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INFLUENCE OF SALINITY VARIATIONS ON THE EMBRYONIC AND EARLY LARVAL DEVELOPMENT OF LONG-SPINED BLACK SEA URCHIN (*DIADEMA SETOSUM)*

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ABSTRACT

Effects of salinity on fertilization, embryonic, and early larval development and growth performances of long-spined black sea urchin (*Diademasetosum*) were investigated in a controlled laboratory condition. The experiment was carried out with seven salinity treatments (22, 25, 28, 31, 34, 37 and 40ppt), each of which was triplicated. Significantly highest fertilization success was achieved at 31 ppt (97.33%), followed by those at 34, 37, 28, 25 and 40 ppt, and the lowest value at 22 ppt, decreased with increasing and decreasing salinities ($P< 0.05$).The time required to reach these embryonic and larval stages was increased with the salinity deviations from 31 followed by 34, 37 and 28 ppt. Survival (%) of the early larval stages (from prism to 4-arm pluteus) followed the same trends as fertilization rates. No significant differences ($P> 0.05$) were recognized among these four salinity levels on prism larval length and width. However, significant differences ($P> 0.05$) were noted in morphometric characteristics of 2-arm and 4-arm pluteus larvae. The finding of the study indicated that *D.setosum* is a stenohaline echinoid that could not be able to survive and develop if the salinity range is less than28 or more than 37 ppt.

Keywords: Sea urchin, *Diademasetosum*, Salinity, Embryo, Larvae, Development.

INTRODUCTION

Sea urchins are the high-valued marine invertebrates that have been used as raw material to produce foodstuff, in particular, the product of processing gonads known as "Sea urchin Roe or Uni" (Kaneniwa and Takagi, 1986; Oshima *et al*.,1986; Ichihiro, 1993).It has also been considering as a prized delicacy in Asia, Mediterranean countries, and Western Hemisphere countries such as Barbados and Chile (Lawrence *et al*., 1997;Yur'eva *et al*., 2003). Peoples in the Asian Pacific Region have used sea urchin gonads for many years as a remedy for improving health condition, treatment for a number of diseases and also for increasing the sexual potency of the middle-aged men (Yur'eva*et al.*, 2003). Gonads of sea urchins have long been considered as one of the luxury food in Japan (Shimabukuro, 1991). Some studieshave proved that sea urchin gonads are rich in valuable bioactive compounds, like polyunsaturated fatty acids (PUFAs) and *β*-carotene (Dincer and Cakli, 2007). Sea urchin fisheries have expanded so greatly in recent years that the populations of sea urchins around the world have been overfished (Andrew *et al*., 2002, 2004). Not surprisingly, the decrease in supply and the continued strong demand have led to a great increase in interest in aquaculture of sea urchins, particularly in those areas where their populations have been depleted (Lawrence *et al*., 1997, 2001; Robinson, 2004).

Diademasetosum (Leske, 1778) (Echinometra: Echinoidea: Diadematidae) also known as long-spined black sea urchin, is one of the most common echinoids that widely distributed in the Indo-West Pacific Ocean especially in coral ecosystems,and have profound biological, ecological and aquacultural significance (Lessios *et al.*, 2001; Rahman *et al.*, 2012a). This black sea urchin can be found in tropical (Hori *et al*., 1987; Grignard *et al*., 1996; Ruengsawang and Yeemin, 1999;Carreiro-Silva and McClanahan, 2001)and also in temperate water areas(Yoshida, 1956;Mokady*et al*., 1996).It has small test with very long spines (some can also reach 30 cm long). *Diademasetosum* have all black but some have grayishspine.It hasbeen known as prolific grazer and mainly feed on various algal species that can be found on coral reef, sand flat and sea grass beds.

According to Lepage *et al.* (1992), embryogenesis was described as immediate cleavage of fertilized eggs into a greater number of small cell formations. After fertilization process the eggs develop into 2-cell, 4-cell, 8-cell, 16-cell stages and so on until a blastula stage (128-cell stage) is formed (Sewel and Young, 1999). Lepage *et al*.(1992) also reported that the fertilization envelope may be thinner and finally vanish

as the organism secretes hatching enzyme to digest it. After hatching, it will be considered as a free swimming blastula. Followed by gastrulation, blastula will be changed into pluteus larval stage where it exhibits the sea urchin characteristics. According toMetaxas (1998),there are five stages that will be passed through by sea urchin and these are: prism, 2-arm, 4-arm, 6-arm and end up with 8-arm pluteus.

