Determination of developmental stages of embryo in the Sea Urchin, *Echinometra mathaei*

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Abstract

Sea Urchin is one of the most useful tools in developmental biology studies because this organism has the simplest kind of developmental stages. We aimed to determine developmental stages and timetable of *Echinometra mathaei* embryo (the species of Persian Gulf). The spawning of *E. mathaei* was induced by 0.5M KCl injection (1ml) into the coelomic cavity. After fertilization, embryos were placed in beakers and were incubated at 29°C and a salinity of 39 ppt until embryos reached the pluteus stage. The developmental stages of embryos and the timing of each stage including cleavage, morulae, blastula, gastrula, prism and pluteus larvae were studied under the microscope. Our results showed that after 30 hours from fertilization time, the embryos developed to pluteus larvae. *E. mathaei* had the shorter development time in comparison to the other Sea Urchin species. Therefore, it may be appropriate as a model organism in biological researches.

Keywords: Sea Urchin, *Echinometra mathaei*, Biological Model, Development, Timetable, Persian Gulf

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Introduction

Sea Urchins have an annual reproductive cycle which can be affected by various factors (Brooks and Wessel, 2004; Walker et al., 2005). Sea Urchins have separate male and female sexes. Sperms and eggs are released into the water and after fertilization, zygotes are developed by radial holoblastic cleavage. The first and second cleavages are both meridional and are perpendicular to each other. The third cleavage is equatorial and the fourth divides the zygote unequally to produce mesomeres, macromeres, and micromeres (Gilbert, 2003). After successive cleavage, the embryo is developing to blastula stage. By this time, the cells start to occupy the periphery while leaving a central space and gradually form a central cavity called blastocoel (Briggs and Wessel, 2006). Then Gastrulation is initiated by invagination of the thickened vegetal plate into the blastocoel that ultimately gives rise to the archenteron (Kominami and Takata, 2000). Finally, embryo develops to the pluteus larva which starts the feeding and its arms begin to develop (Yajima and Kiyomoto, 2006).

In general, Sea Urchins live in a vast tidal spectrum and have a major effect on the structure and dynamic of their habitats (Williamson et al., 2000; Williamson and Steinberg, 2001). The Sea Urchin, *Echinometra mathaei* is a member of the phylum Echinodermata and the class Echinoidea (Lawrence, 1987). This species is generally found in shallow waters of the Persian Gulf and Oman Sea (Shahri et al., 2008). Some of the features like the ability of spawning induction, artificial fertilization, coordinated and rapid development, optical clarity of embryos have made the Sea Urchin a candidate of the suitable tool for research about the fertility, early embryonic development and also biological tests (Conway et al., 1984; Semenova et al., 2006). In spite of the vast research on the development of Sea Urchins in the world, there are no reports about *E. mathaei* from south coasts of Iran, except determination of reproductive cycle (Shahri et al., 2008) and recent studies about toxicity effects of metals, Hg, Cu, Pb, Cd on embryo – larval development of this species.

The objective was to determine the developmental stage and timetable of *E. mathaei* embryo (The Persian Gulf species) which may be used as a bioindicator in biological research such as toxicity bioassays and other ecotoxicology researches.

Materials and methods

Biological materials

About 20 samples of adult *E.mathaei* were collected from Bostane costs (26˚31S, 54˚39E) by snorkeling. Then samples were transferred to the laboratory of Persian Gulf and Oman Sea Ecological Research Institute. To induce spawning, 0.5 M potassium chloride (1 ml) was injected into the celomic cavity of individual Sea Urchins. Spawning females and males were inverted (oral side up) over beakers of filtered seawater (0.5 and 1 μm filters) with a temperature of 29˚C so that spawned eggs and sperms were allowed to settle in the base of the dish and then were collected separately. Then gametes were observed using a microscope to check their

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maturity (spherical eggs and mobile sperm). In order to fertilize a few amounts of sperm solution were added to the suspension of eggs and it was carefully stirred to allow fertilization. To ensure the fertilization (formation of fertilization membrane) has been occurred, samples were observed using a microscope and subsequently the fertilization eggs were diluted to a density of 200 eggs per ml filtered seawater (Bielmyer et al., 2005).

