Effects of Salinity on Embryonic and Early Larval Development of a Tropical Sea Urchin, *Salmacis sphaeroides*

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Abstract

Effects of salinity on fertilization, embryonic stage, and early larval development and growth performances of short-spined white sea urchin, *Salmacis sphaeroides* were conducted under a controlled laboratory condition. The experiment was carried out with seven salinity treatments (15, 20, 25, 30, 35, 40 and 45 PSU), each of which was triplicated. Significantly highest fertilization success was achieved at 30 PSU, followed by those at 25, 35, 20, 40 and 45 PSU, while the lowest value was obtained at 15 PSU, decreased with increasing and decreasing salinities ($p<.05$).The time required to reach these embryonic and larval stages was increased with the salinity deviations from 30 till the extent to 25 and 35 PSU. No significant differences ($p>.05$) were noted among these three salinity levels on prism larval length and width. However, significance differences ($p>.05$) were noted in morphometric characters of 2-arm and 4-arm pluteus larvae. The findings of the this study indicate that *S. sphaeroides* is stenohaline and do not survive and develop out of the range from 25 to 35 PSU.

Keywords: *Salmacis sphaeroides*, Sea urchin, Salinity, Embryo, Larvae development

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Introduction

Salmacis sphaeroides (Linnaeus, 1758) (Echinodermata: Echinoidea:Temnoplueridae), commonly known as short-spined white sea urchin,is one of the rare species of regular echinoids, although it can be found in the Indo-Pacific area. It has almost cloudy while test (5.0 to 8.0 cm diameter) with numerous short white spines (1.0 to 1.5 cm). Some may have white spines with maroon bands, others with all maroon spines, and yet others with green and maroon bands. It can be found in the warm temperate regions including Johor Straits, between Malaysia and Singapore (Tan and Ng, 1988; Rahman et al., 2012). This species is recognized to present in shallow seagrass beds and coral reef areas (Schoppe, 2000). It frequently feeds on algae that grow on the substrates. This species is also well known by covering themselves with dead coral or detritus.

Embryogenesis is characterized by the immediate cleavage of the fertilized eggs into a greater number of small cell formations (Lepage et al.,1992). The fertilized eggs will establish fertilization envelope and undergo several cleavages including 2-cell, 4-cell, 8-cell, 16-cell stages and so on until a blastula stage (128-cell stage) is formed (Sewell and Young, 1999). During hatching, the fertilization envelope is observed to be thinner and finally vanish as the organism secretes hatching enzyme to digest it (Lepage et al.,1992). After hatching, it is then considered as free swimming blastula. In gastrulation, blastula will be established to the pluteus larval stage, which subsequently exhibits sea urchins' characteristics. Furthermore, during larval development, sea urchins pass through five stages: prism, 2-arm, 4-arm, 6-arm and end up with 8-arm pluteus stage (Metaxas, 1998).

Bressan et al. (1995) suggested that among the abiotic factors, temperature and salinity are considered as the most vital factors on the embryonic development of purple sea urchin, *Paracentrotus lividus*. However, according to some previous studies, the results showed 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) reported that increasing salinities have affected the time needed for embryonic development of *Echinocardium cordatum* in the same temperature. Metaxas (1998) reported that reducing salinities slowed larval development of *Echinometra lucunter.* Allen and Pechenik (2010) suggested that fertilization envelope of eggs seldom rise and even successfully fertilized eggs do not cleave after introducing to low salinity seawater. Salinity tolerance ranges for larvae can be wider or narrower than their adults. Larvae of Atlantic sea urchin, *Echinometra lucunter* are more sensitive to salinities and also can tolerate narrower salinity ranges than the adults (Metaxas, 1998). Low salinity condition reduces feeding rate, decreases growth and therefore limits the size of ectoderms (Forcucci and Lawrence, 1986). Lawrence (1975) reported that decreased salinity causes the reduction of viability and thus results in mass mortality of adult sea urchin, *Lytechznus varrigatus* at Florida. However no such studies have yet been done

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in the tropical species of *S. Sphaeroides.* Therefore, the present work have been undertaken to investigate the effect of salinity on the embryonic and early larval development of this sea urchin in a controlled laboratory condition.