Studies conductedon the embryonic development of purple sea urchin, *Paracentrotuslividus*) showed that most critical factors among the abiotic factors are temperature and salinity(Bressan *et al*., 1995). Nevertheless, in other previous studies alsoshowed that salinity has the greatest effects on the survival, embryonic as well as larval development of sea urchins (Roller and Stickle, 1993; Metaxas, 1998; Forcucci and Lawrence, 1986). Kashenko (2007)documented that increasing salinities in the same temperature have affected the time needed for embryonic development of *Echinocardium cordatum*. However, larval development of *Echino metralucunter* was found to be slowed considerably in reducing salinities (Metaxas, 1998). Allen and Pechenik (2010) reported that fertilization envelope of eggs seldom rises and even successfully fertilized eggs do not cleave after introducing to low salinity seawater. Compared to their adult, larvae of sea urchin may have salinity tolerance range and it can be wider or narrower.According to Metaxas (1998) larvae of Atlantic sea urchin, *Echino metralucunter* are more sensitive to salinities and also can tolerate narrower salinity ranges than their adults. Low salinity conditionreduces feeding rate, decreases growth and therefore limits the size of echinoderms (Forcucci and Lawrence, 1986). Decreasing salinity affected the viability and also causedthe mass mortality of adult sea urchin, *Lytechznusvarrigatus* at Florida(Lawrence, 1975).However, such types of studies are still lacking in the tropical species of Diadematid sea urchins. Therefore, the present work have been undertaken to investigate the effects of salinity variationson the scheduled embryonic and early larval development of the high-valued sea urchin, *D. setosum* in a controlled laboratory condition.

MATERIALS AND METHODS

Samplecollection and site: Around50 mature adults of *D.setosum*, weighing from 60 to 130g and diameter from 45 to 80 cm were collected from PulauTelur (5.7667°N, 100.2833°E) in Kedah, Malaysia at low tide during their natural breeding season from June to December, 2013. Live samples were then transported to the Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia (UPM), where they were maintained in aerated closed aquaria and used within 3-4 days of collection.

Spawning: Three pairs of matured adults of*D. setosum*, weighing from 90 to 130 g and diameter from 50 to 80 cm were used for breeding. Chemical induction was done for sea urchin breeding by injecting 2.0 ml of 0.5 M KCl into the celomic cavity of female urchins (Rahman *et al*., 2000, 2005, 2012b). The eggs were collected by inverting the female urchin on a glass beaker filled with 2.0 µm filtered seawater (FSW). Before fertilization process, egg quality and maturity were checked under a compound microscope (Zeiss Axioskop 2). Good quality eggs, having distinct nucleus with uniform shape were used for fertilization experiment (Rahman and Uehara, 2004). After complete shedding, debris and immature eggs were removed from the egg-mass by 3-4 consecutive washes with FSW (Giudice, 1973). Sperm motility was observedfrom each male urchin under a compound microscope (Rahman and Uehara, 2004). Only high motility sperms were selected for fertilization trials because it can enhance the fertilization success.

Insemination and fertilization: Fertilizationexperiment was done at room temperature (26 to 28°C). One or two drops of diluted sperm solution were pipetted into a small bowl containing egg suspensions. Mixed solution (containing sperm and egg) were left for at least 10 minutes to make sure that all the eggs were encountered by sperms during fertilization process. Overload sperms and debris in the mixed solution were then removed from the inseminated eggs by 3 to 4 consecutive washes with FSW (Rahman and Uehara, 2004).

Rearing of embryos and larvae: Approximately 500 inseminated eggs were transferred into extraction plastic tubes containing 50 ml artificial seawater (Instant Ocean, Aquarium Systems, Sarrebourg, France)having 7 different salinities (22, 25, 28, 31, 34, 37 and 40 ppt). In this experiment, 31ppt was set as a control treatment, using normal sea water. Each treatment was conducted with three replicates. Temperature was set up at 26±2°C for the whole experiment. The salinity level in each experimental tube waswell-maintained by frequent checking with a refractometer and topping up the evaporation losses regularly when needed. First 100 eggs that encountered were classified as fertilized if they had reached 2-4 cell stage at 1.25 to 1.5 h post-insemination (Rahman and Uehara, 2004).