Optimal conditions for growth of the embryos

In this study, several factors such as temperature, salinity and the type of container required for embryos growth under laboratory conditions were analyzed. According to Fernandez and Beiras (2001) and Kurihara and Shirayama (2004), the polypropylene vials, beaker and Petri dish were used to determine the suitable container for embryos growth. For this test, approximately equal numbers of fertilized eggs (n=120) were placed in beakers, vials and Petri dishes and three replicates per container were assayed. The containers containing the fertilized eggs were incubated at acceptable average from temperature and salinity range for this species (temperature and salinity were 29°C and 37 ppt, respectively). All the larvae that had developed in each container were counted. The numbers of larvae found in each container were compared to assess any differences between the containers. After the selection of suitable containers, three temperatures and salinities were tested within the range of acceptability for the species to determine optimal temperature and salinity. However, the percentage of pluteus larval development was assessed in three different temperatures (27, 29 and 31°C) in each of which, three different concentrations of salinity (35, 37 and 39 ppt) with three replicates were selected.

The developmental stage and timetable of E. mathaei

For determination of the developmental timetable, after fertilization, the embryos were added to each of the test container containing filtered seawater to attain a final density of 20 embryos per ml. Then samples were incubated under obtained optimum conditions until embryos reached the pluteus stage. To reduce the error rate, experiments were performed with 3 replicates. For the timing of each developmental stage (early cleavage, morulae, blastula, gastrula, prism and pluteus larvae) the samples were tested at first in short timing intervals (~30 minute) and in advanced developmental stage they were checked in long timing intervals (1 up to several hours) by using the microscope. The timing point was recorded when clear characteristics of each stage were observed.

Data analysis

The statistical procedure was conducted in two steps. In the first test, the number of developed 4-arm pluteus larvae in three containers were compared using One Way analysis of variance (ANOVA), followed by a Bonferroni test. In the secondary test, the percentages of pluteus larval development were compared by two-way ANOVA and a Tukey HSD (honestly significant difference) multiple mean comparison test (effects: temperature and salinity). Also homogeneity of the variance test was used prior to ANOVA.

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Significant differences were considered at the 95% level.

Results

Optimal conditions for growth of the embryos

In the first step of analysis, homogeneity of variance test showed that all three container variances were similar to each other ($F_{(2,6)}$ □□0.238, $p > 0.05$). So, one-way ANOVA test was carried out and these results illustrated that significant differences existed between the numbers of developed 4-arm pluteus larvae in the aforementioned groups. Subsequently, Bonferroni test indicated significant differences on the developmental rate of pluteus larvae for both beaker and vial when compared to the Petri dish but the difference between beaker and vial was not significant (Table 1).

Table 1: Mean number (M) and standard deviation (SD) of the developmental rate of pluteus larvae in three containers, mean comparison, one-way ANOVA, Bonferroni test, p < 0.05

	Beaker		Vials		Petri dish		*F*-Value (df 2,6)	*p*	B-V	B-P	V-P
	M	SD	M	SD	M	SD					
number of developed larvae	94.33	4.72	82.66	4.04	41.66	6.03	91.818	<0.001		*	*

Because of the developmental rate of pluteus larvae was higher in beaker than vial and Petri dish, beaker was selected for the next step of our experiment. In the second step of analysis, beakers were used for comparing results of the percentage of pluteus larval development in different levels of temperature and salinity. Table 2 shows experimental results in different temperatures and salinities.