Materials and Methods

A totalof 60 mature adultsof *S. sphaeroides*weighing from 80 to 150g were collected from Merambong shoal off Tanjung Kupang(01°34' N; 103°60' E), Johor, Malaysia at low tide during their natural breeding season from May to August, 2012. Specimens were then transported to 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.

In total,9 male and 9 female adults of sexually matured *S. sphaeroides* weighing from 120 to150g were used for spawning. The spawning was done by injecting 2.0ml of 0.5 M KCl into the celomic cavity of female urchins (Rahman et al., 2000, 2005, 2012). The eggs were collected by inverting the female on a glass beaker filled with 2 µm filtered seawater (FSW). Egg condition and maturity were checked under a compound microscope (Zeiss Axioskop 2) before fertilization. Only eggs having distinct nucleus with uniform shape were used for fertilization experiment (Rahman and Uehara, 2004). After complete shedding, the eggs were washed consecutively with FSW for 3-4 times to remove the debris and immature eggs by sucking out the supernatant seawater (Giudice, 1973). Sperm from each male urchin was observed under a compound microscope to determine their motility (Rahman and Uehara, 2004). Only high motility sperms were used for fertilization trials because it can improve the fertilizationsuccess.

Fertilization was done at room temperature (26 to 28°C). Few drops of diluted sperm solution were pipetted into a small bowl containing egg suspensions. Sperms were left for at least 10 minutes to ensure that all the eggs were encountered by sperms during fertilization process. Excess sperms and debris were then removed from the inseminated eggs by 3 to 4 consecutive washes with FSW (Rahman and Uehara, 2004).

Around 500 inseminated eggs were transferredinto transparent plastic tubes containing 50 ml artificial seawater (Instant Ocean, Aquarium Systems, Sarrebourg, France)each with 7 different salinities (15, 20, 25, 30, 35, 40 and 45 PSU). For this experiment, 30 PSU was set as a control treatment, using normal sea water. Each treatment was conducted for three replicates. Temperature was maintained at 26±2°C for the entire experiment. The first 100 eggs 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).

In each salinity treatment, cleavage (cell division) and early larval stages were observed under microscope as above. Number of embryos that reached to the particular stage was determined. Development rate was then investigated by assessing the time required for certain cell stage (2-cell, 4-cell, 8-cell, 16-cell, morula and blastula stage) to be achieved

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(Figure 1). These stages were checked under a compound microscope(Zeiss Axioskop 2) at hourly interval for at least 50% embryos to achieve the particular stage (Fujisawa, 1993; Rahman et al., 2002). Once the blastula attained the pluteus larva through gastrula and prism stages (Figure 2), the culture was examined daily andnumbers of larva developed into each 2- and 4-arm pluteus stage (Figure 3) were counted by sub-sampling techniques. The time required for both developmental stages (2- and 4-arm pluteus) to be achieved, was also estimated by the duration taken for at least 50% larva to achieve the particular stage (Fujisawa, 1993; Rahman et al., 2002).

Morphometric characteristics of larvae at different salinity levels were measuredand comparedamongthe treatment. These are: larval length(LL), larval width(LW), body length(BL), post oral arm length (POA)and anterolateral arm length(ALA) (Figure 4).Prior to observation, survived embryos and larvae at each stage under different salinities were preserved in eppendorf tube with 10% buffered formalin.They were then placed on microscope slides with cover slip for final morphometric measurements and photographing, using adigital microscope (Keyence VH-S30K).