Embryonic and early larval development: In each salinity treatment, embryonic stages like cell division (cleavage) and early larval stages were observed under a compound microscope as above. Number of embryos that reached to the particular stage was determined. Development rate was carried out by assessing the time required for certain cell stage (2-cell, 4-cell, 8-cell, 16-cell, morulla and blastula stage) to be accomplished (Figure1). Every stagewas checked under a compound microscope (Zeiss Axioskop 2) at hourly intervals for at

least 50% embryos to attain the particular stage (Fujisawa, 1993; Rahman *et al*., 2002). Once the blastula reach the pluteuslarval phase through gastrula and prism stages (Figure2), the culture was examined daily and numbers of larva developed into each 2- and 4-arm pluteus stage (Figure2) were counted by sub-sampling techniques. The time required for developmental stages (prism, 2- and 4-arm pluteus) to be completed, was also estimated by the duration taken for at least 50% larva to achieve the particular stage (Fujisawa, 1993; Rahman *et al*., 2002). Survival of larvae at each stage under different salinity levels were also estimated and compared among the treatments.

Measurement of Larvae: Morphometric characteristics of larvae were measured and compared among the salinity treatments. These includes: larval length (LL), larval width (LW), body length (BL), post oral arm length (POA) and anterolateral arm length (ALA) (Figure3).After thorough observation,every stage of survived embryos and larvae under different salinities were preserved in small plastic tube with 10% formaldehyde. They were then placed on microscope slides with cover slip for final measurements and photographing, under a computerized digital microscope (Keyence VH-S30K).

Statistical Analysis: All data collected from the fertilization experimentsas well as larval development and growth of *D. setosum* were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's New Multiple Range Tests (Duncan, 1955).Data analysis was accomplished using computerized statistical package "SPSS" version 20.Significance level was set at 0.05.

RESULTS

Fertilization success: Fertilization percentage at different salinity levels is shown in Figure 4. The mean fertilization (%) was highest at 31ppt, followed by 34, 37, 28, 25, 40 and the lowest at 22ppt, decreased with increasing and decreasing salinities ($P \leq 0.05$). It could be observed that the fertilization rateof *D. setosum* is largely affected by salinity fluctuations.

Early development: Salinity effects on embryonic and larval development of *D. setosum* are shown in Table 1.Since the embryos at the salinity levels of 22, 25and 40ppt were cleaved unequally, developed abnormally or died at the beginning of the experiment, they were not analyzed statistically. The 2-cell stage attained within 1.38, 1.21, 1.28 and 1.34 h post-insemination at 28, 31, 34 and 37ppt, respectively. Development times of 4-, 8- and 16-cell stages at 28 and 31ppt showed significant differences ($P \leq 0.05$) than those at 35 ppt. Besides that, further cleavages into morulla, blastula, gastrula, early prism, 2- and 4-arm pluteus stages showed significant differences ($P \leq 0.05$) in development times among these four salinity levels. The time taken to reach these stages was increased with the salinity deviations from 31 till the extent to 34, 37 and 28ppt in that order. The development times for every stage of the embryo and larva were more or less similar for 31 and 34 ppt.

Table 1. Effects of salinity variations on developmental time of *D. setosum*: Times taken for 50% embryo and larvae to reach each stage. Three replicate experiments were conducted for each breeding trial. Each value indicates mean ± SE in hour

Stages	Salinity (ppt)			
	28	31	34	37
2-cell	1.38 ± 0.02^{d}	1.21 ± 0.02^{a}	1.28 ± 0.06^{b}	1.34 ± 0.06^{c}
4-cell	2.27 ± 0.02^{d}	2.13 ± 0.01^{a}	2.18 ± 0.01^{b}	2.24 ± 0.04^{c}
8-cell	3.09 ± 0.08^{d}	2.42 ± 0.04^{a}	2.52 ± 0.02^{b}	3.03 ± 0.02^{c}
16-cell	3.28 ± 0.12^{d}	3.08 ± 0.09^{a}	3.17 ± 0.57^{b}	3.21 ± 0.22^{c}
32-cell	3.53 ± 0.18^{c}	3.31 ± 0.16^{a}	3.39 ± 0.17^{b}	3.45 ± 0.20^{b}
Morulla	4.11 ± 0.11^{d}	3.50 ± 0.20^{a}	3.58 ± 0.02^{b}	4.04 ± 0.04^{c}
Blastula	9.53 ± 0.20^{d}	9.13 ± 0.12^{a}	9.21 ± 0.08^{b}	9.38 ± 0.18^{c}
Gastrula	17.56 ± 0.15^{d}	16.37 ± 0.12^{a}	16.54 ± 0.20^{b}	17.10 ± 0.26^{c}
Early prism	24.54 ± 0.15^{d}	22.53 ± 0.11^{a}	22.61 ± 0.17^{b}	23.26 ± 0.28^{c}
2-arm pluteus	36.40 ± 0.09^{d}	34.36 ± 0.20^{a}	34.54 ± 0.28^{b}	35.22 ± 0.43^{c}
4-arm pluteus	50.53 ± 0.32^{d}	48.10 ± 0.43^{a}	48.46 ± 0.44^{b}	49.27 ± 0.28^{c}

Mean values in the same row with the same superscript are not significantly different ($P > 0.05$).

