favorable, and the market prices are reasonable (Sadek and Moreau, 1996). Even though the general embryogenesis was well known, only a few works, on the embryonic morphology of species of *Marcobrachium* were available in any of those schemes of description.

Previous studies dealt with *M. rosembergii*, *M. olfersii*, *M. acanthurus* and *M. americanum* (Caceci et al., 1996; Muller et al., 2004, 2007; Garcia-Guerrero and Hendrickx, 2009), respectively. Other studies compared the embryogenesis of four Palaemoidae, *M. olfersii*, *M. potuina*, *P. pandaliformis* and *Palaemonetes argentinus* (Muller et al., 2004).

Females of decapods carry their eggs under the abdomen in a broad pouch until hatching. The eggs are rich in yolks which are used as embryonic development progresses. Protein is considered as one of the main components of yolk which plays an important role in both morphogenesis and energy supply to the developing embryos (Luo et al., 2004). Lipid content is relatively high in decapod eggs and is one of the main energy sources. Some studies have examined biochemical metabolism during the embryonic development of crustaceans (Petersen and Anger, 1997; Chen et al., 1998; Wehrtmann and Kattner, 1998; Gimenez and Anger, 2001; Yao et al., 2006).

Going through the literatures, little studies on the embryology of *M. rosenbergii* and information regarding changes in the biochemical composition of the eggs during the embryonic development of *M. rosenbergii*. Thus, this work was undertaken to describe the embryonic developmental stages of *M. rosenbergii* during cultivation in the laboratory. Also, the present study was designed to investigate the histological and biochemical composition of the eggs during embryonic development of the investigated species.

Materials and methods

Sampling

Adult prawns of *M. rosenbergii* were obtained from Mariout Fish Farming Company at El-Amreia, Alexandria) during May 2009. Prawns were transported in plastic bags supplied with oxygen, to the invertebrate laboratory at Fish Research Station belonging to the National Institute of Oceanography and Fisheries, at El-Qanater El-Khayria City, Egypt. They were placed in rounded fiber glass tanks (Diameter 1.25 m) filled with aerated tap water with a depth of one meter. Polyvinyl chloride tubes (10 cm diameter, 20 cm length) were placed as a shelter. Sex ratio 1:3 male: females. Prawns were fed every day with commercial shrimp pellet (35% crude protein, 8% lipid). Water parameters were measured weekly. Temperature by using a graduated thermometer, hydrogen ion concentration (pH) was measured by using pH meter (Digital Mini-pH Meter model 612JENCO). Dissolved oxygen (DO) by the digital Conductivity DO meter (HANNA HI 8043) and expressed as (mg/l).

The detected gravid females were separated and placed in 40 l plastic aquariums with water at 28 °C ± 0.5 and with continuous aeration. The berried females were separated and sampled. The first sample (three females) which corresponds to the newly eggs (orange eggs) was taken; eggs were separated by using a forceps and weighed by using electronic digital balance (ModelMR-220). A clutch of eggs were fixed in 10% formalin for observing the morphological features and for the histological studies. Another sample was preserved in a deep freezer (−25 °C) for biochemical analysis. Length and weight of used females were determined (Total length of females was measured from the rostrum to the end of the telson by using a Vernier caliper), (total length ranged from 8.5 to 13.5 cm, and weight ranged from 5.2 to 28.95 g). The same steps were applied on the other embryonic developmental stages (about three stages after orange color eggs, after about one week, the eggs take a deep yellow color, then changed into pale brown and finally deep brown as the embryo progress in age). Embryonic developmental stages were identified by examining them under a tri-occular florescent microscope (Olympus) by observing the appearance of new embryonic structures, according to the guidelines described by Muller et al. (2003). Photographs were taken with a digital camera attached to the microscope for each developmental stage. Also, some embryonic developmental stages were drawn using Meiopta microscope provided with Camera Lucida. The diameter of eggs (narrow and width diameters) was determined by using an objective micrometer.

Histological study

Eggs saved in 10% formalin were dehydrated in ascending series of ethyl alcohol, cleared in terpineol and embedded in paraffin wax. The blocks were sectioned serially at 4–6 μm. Sections were stained with three standard stains: (1) hematoxylin and eosin to study the gross qualitative changes of the egg; (2) sudan black to quantify lipid and (3) periodic acid-schiff stain (PAS) to quantify carbohydrates of eggs. Slides were examined by light microscope and images were captured using a digital image system (Olympus CX 31) connected to a computer.