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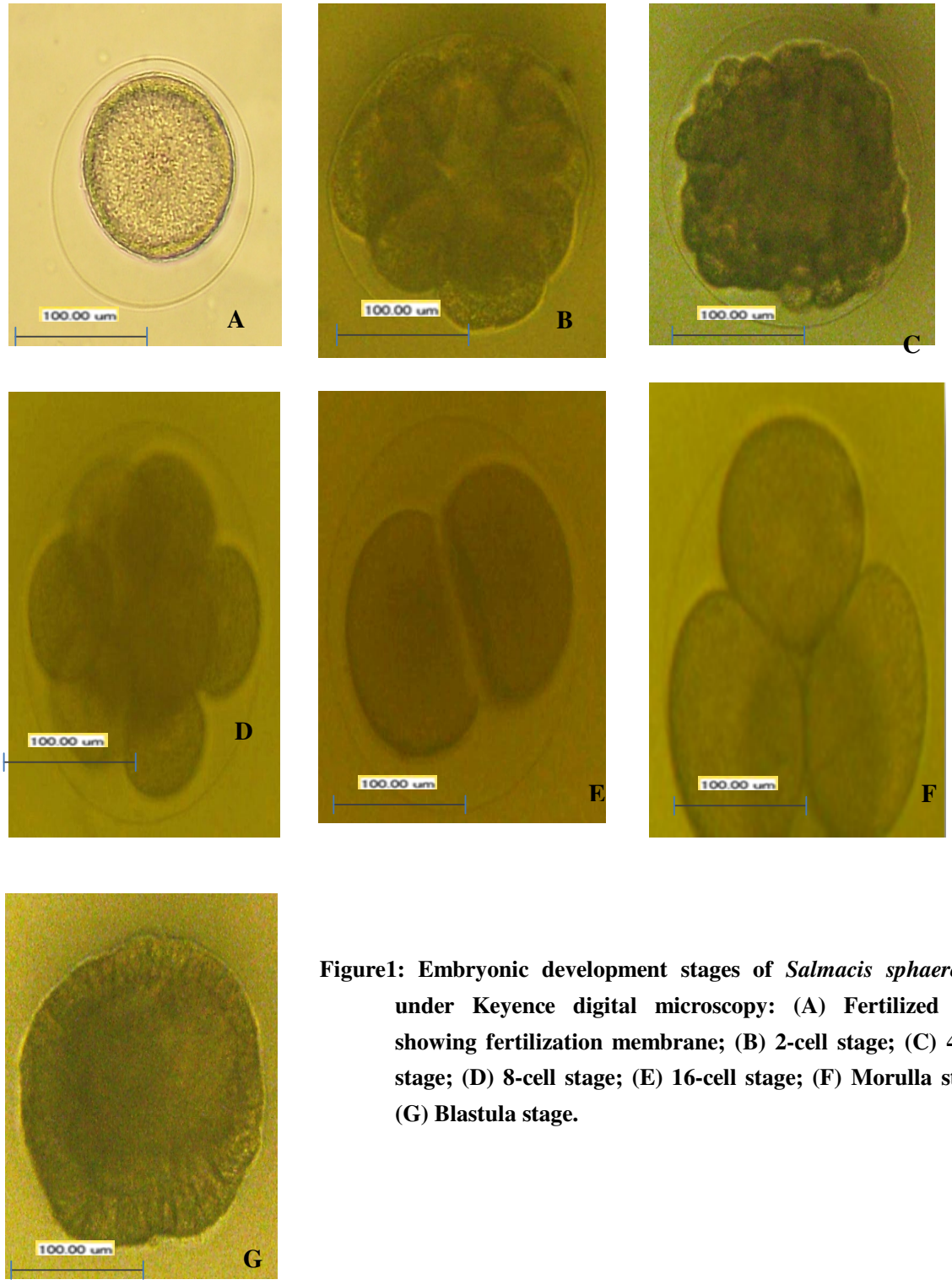

Figure1: Embryonic development stages of *Salmacis sphaeroides* under Keyence digital microscopy: (A) Fertilized eggs showing fertilization membrane; (B) 2-cell stage; (C) 4-cell stage; (D) 8-cell stage; (E) 16-cell stage; (F) Morulla stage; (G) Blastula stage.