Table 2: Mean percentage (M) and standard deviation (SD) of pluteus larval development at different temperatures and salinities

Temperature (°C)	Salinity (ppt)					
	35		37		39	
	M	SD	M	SD	M	SD
27	5%	1.65	15%	2.08	21%	3.11
29	54%	2.25	78%	1.93	98%	1.85
31	49%	3.16	56%	2.51	74%	2.55

The homogeneity of variance test showed there is no reason to believe that the equal variances assumption is violated ($F_{(8,18)}$, 0.332, $p > 0.05$) so two-way analysis of variance (ANOVAs) test was carried out with temperature and salinity as factors. There was significance in the percentage of pluteus larval development by temperature ($F_{(2,18)}$ □□1659.36, $p < 0.001$) and salinity ($F_{(2,18)}$, 314.37, $p < 0.001$). Also the Tukey HSD test indicated that significant differences existed amongst all salinities and temperatures, each of which yielded significantly unique sets of data (all $p < 0.05$).

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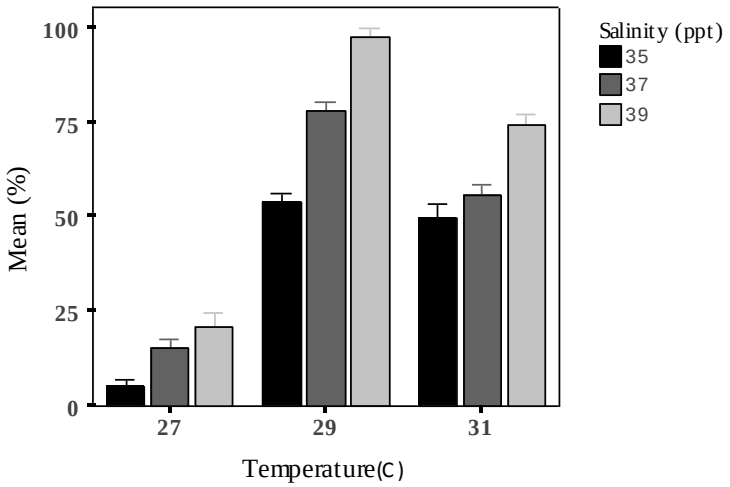


Figure 1: Comparison between mean percentage of pluteus larval development at different temperatures and salinities. [Error bars=SD]

As illustrated in Figure 1, with regard to the results of ANOVA test, temperature of 29°C and salinity of 39 ppt were more suitable than the others for growth and development of embryos, in these conditions 98% of embryos developed to pluteus larvae (Table 2).

The development of E. mathaei

The sperm morphology in *E. mathaei* was longer than the other echinoderms and its egg was completely round. When sperm and egg were close together (Fig. 2A), sperm penetrated into the egg and fertilization occurred 2 minutes after contact of sperm and egg. The obvious characteristic of fertilization was the formation of fertilization membrane around the egg (Fig. 2B). 60 minutes after fertilization, the first meridional cleavage occurred which leaded to formation of two equal size cells (Fig. 2C). The plane of the first cell division was possessed through the animal-vegetal axis of the egg. After a few hours, second meridional and then an equatorial cleavage generated four and eight blastomere of equal size, respectively (Figs. 2D and E). The fourth cleavage generated a 16-cell embryo in which four vegetal cells divide unequally to form 4 micromeres and 4 macromeres, while four animal cells divided into 8 equal mesomeres longitudinally (Fig. 2F).

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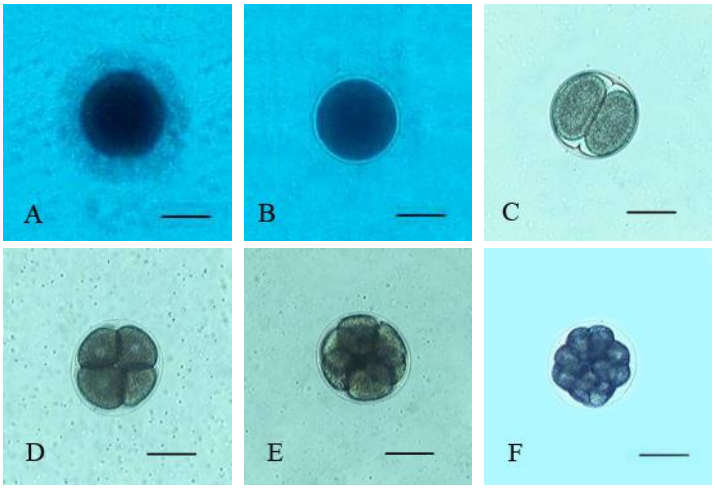