Proximate composition

For the biochemical analysis of egg, samples of approximately 100 eggs from each female were taken at every stage of embryonic development and were homogenized in a saline solution

(1.2% NaCl). To quantify protein, the homogenate was first digested with 0.5 N NaOH. The concentration was determined using the Bradford (1976) method using albumin as standard and absorbance was read at 595 nm using Spectrophotometer (Spectronic 21D). The content of carbohydrates was determined by precipitated proteins with 20% trichloroacetic acid and centrifuged at 1200 for 10 min. Then, the carbohydrates were quantified from the supernatant using the Anthrone method (Van Handel, 1965), using glucose as standard, and absorbance was read at 620 nm using the same equipment. Total lipids were extracted according to Bligh and Dyer (1959), an aliquot of the homogenate was mixed with pure H_2SO_4 and incubated at 80 °C for 10 min. The acid solution obtained was mixed with the phosphovanillin reagent and the absorbance was recorded using a Biorad reader at 560 nm, using a mixture of TAGS (12 mg ml^{-1}) and cholesterol (8 mg ml^{-1}) as standard. Water content was determined by determining the weight of egg and by relating it to the wet weight of samples (Lafmm, 1979).

Statistical analysis

Only proximate composition of eggs was statistically analyzed using one-way ANOVA, using the software Statistic (Version 4.5). Duncan's comparison procedure was applied if ANOVA indicated significant differences ($P < 0.05$).

Results

Egg coloration and morphology during embryonic development

At the laboratory conditions (28.5 ± 0.45 °C, 6.2 ± 0.438 mg/l dissolved oxygen, 7.8 ± 0.302 pH and 0.33 ± 0.045 mg/l ammonia), it was found that eggs of *M. rosenbergii* completed their development in 20 days. Measuring of the maximum lengths and widths of eggs at different embryonic stages showed a significant increase in the egg diameter during the embryonic development (Table 1).

The eggs are slightly elliptical in shape and initially bright orange to yellow in color, then the color is gradually changed to deep brown in a few days before hatching (Fig. 1). An increase in the cell number of the fertilized eggs was observed on the first 2 days after spawning. Regions with high density of cells were observed as the blastopore area which appeared on the egg surface. No other differentiated structures are recognized in (Figs. 2A and 3A). On day 4, a clear region at one pole of the embryonic mass was easily observed (Fig. 2B). On day 6, the clear region was extended lengthwise forming the trunk of the growing embryo (Fig. 2C). After 8 days, a pair of small dark eye spots was developed on the yolky mass (Figs. 2D and 3B). By day 10, the clear region which developed into trunk and caudal portion occupied about 2/3 of the embryo mass (Fig. 2E). After 12 days, the eyes of the embryo were enlarged and became oval in shape. Also, the caudal papilla extended as a lappet of tissue across the median portion of the egg (Figs. 2F and 3C and D). Fig. 2G, show that the appendages are formed beneath the clear trunk region and the eyes are enlarged, became oval and surrounded by striation on 14 day-old embryo. On 18 day-old embryo, the eyes were dark rounded, striation was obvious and the translucent globules became enlarged and occupied most of the dorsal area of the yolky mass and the embryo was found to occupy all the available space inside the egg and traces of yolk remain in most embryos. Rudiments of appendages were also visible (Figs. 2G and 3E). On 22 days-old embryo, telson and rudiments of uropod (folded below the compound eyes) unfolded and segmented abdomen was appeared (Fig. 2H and I and Fig. 3F). Then hatching normally occurs in the night and may last for 2–3 days, egg shell breaks and the newly hatched larvae appeared (Fig. 2J).

Table 1 Measurement of eggs diameters (width and narrow side) during different embryonic development of *M. rosenbergii* (X ± SD, $n = 6$).

Embryonic stages	Wide side (μm)	Narrow side (μm)
Orange eggs	681.7 ± 49.60	563.3 ± 76.10
Yellow eggs	696 ± 63.50	658 ± 42.07
Pale brown eggs	735 ± 46.30	680 ± 55.14
Deep brown eggs	797.5 ± 31.60	710 ± 27.75

Histological studies on the eggs of M. rosembergii during embryonic developments

The surface of the embryo was surrounded by the primary and secondary egg membranes. The secondary egg membrane (vitteline envelope) was homogenous and structures less and connected the eggs to each other and to the pleopods (Fig. 4A). It was PAS positive (Fig. 4B). A lot of wrinkles and mucus were found on the surface of the embryo which called primary egg membrane (Fig. 4B). The fertilized egg was full of yolk granules which were homogeneously distributed in the egg. A cluster of primordial germ cells (PGCs) occupied the dorsmedial region behind the yolky portion of 6 day-old embryos (Fig. 4C).