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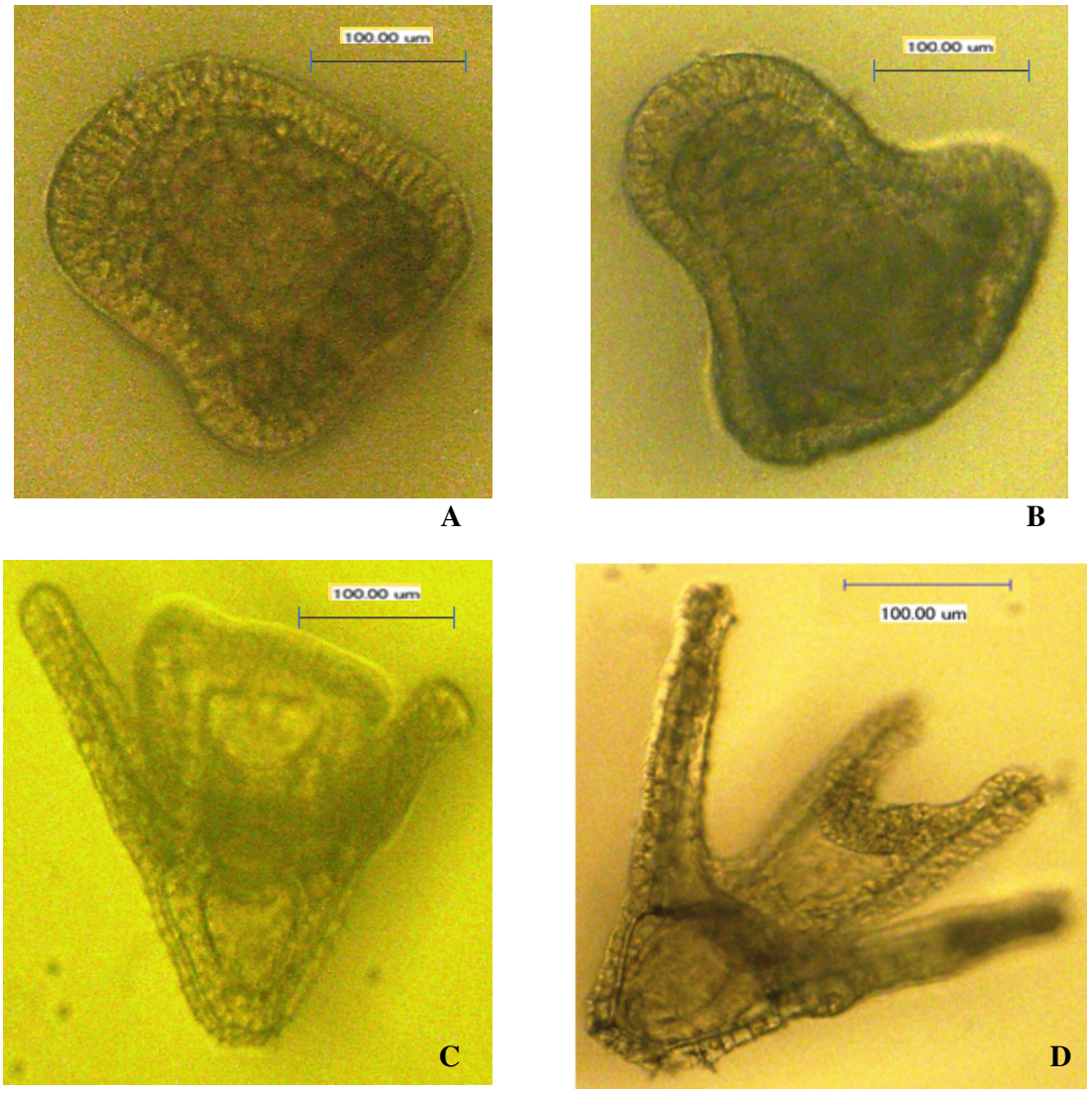

Figure 2:Early larval stages of *S. sphaeroides*under Keyence digital microscopy: (A) Gastrula stage; (B) Prism stage; (C) 2-arm pluteus and (D) 4-arm pluteus