Larval growth performances: Impacts of salinity on early larval growth performances are presented in Table 2, 3 and 4. After 24 h of insemination (Table 2), only four salinity levels (28, 31, 34 and 37 ppt) had larvae that still alive and reached to prism stage. The highest length and width of prism larvae at 31 ppt were 114.51 and 76.33 µm, while the lowest values of 84.66 and 51.22 µm were observed at 28 ppt. There were no significant differences ($P > 0.05$) recognized between these four salinity levels. Morphometric variations of 2-arm pluteus larvae at

different salinities were also investigated (Table 3). In this stage, larvae obtained both the highest larval length (206.75 µm) and body length (131.19 µm) at 31 ppt. The lowest larval and body length of 166.53 and 114.09 µm were observed in 2-arm pluteus at 28 ppt. Larval lengths among the four salinity levels were differed significantly ($P \leq 0.05$). The comparison of four morphometric characteristics in 4-arm pluteus larvae of *D. setosum* at different salinity concentrations are presented in Table 4. The results showed that 4-arm pluteus at 31 ppt attained the highest larval, body, post oral and anterolateral arm length of 250.87, 171.31, 131.23 and 89.10 µm, respectively. Among the salinity treatments, 4-arm pluteus larvae at 28 ppt had the lowest larval, body and post oral arm length of 197.75, 154.32 and 113.97 µm, respectively. Lowest anterolateral length of 48.29 µm was observed in 4-arm pluteus at 28 ppt. The body length and anterolateral arm length showed significant differences ($P \leq 0.05$) among the four salinity levels examined (Table 4). Survival of larvae (Prism to 4-arm pluteus) under different salinity levels are shown in Tables 5. Similar to the fertilization rates, significantly highest ($P \leq 0.05$) survival (%) of all the three larval stages (prism, 2-arm and 4-arm pluteus) of *D. setosum* was obtained at 31 ppt, followed by 34, 37 and the lowest at 28 ppt(Table 5).

Table 2.Comparison of two morphometric characters of the larvae of *D. setosum* at prism stage under different salinity levels. Thirty larvae were measured for each replicate in each treatment. All values represent mean ± SE in µm

Morphometric characters	Salinity (ppt)			
	28	31	34	37
Larval length	84.66 ± 3.82^a (77.35–90.34)	114.51 ± 5.91^b (103.74–124.11)	114.28 ± 8.53^b (100.19–129.67)	85.29 ± 4.58^a (76.94–92.73)
Larval width	51.22 ± 5.18^a (41.52–59.21)	76.33 ± 4.96^b (71.08–86.25)	73.68 ± 5.18^b (61.20–85.18)	52.33 ± 5.51^a (42.98–62.04)

Mean values in the same row with the same superscript are not significantly different ($P > 0.05$).

Table 3.Comparison of three morphometric characters of the larvae of *D. setusom* at 2-arm pluteus stage under different salinity concentrations. Thirty larvae were measured for every replicate in each treatment. All values represent mean ± SE in µm

Morphometric characters	Salinity (ppt)			
	28	31	34	37
Larval length	166.53 ± 5.59^a (156.95–176.32)	206.75 ± 1.55^b (200.65–209.77)	200.90 ± 9.12^b (187.65–218.38)	174.11 ± 4.28^a (165.95–180.42)
Post oral arm length	69.20 ± 0.94^a (67.58–70.82)	87.22 ± 0.46^b (82.43–98.03)	86.78 ± 5.21^b (80.97–97.18)	71.31 ± 1.99^a (68.54–79.16)
Body length	114.09 ± 2.46^a (110.98–120.94)	131.19 ± 4.36^b (121.19–139.67)	130.78 ± 1.03^b (119.74–132.84)	119.19 ± 3.10^a (114.25–124.89)

Mean values in the same row with the same superscript are not significantly different ($P > 0.05$).