Some organ analogs such as thoracico-abdominal fold were observed during 8 day-old embryos (Fig. 4D and E). Fig. 4F shows that the compound eye analogs were observed in 10 day-old embryos and the yolk granules near organ analogs were different from those observed in the fertilized egg stage. On day 14 of development, mass of PGCs, which coalesce with the primodrium of the gonad, was observed posterior to the heart (Fig. 4G and H).

The morphology of PGCs at 14 days of development was slightly different from that of the early stage. These germ cells were relatively smaller and are not as strikingly different from somatic cells as those of the early stages.

In 22 days old embryo, formation of the eyestalk and internal organs and elongated body was noted (Fig. 4I and J). Moreover, some yolk granules were still observed.

Biochemical composition of eggs of M. rosenbergii during embryonic development

Biochemical composition of eggs during embryonic development is given in Table 2. During the embryonic development, the protein content of the bright orange eggs was 26.5 ± 1.47 μg/egg and increased significantly to 30.9 ± 1.137 μg/egg in deep brown eggs just before hatching. On the contrary, total lipid in eggs was significantly decreased during the embryonic

development. The lipid level was 18.3 ± 0.954 μg/egg in bright orange eggs. Meanwhile, it was significantly decreased to 16 ± 0.306 μg/egg, 9.7 ± 0.473 μg/egg in pale and deep brown eggs, respectively. The carbohydrate content in bright orange eggs was 3.4 ± 0.153 μg/egg and decreased significantly to 2.1 ± 0.306 μg/egg pale brown eggs and continued to decrease in deep brown eggs to 1.9 ± 0.153 μg/egg. The lowest water content was found in the bright orange eggs (49.23%) and reached the highest level (76.23%) in the deep brown eggs. It was noted that the increase in the water content was correlated to the increase in the egg diameters.

Discussion

Embryonic development is a complex process in which cellular differentiation and proliferation occur simultaneously but at different rates (Gould, 1977). Both organogenesis and somatic growth are controlled by enzymatic activities. Embryonic development of ectotherms mainly depends on the differential expression of certain genes and temperature (Ojanguren and Brana, 2003) and the rates of their biological functions are critically depending on environmental temperature. Crustaceans from warmer water environments typically have shorter embryonic development on the order of several days to weeks (Yamaguchi, 2001). In the current investigation, the eggs of *M. rosenbergii* hatched out in 20 days at 28 °C. These results were in agreement with the earlier reports of Ogasawara (1984) who indicated that the eggs of *M. rosenbergii* hatched out in 25 days at 26 °C, 20 days at 28 °C, and in 17 days at 32 °C. Also, Manush et al. (2006) found a direct linear relationship between rates of development of *M. rosenbergii* embryos with incubation temperature.

The present study deals with a species of palaemonidae, as do those of Nazari et al. (2000) and Muller et al. (2003), but follows the method initially proposed by Sandeman and Sandeman (1991) for a species of Astacidae. This method is indeed considered more accurate and easier to apply in decapods embryology (Sandeman and Sandeman, 1991; Garcia-Guerrero et al., 2003), since it presumed that embryonic development should be analogous in all *Macrobrachium* species, as in other congeneric species of decapod crustaceans.

The present investigation showed that the embryonic development of *M. rosenbergii* based on eight different major events, called stages which are separated by some morphological