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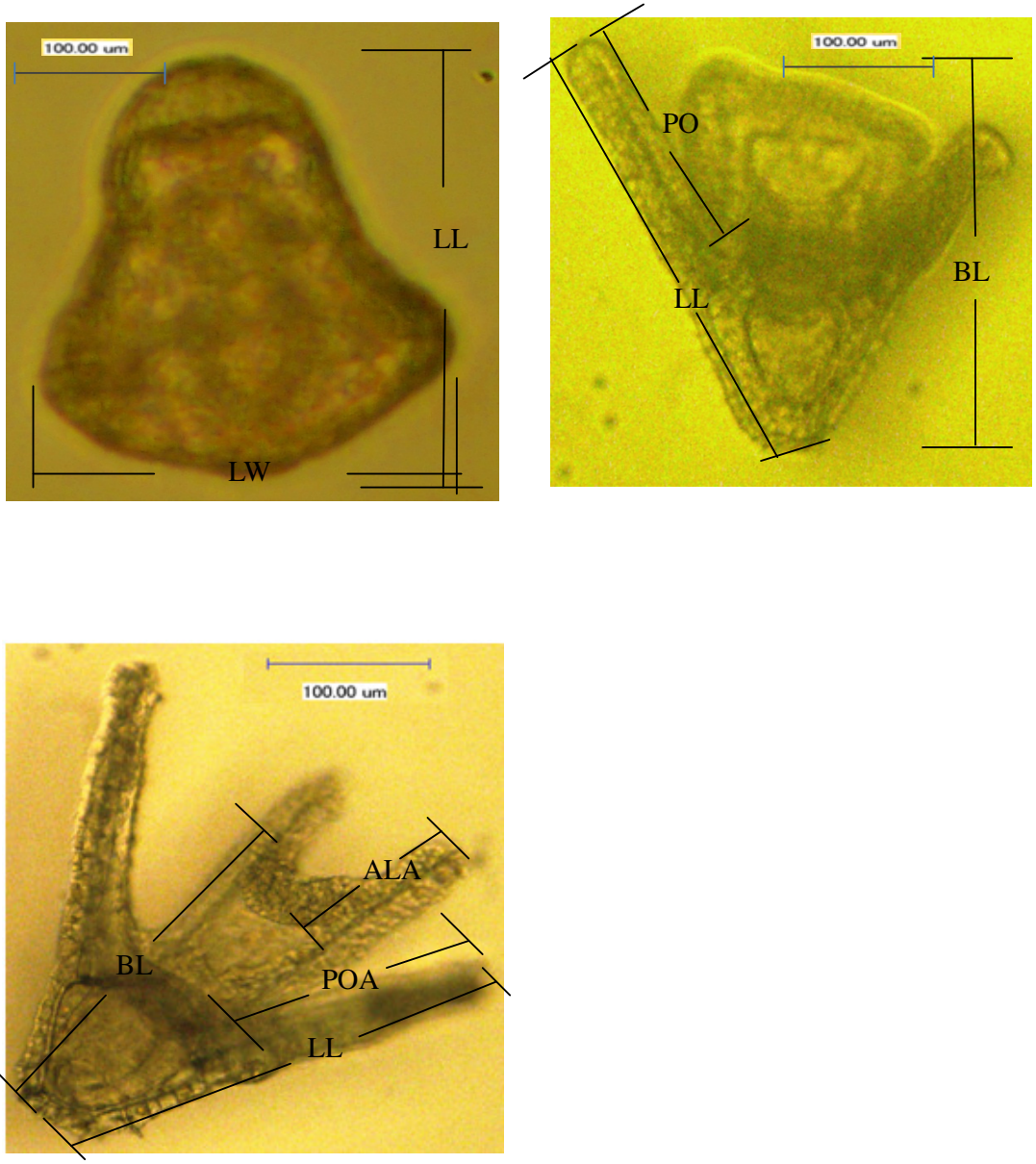

Figure 3: Morphometric measurements of early larval stages of *S. sphaeroides* under Keyence digital microscopy: LL= larval length, LW= larval width, BL = body length, POA= post- oral arm length, ALA= anterolateral arm length