Table 4.Comparison of four morphometric characters of the larvae of *D. setosum* at 4-arm pluteus stage under different salinity levels. Thirty larvae were measured for each replicate in each treatment. All values were measured in µm and represent mean ± SE

Morphometric characters	Salinity (ppt)			
	28	31	34	37
Larval length	197.75 ± 8.40^a (178.43–206.33)	250.87 ± 8.22^b (236.51–264.98)	243.89 ± 12.41^b (221.45–264.31)	200.13 ± 6.33^a (118.46–210.22)
Post oral arm length	113.97 ± 2.42^a (101.89–118.75)	131.23 ± 4.07^b (120.75–139.18)	130.41 ± 1.27^b (118.77–132.91)	119.23 ± 2.84^a (104.5 –124.39)
Body length	154.32 ± 2.24^a (140.76–158.46)	171.31 ± 4.19^b (160.94–179.56)	171.18 ± 0.92^b (159.41–172.48)	159.38 ± 2.72^a (144.8 –164.28)
Anterolateral arm length	48.29 ± 1.28^a (36.28–50.67)	89.10 ± 1.54^c (76.52–91.85)	87.79 ± 1.33^c (75.19–89.54)	64.70 ± 2.33^b (50.87–68.92)

Mean values in the same row with the same superscript are not significantly different ($P > 0.05$).

Table 5.Survival (%) of early larval stages of *D. setosum* at different salinity levels. All values represent mean ± SE and ranges in parentheses

Larval stages	Salinity (ppt)			
	28	31	34	37
Prism	68.95 ± 4.38[a] (61.48-76.65)	91.24 ± 3.15[b] (87.64-97.52)	88.55 ± 4.34[b] (80.54-95.47)	80.13 ± 1.84[ab] (77.19-83.52)
2-arm pluteus	69.35 ± 5.18[a] (60.28-78.21)	90.64 ± 3.54[b] (85.12-97.26)	88.64 ± 3.83[b] (81.29-94.18)	79.94 ± 2.77[ab] (75.32-84.91)
4-arm pluteus	70.23 ± 5.61[a] (59.87-79.15)	91.89 ± 2.30[b] (88.72-96.37)	87.85 ± 4.25[b] (79.38-92.65)	80.34 ± 0.83[ab] (78.69-81.37)

Mean values in the same row with the same superscript are significantly different ($P \leq 0.05$).