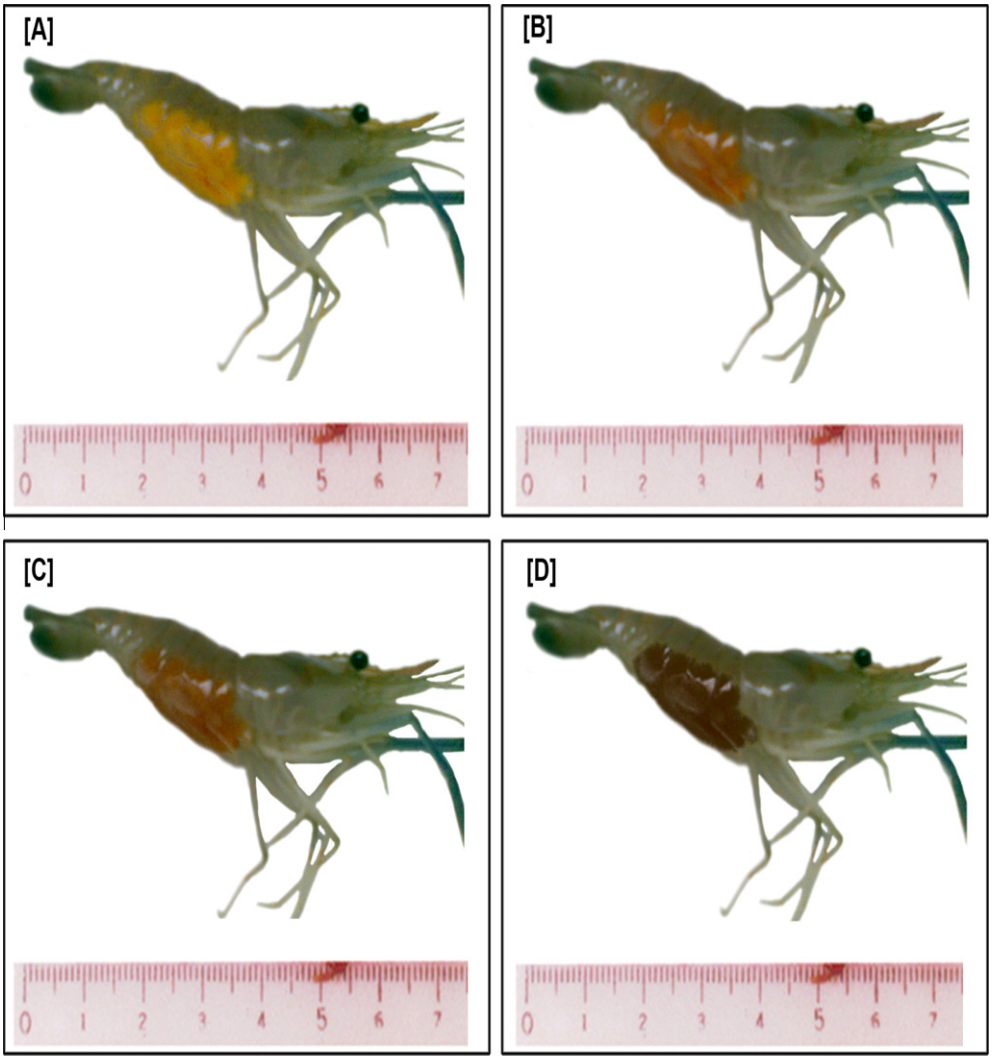

Figure 1 Photographs showing berried females of *M. rosenbergii*: (A) bright orange eggs, (B) pale yellow eggs, (C) dark yellow eggs and (D) brown eggs before hatching.

events. This finding was in agreement with Muller et al. (2003) who described the embryonic development of *M. offersii* .The separation in period's equal duration is more accurate when describing a continuous embryonic development. The same finding was reported by Sandeman and Sandeman (1991), Garcia-Guerrero et al. (2003), Garcia-Guerrero and Hendrickx (2004, 2006). This is due to the fact that the separation between major events of embryogenesis in crustaceans is not always distinguished, and some events may start before ending of the previous one, making the separation into events unclear when the process is continuum.

PGCs of the giant freshwater prawn, *M. rosenbergii* are separated from the somatic cell lines early in the embryonic stage. Predetermination of the PGCs during early stages of development is common in invertebrates. They are generated within the first hour after the onset of the cleavage in nematode, *Caenorhabditis elegans* (Schierenberg, 1989) and to the separated cells prior to the formation of somatic cells in *Drosophila melangaster* (Mahowald, 1971). During the early stages of embryonic development, PGCs of the freshwater prawn, *M. rosenbergii* are easily discrenable. Their morphology is similar to those of other vertebrates and invertebrates described so far (Fujimoto et al., 1976, 1977; Gimsburg, 1997; Ginsburg et al., 1990). They are large round cells containing large nuclei with conspicuous nucleoli, this is in agreement with the observations made by Damarongphol and Jarosenstraraks (2001). In this study, PGCs of *M. rosenbergii*, the cytoplasm are granulated, resembling those observed in the insect, *D. melangaster* (Mahowald, 1971; Tajima et al., 1998), quails (Yoshinaga et al., 1993; Praneet and Pleanphit, 2001). In quails, PGCs contain