Figure 2: The early development of *E. mathaei* occurs rapidly; (A) sperm around the egg; (B) ovum after fertilization; (C) 60 min after fertilization, 2-cell stage; (D), (E) and (F) < 4 hr after fertilization, 4-cell stage, 8-cell stage and 16-cell stage, respectively. Scale bar= 50 µm for A-F.

Cell division continued repeatedly and after 4 hours, the embryonic cell mass converted to the morulae (Fig. 3A). After 6 hours, the seventh cleavage generated a blastula. In this stage, the cells formed empty spheres that surround the central blastocoel. The most important morphological features in this stage were the formation of cilium around the cell mass and the mobility of embryo which was observed microscopically (Fig. 3B). Gastrula formed 14 hours after fertilization through invagination at the vegetal pole (Fig. 3C). The invaginated region is called the archenteron that eventually made contact with the blastocoel's wall and formed the mouth. Then continuous digestive tube was formed by connecting the mouth to the archenteron. Almost 20 hours after fertilization, embryos entered to prism stage and formed the skeletal elements. In this stage the embryo was prismatic and the primitive gut was observed clearly (Fig. 3D). Finally, 30 hours after fertilization, the prism developed into 4-armed pluteus larvae. At this time, a pair of frontal short arms and a pair of dorsal long arms of larvae could be observed. Furthermore, several pigmented cells were visible under the light microscope (Fig. 3E). A developmental timetable which was observed in the laboratory is given in Table 3.

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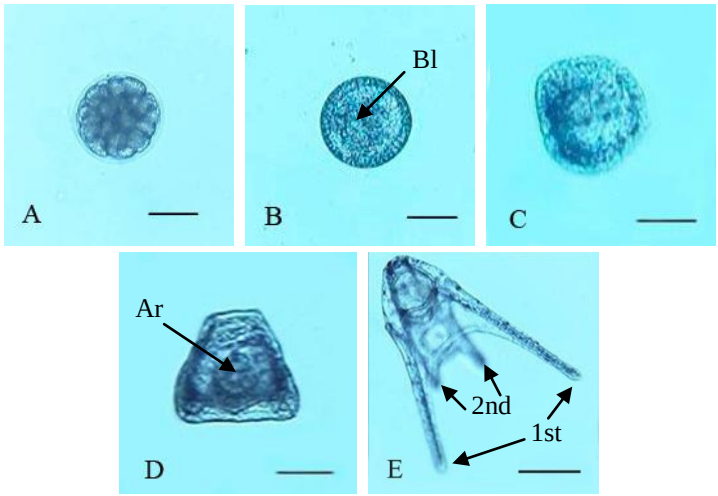


Figure 3: The developmental stages of morulae to 4-armed pluteus larvae

Ar, primary gut or archenteron; Bl, blastocoel. (A) 4 hr after fertilization, morulae stage, (B) 6 hr after fertilization, blastula stage, showing blastocoel (C) 14 hr after fertilization, gastrula stage, archenteron not visible clearly in this image, (D) 20 hr after fertilization, prism stage, showing primitive gut, (E) 30 hr after fertilization, 4-armed pluteus larvae, showing first (1st) and second (2nd) pairs of arms. Scale bar= 50 µm for A-D. Scale bar= 100µm for E.

Table 3: Developmental timetable of *E. mathaei*.