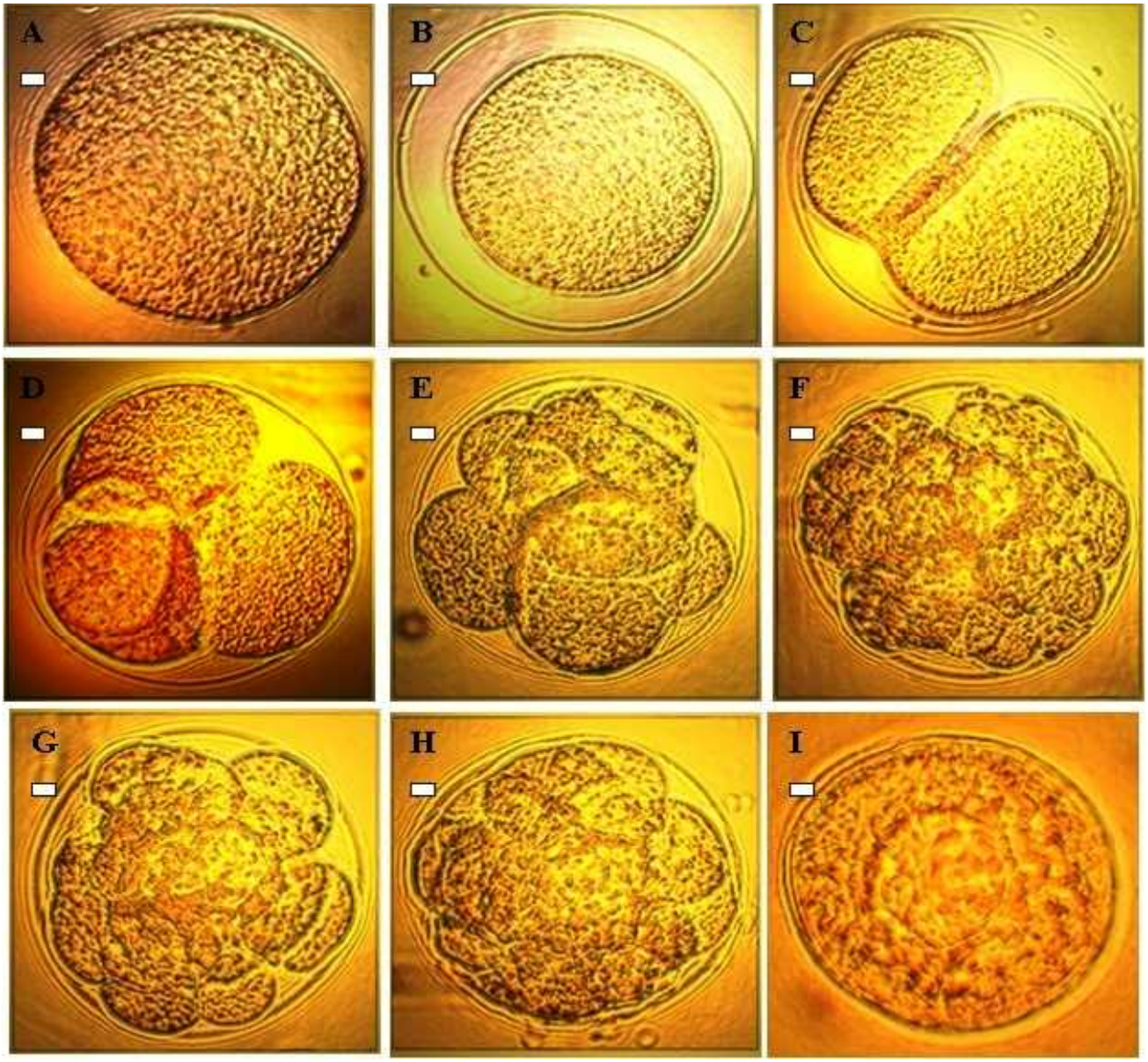

Figure 1.Embryonic developmental stages of *D. setosum* under a compound microscope. A. Fertilized egg showing fertilization membrane, B. Fertilized egg with complete fertilization membrane C. 2-cell stage, D. 4-cell stage, E. 8-cell stage, F. 16-cell stage, G. 32-cell stage, H. Morulla stage enclosed with fertilization membrane, I. Blastula.

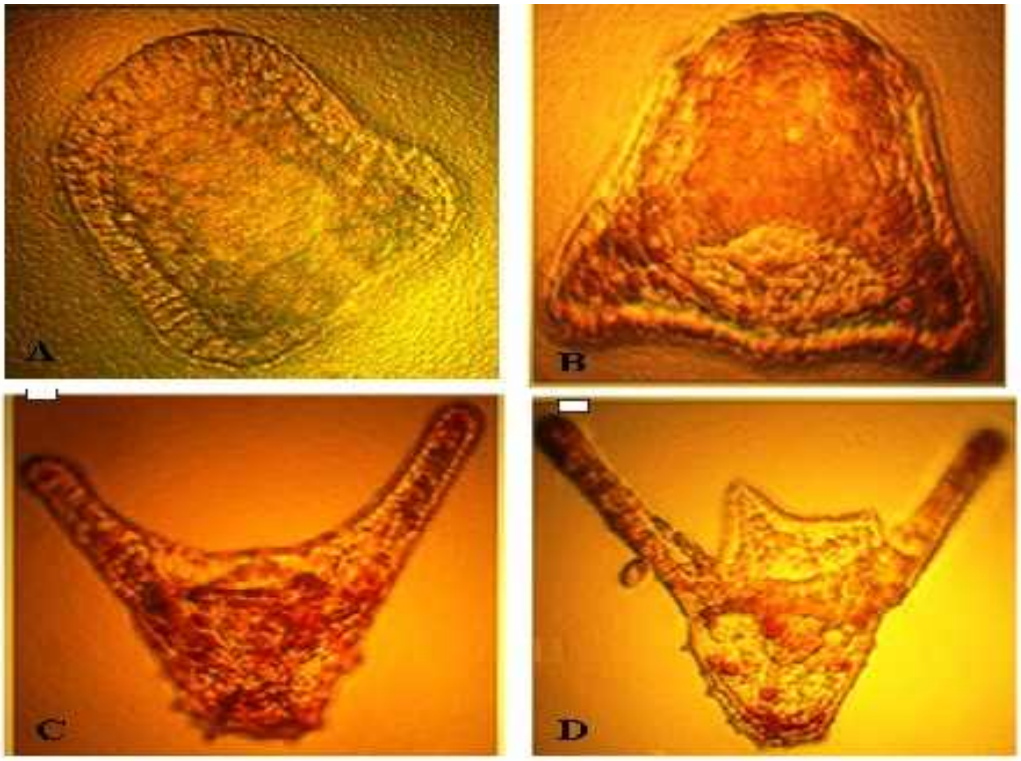

Figure 2: Early larval stages of *D. setosum*under Keyence digital microscope: (A) Gastrula stage; (B) Prism stage; (C) 2-arm pluteus and (D) 4-arm pluteus.