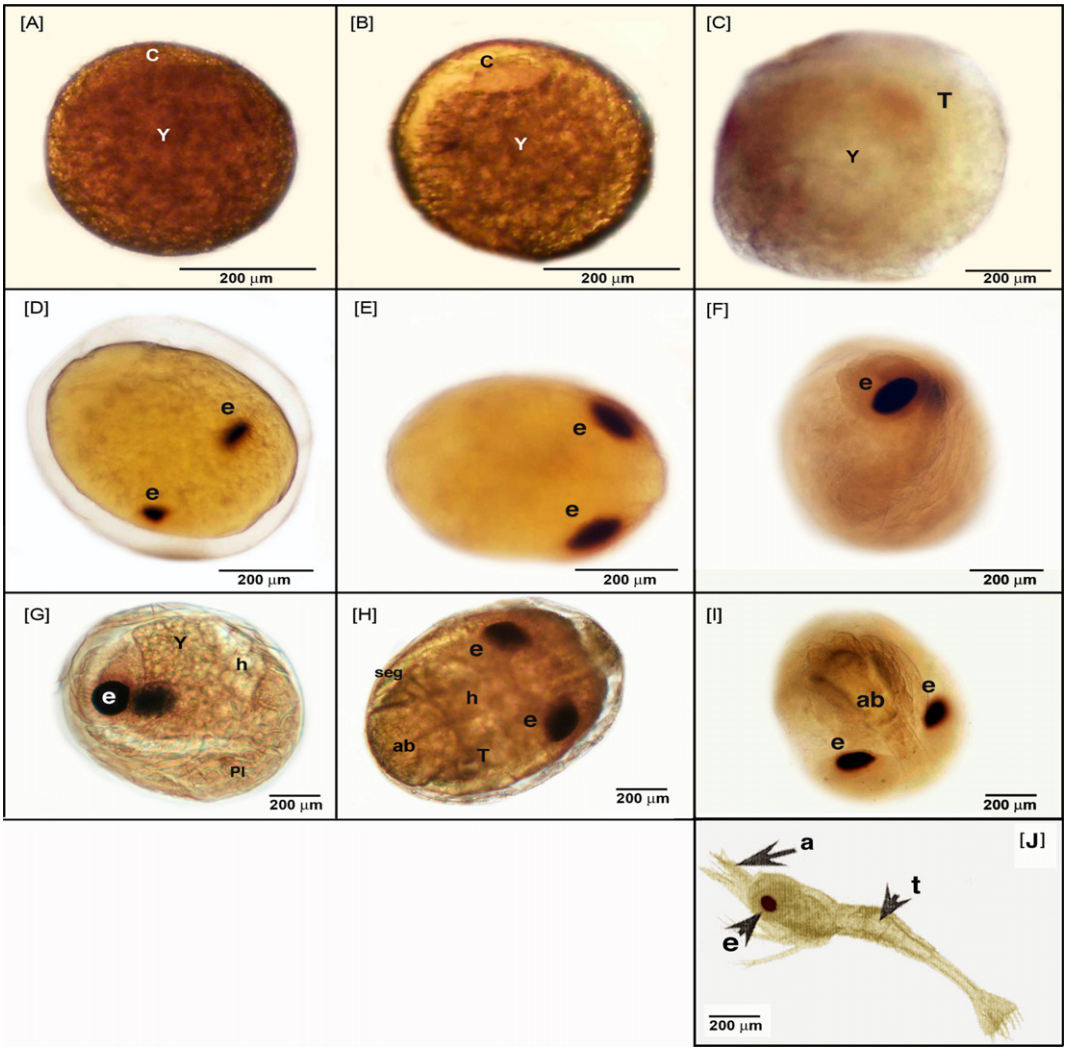

Figure 2 Bright-field micrographs of developing embryos of *M. rosenbergii*, (A) 2 days-old embryo showing a small translucent region © at one pole and the yolky portion (Y), (B) side view of 4 days-old embryo, (C) 6 days-old embryo showing transparent trunk (T), (D) 8 days-old embryo with developing 2 eyes spots (e) in the yolky mass (Y), (E) 10 days-old embryo showing enlarged oval-shaped eyes (e), (F) 14 days old embryo showing large oval-shaped eyes (e) surrounded by striation, (G) 18 days-old embryo with prominent dark relatively rounded eyes (e) , the heart region (h) behind the yolky portion (Y) and the transparent trunk (t) curved forward at the ventral side, (H) top view of 20 days-old embryo showing a pair of rounded eyes (e), abdomen which curved forward, (Ab), segmented abdomen (Seg) and heart region (h), (I) ventral view of the 20 days-old embryo, (J) newly hatched larvae showing the eyes (e), the antenna (a) and the trunk (t).