Developmental stages	Time after fertilization
Formation of fertilization membrane	2 min
First cleavage	60 min
Morulae	4 hours
Blastula	6 hours
Gastrula	14 hours
Prism	20 hours
4-armed pluteus larvae	30 hours

Discussion

For determining the optimum conditions, the experiment was conducted in different containers. Because the type of the container can affects the embryos growth in laboratory conditions (Lera et al., 2006), we used different containers including polypropylene vials, Petri dish and beaker. Our results showed the effect of container on developmental rate of pluteus larvae was significant and the mean of larval development in beakers was higher than vials and Petri dish so beaker was selected as a suitable container for experiment. The results showed that our optimal temperature and salinity were higher than the other similar researches. Fernandez

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and Beiras (2001) reported the optimal temperature for embryos growth of the Sea Urchin *Paracentrotus lividus* at 20ºC. Cesar et al. (2004) also reported the optimal temperature of 22ºC for this species. Bielmyer et al. (2005) showed that optimal salinity for *Diadema antillarum* was 33 ppt and King and Riddle (2001) reported an optimal salinity of 34 ppt for *Sterechinus neumayeri* embryos. Probably difference of optimal temperature and salinity for embryos growth is related to the difference in the habitat of studied species. The optimal temperature for growing *E. mathaei* embryos that live in the warm area of Iran (Bandar Abbas) was 29 ºC and optimal salinity for this species was 39 ppt.

According to table 3, 60 minutes after fertilization, the first cleavage occurred similar to *Arbacia punctulata* Sea Urchin (Shimek, 2003). Also, King and Riddle (2001) showed that in *Sterechinus neumayeri* the embryos develop into the blastula 2-3 days after fertilization, while that occurs after 12 and 6 hours in *Paracentrotus lividus* (Russo et al., 2003) and *E. mathaei* (in the present study), respectively. In the present study, developing into the gastrula was 14 hours after fertilization while King and Riddle (2001) showed that *Sterechinus neumayeri* embryos developed into the gastrula after 9-10 days and that occurred in *Arbacia punctulata* after 24 hours (Shimek, 2003). Prism occurred 20 hours after fertilization in *E. mathaei* whereas Shimek (2003) reported that embryos develop into prism after 24 hours. Finally, the researches reported that *Paracentrotus lividus* and *Sterechinus neumayeri* spend 48 hours and 20 days after fertilization to reach the pluteus larvae, respectively (Russo et al., 2003; King and Riddle, 2001) while in *E. mathaei* that occurred after 30 hours. Our results showed *E. mathaei* has a shorter developmental timing and the pluteus larvae developed in a shorter time compared to the other species of Sea Urchins. Probably the environmental conditions that lead to the different adaptation of the species to the environment resulted in different developmental timing. Whereas this experiment was carried out in the warm area of Iran, thus, one of the important environmental factors was temperature. King and Riddle (2001) indicated that Sea Urchin embryos in cool regions reach to pluteus larvae sooner than similar species from warmer regions.

In conclusion, we demonstrated that *E. mathaei*, as other species of warm regions, has a shorter developmental timing compared to other studied species. Researches showed that Sea Urchins are a suitable model for biological studies due to some of features such as ability of spawning induction and artificial fertilization, rapid development and also optical clarity of embryos (Conway et al., 1984; Semenova et al., 2006). As a result,

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Sea Urchin embryos have been widely used for embryo– larval toxicity bioassay (Fernandez and Beiras, 2001). Bielmyer et al. (2005) for investigating the effects of metals (Cu, Ag, Ni, Se) on embryo-larval stages used the *Diadema antillarum* Sea Urchin that develops into pluteus larvae after 40 hours. Also, Fernandez and Beiras (2001) examined toxicity of metal Hg, Cu, Pb and Cd using the 48 hour larvae of *Paracentrotus lividus* as a bioindicator. Our results showed *E. mathaei*, the species in the south coast of Iran, has especially short developmental timing that allows performing several exercises in a short time. Therefore, this species may be used as a bioindicator for biological tests, including toxicity bioassays and other ecotoxicology tests. In the next studies, we used *E. mathaei* of the south coast of Iran as a model organism for studying the toxicity effects of Hg, Cu, Pb and Cd metals on embryo- larval development.

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