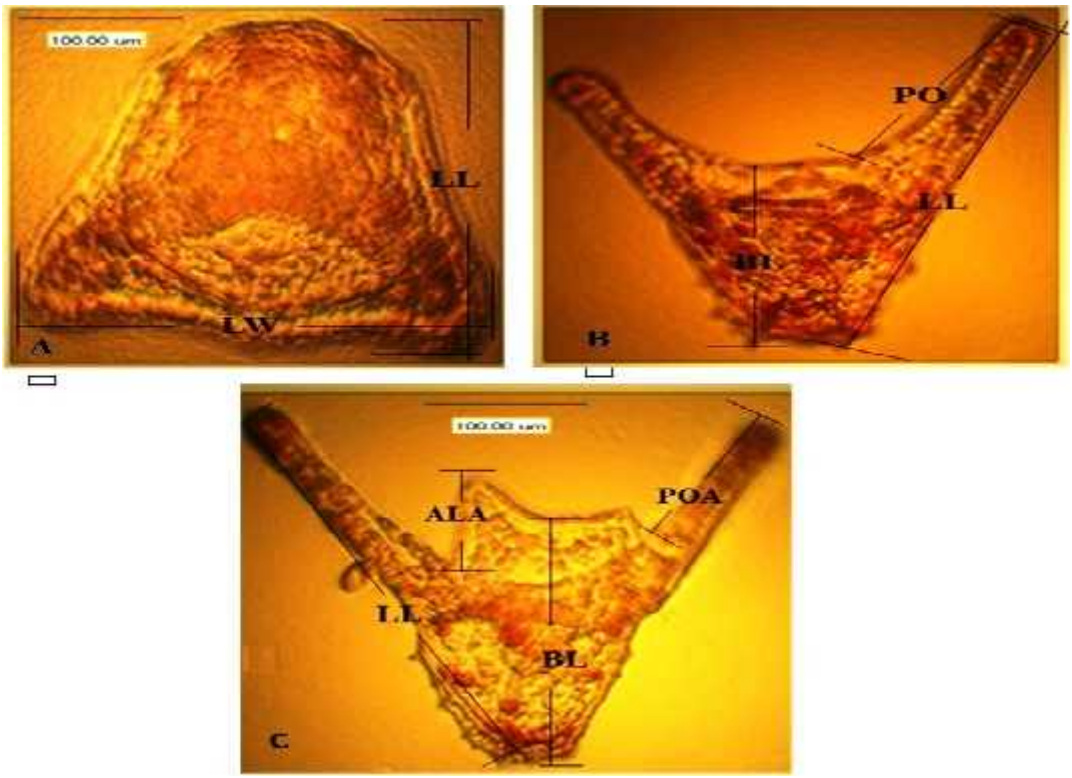

Figure 3: Morphometric measurements of early larval stages of *D. setosum*underKeyence digital microscope: LL=larval length, LW=larval width, BL=body length, POA=post-oral arm length, ALA=anterolateral arm length. (A=Prism; B=2-arm pluteus; C=4-arm pluteus stage).