granulated cytoplasm and showing a negative reaction with PAS indicating a lake of glycogen content (Ginsburg et al., 1989; Yoshinaga et al., 1993). The PGCs of the giant freshwater prawn, *M. rosenbergii* also show granulated cytoplasm similar to that of the quails PGCs. This may implicate the similarity in mechanism of the PGCs migration to the low level of glycogen, reflecting the energy source. In the mouse, PGCs have been suggested to be the cause of their passive migration along with the morphogenetic movement during gastrulation (Jeon and Kennedy, 1973). PGCs migrate across the gut primordium and assemble into the gonad primordium where active increase in the number by mitotic division occurs (Godin et al., 1990).

Egg size appears to be species specific among decapods. The size of eggs correlates with the stage of development and serves as an indicator of energy content (Herring, 1974). Generally, species with large size eggs contain more yolk nutrients and their embryonic development time is longer. In the present study, egg size of *M. rosenbergii* increased gradually during embryogenesis from 563.3 to 710 μm (narrow side) .and from 681.7 to 797.5 μm (wide side) and can be considered as small, typical for *M. rosenbergii* species. This finding was in accordance with Lara and Wehrtmann (2009) on *M. carcinus*. In comparison with other crustacean species, it was found that eggs of the investigated species are much smaller than that of

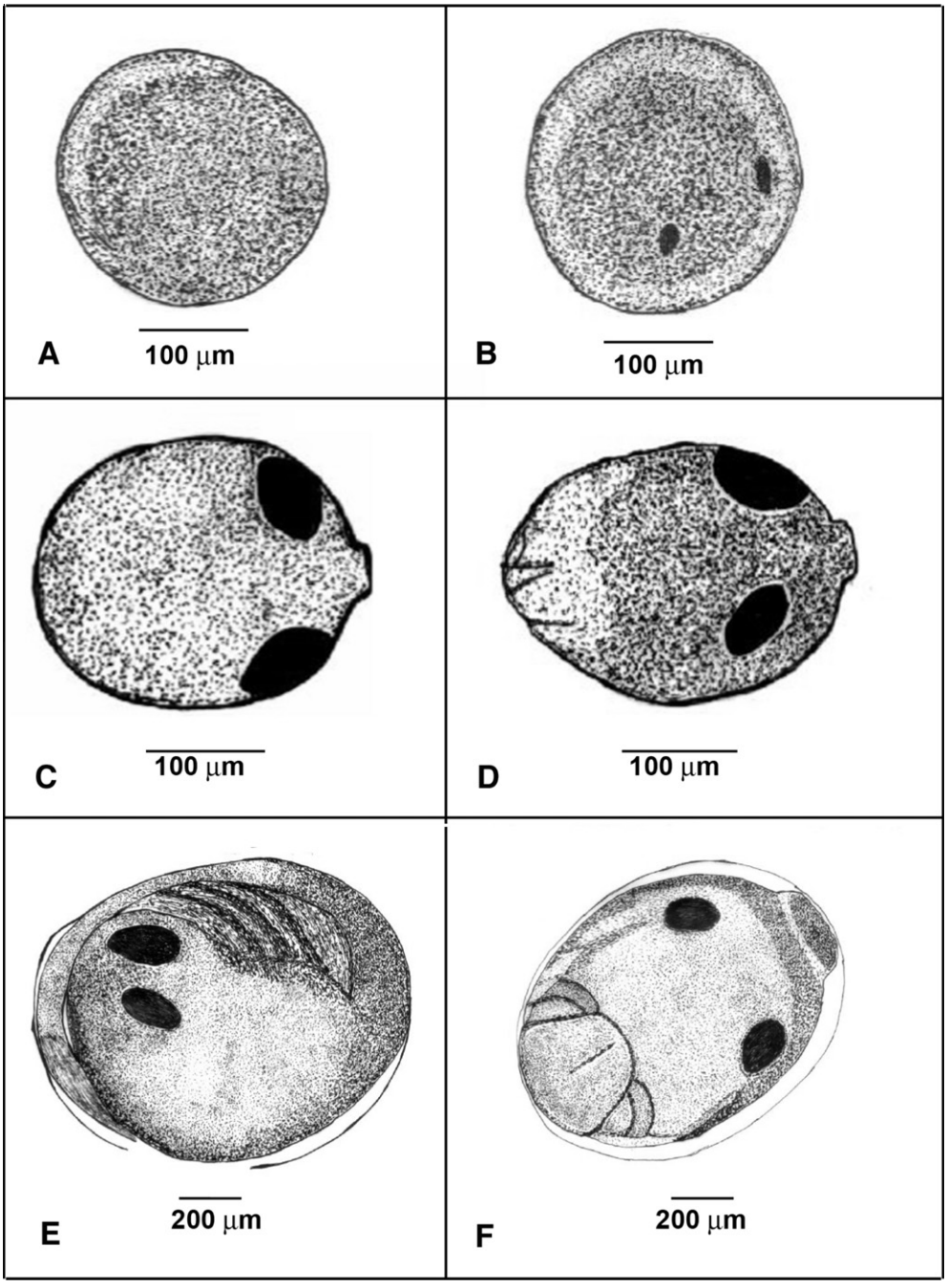

Figure 3 Camera Lucida drawings of the embryonic development of *M. rosenbergii*.