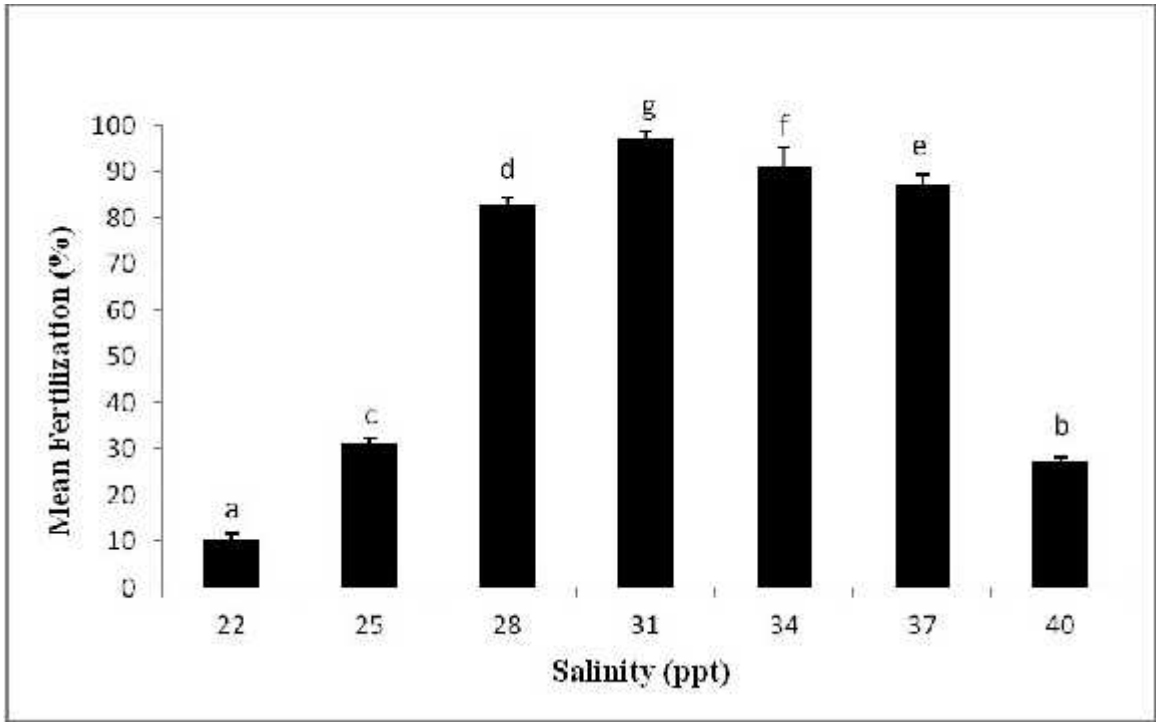

Figure 4: Comparison of fertilization (%) of *D. setosum* eggs at different salinity levels; mean±SE, *n*= 6. Columns with different letters represent means that are significantly different (*P*≤ 0.05).

DISCUSSION

Effect of salinity fluctuation on several sea urchin species have been studied to find out the best salinity level for optimum development and growth of embryo and larva. Most of the studies revealed that larvae of many sea urchin species are stenohaline, where their survival and growth are mostly influenced by salinity changes (Bressen *et al*., 1995; Cowart *et al*., 2009; Allen and Pechenik, 2010).This study represents the first time investigation of salinity effect on fertilization, embryonic and larval development in long-spined black seaurchin *D. setosum*. Results obtained from the present study showed that embryos were successfully survived and then developed through fertilization within the salinity levels from 28 to 37ppt. However, at salinities lower than 28 ppt or greater than 37 ppt, the embryos were developed abnormally or died. Exposure to the extreme salinity levelsmay cause high stress during embryogenesis followed by abnormal development(Roller and Sticker, 1993). In our present study, the best developmental time to achieve each stage of *D. setosum* was at 31ppt compared to other three salinities tested. Slowest development was occurred at lowest salinity levels of 28 pptas was also found in other sea urchin species(Metaxas, 1998; Cowart *et al*., 2009).Bressan *et al*. (1995)reported thathigher salinity level increased the larval development rate. However, in this study the increasing of salinity slowed the development rate by increasing the time taken for each stage to be completed. The highest development rate at 31 ppt salinity level may be explained by the acclimation of gametes to fertilize and cleave well in their naturally adapted water salinity, in which more or less the same salinity level of 32 ppt is maintained at the sampling site. Apart from that, Echinoderms are commonly regarded as stenohaline and are restricted to particular site of high salinity seawater, yet some of them have been found to occur in estuarine habitats. Therefore, some species of sea urchin gametes are able to tolerate to the salinity alteration (Allen and Pechenik, 2010). This further revealed from our study thatthe larvae of *D. setosum* could successfully grow and develop within the salinity range between 28 and 37 ppt.

The length and width of prism larvae did not show any significant differences among the salinity levels tested. However, the highest length and width of larvae were observed at 31ppt. Highest morphometricmeasurements of 2- and 4-arm pluteus were exhibited by larvae at 31ppt, while the lowest values were found at 28ppt. Referring to study that had been done by Roller and Stickle (1993), slightly higher salinity resulted in abnormal development of *Lytechinusvariegatus* larvae in later life. In our study,the growth rate of 2- and 4-armpluteuslarvae of *D. setosum* was decreasing and slowing as they may come to abnormal development or no development until the next few stages before undergoing metamorphosis. Some researchersreported that larval survival and developmental rate

werereducingat lowersalinity levels (Roller and Stickle, 1993; Cowart *et al*., 2009; Allen and Pechenik, 2010).Nonetheless,salinity decreasing within the tolerance range; but not at salinity extreme may improve the growth of larval length as salinity shock induces the pluteus to grow further(Roller and Stickle, 1993; Allen and Pechenik, 2010).

Until now, this is the first trial to investigate the influences of salinity variations on larval developments and morphometric characteristics in the widely distributed species of tropical sea urchin, *D. setosum*. The findings obtained from our present study, would not only be helpful towards the understanding of the suitable salinity level for optimum growth and development of embryos and larvae of *D. setosum* but also to facilitate us for the development of captive breeding and seed production of this important sea urchins for commercial aquaculture industry.

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