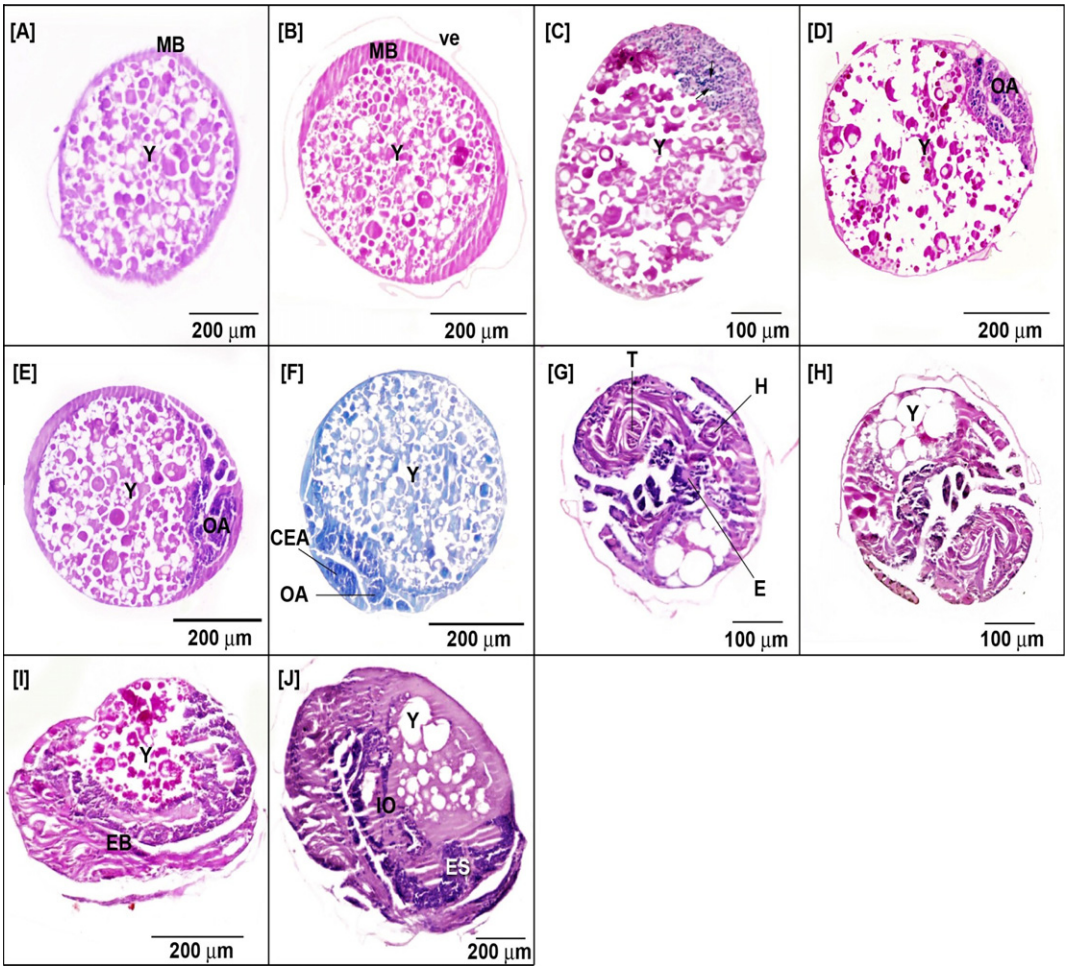

Figure 4 Histological structures of eggs of *M. rosenbergii* during the embryonic development. (A, B) Fertilized egg stage, showing first egg membrane (MB), Yolk (Y) and vetelline membrane(Ve). Hx and PAS stains, respectively. (C) 6 days old embryo, two stands of PGCs (arrow at the dorso-medial region behind the yolk (Y), PAS stain. (D, E) 8 days-old embryo showing organ anlage (OA). PAS and Hx stains. (F) 10 days-old embryo showing eye anlage (CEA). Sudanblack stain. (G, H) 14 days-old embryo showing the heart (H), eyes (E) and the tail muscle (T). Hx and PAS stains, respectively. (I, J) 20 day-old embryo showing the formation of eyestalk (ES), internal organ (IO), elongated body (EB) and presence of yolk (Y).

Table 2 Proximate composition of eggs (µg/egg) of *M. rosenbergii* during the embryonic development (X = SD, $n = 6$).

Embryonic stages	Protein	Lipid	Carbohydrate	Moisture (%)
Orange eggs	26.5 ± 1.47^{B}	18.3 ± 0.95^{A}	3.4 ± 0.153^{A}	49.23 ± 0.757^{D}
Yellow eggs	26.7 ± 1.56^{B}	17.4 ± 0.60^{A}	2.3 ± 0.20^{B}	55.90 ± 3.01^{C}
Pale brown eggs	27.2 ± 1.22^{B}	16 ± 0.31^{B}	2.1 ± 0.32^{B}	68.53 ± 1.65^{B}
Deep brown eggs	30.9 ± 1.14^{A}	9.7 ± 0.47^{C}	1.9 ± 0.15^{B}	76.23 ± 2.64^{A}

Means bearing the same superscript in the same column are not significantly different ($P < 0.05$).

the freshwater crayfish, *Cherax quadricorinatus*, *Alepheus saxidomus* (0.247 mm^3) for both species (Wehrtmann and Graeve, 1998) and *Nephrops norvegicus* (1.406 mm^3) (Rosa et al., 2003), but it is longer than that of *Palaemonetes schmitti* (0.056 mm^3) (Wehrtmann and Graeve, 1998). It seems that egg volume relates little to lipid content. Egg volume of *M. rosenbergii* is one-third of *A. soxidomus*, one-eighteenth of *N. norvegicus*, but larger than *P. schmitti*. However, the eggs of *M. rosenbergii* contained more lipid (30% average) than did *A. soxidomus, P. schmitti Nauticaria mateiianice*, *Betaeus emarginatus* and *N. norvegicus* (>20% dry weight) (Wehrtmann and Graeve, 1998; Rosa et al., 2003) The biochemical analysis in the present study showed that the higher lipid and protein contents in the eggs of *M. rosenbergii* might be the reason for long larval stages in the embryonic development of this species.

The current study revealed that the biochemical changes in the eggs were found to reflect changes in morphogenesis during the embryonic development of *M. rosenbergii*. The present study indicated that the carbohydrate is the main energy source in the early stages of embryonic development. Lipid also serves

as an energy source, and protein served mainly as the structural substance. During the last stage, yolk was hydrolyzed at a high rate and embryological organs were nearly developed, leading to the beginning of physiological functions and the heart began to beat. In the present study, egg still contains some yolk till hatching which ensures the first successful molt and favors their independence of external energy resource while external feeding. These findings coincided with Yao et al. (2006).

Weight of eggs during the last stage of the embryonic development increased in comparison with the fertilized stage, this is probably due to increasing the water content. Water provided a liquid environment for the embryo and the higher mater pressure during the last stage enables the embryo to break the egg membrane in the preparation of hatching. These findings coincided with that of Yao et al. (2006).

The findings of this study contribute to a better knowledge of this species, which having a high nutritive value participates in the food chains.

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