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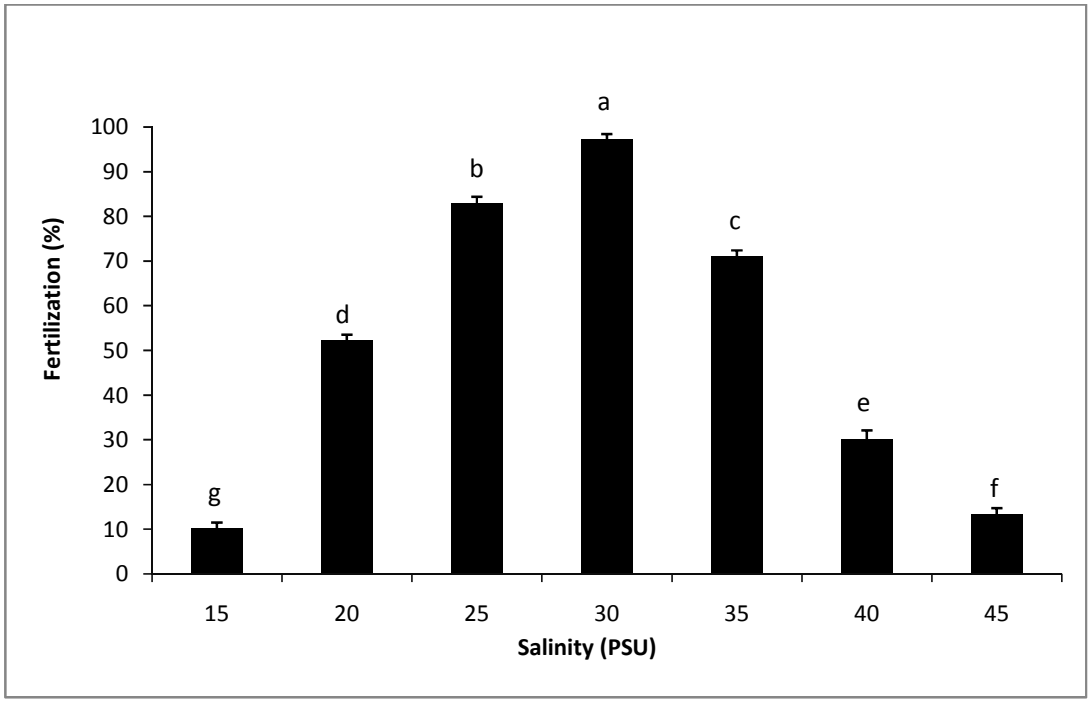

Figure 4: Comparison of fertilization (%) of *S. Sphaeroides*eggs at different salinity levels; mean±SE, *n*= 6. Columns with different letters represent means that are significantly different (*p<.05*)

The data on the fertilization, larval development and growth were analyzed using one-way analysis of variance (ANOVA), followed by Duncan's New Multiple Range Tests (Duncan, 1955). The level for statistical significance was set at 0.05. All statistical analyses were performed by a computerized statistical package "SPSS" version 16.

Results

The percent of fertilization at different salinities is shown in Figure4. The mean fertilization (%) was highest at 30 PSU followed by 25, 35, 20, 40, 45 and the lowest at 15 PSU, decreased with increasing and decreasing salinities (*p<.05*). It could be observed that fertilization success of *S. Sphaeroides* is largely affected by salinity fluctuations.

The effects of salinityon embryonic and larval development of *S. sphaeroides* are shown in Table 1. Embryos at the salinity levels of 15, 20, 40 and 45 PSU were cleaved unequally,developed abnormally or died at the beginning of the experiment, hence they were not analyzed statistically. The 2-cell stage attained within 1.32, 1.19 and 1.41 h post-insemination at 25, 30 and 35 PSU, respectively. The development times of 4-, 8- and 16-cell stages at 25 and 30 PSU showed significant differences (*p<.05*) than those at 35 PSU. Besides that, further cleavages into morula, blastula, gastrula, early prism, 2- and 4-arm pluteus stages showed significant

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differences ($p<.05$) in development times among these three salinity levels. The time taken to reach these stages was increased with the salinity deviations from 30 till the extent to 25 and 35 PSU. Greatest difference in development times was observed in 4-arm pluteus, where the stage occurred within 52.46, 50.19 and 58.11 h at 25, 30 and 35 PSU, respectively.

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

Stages	Salinity (PSU)		
	25	30	35
2-cell	1.32±0.04[a]	1.19±0.04[a]	1.41±0.04[b]
4-cell	1.68±0.09[a]	1.61±0.11[a]	2.32±0.05[b]
8-cell	2.22±0.14[a]	2.18±0.04[a]	3.12±0.06[b]
16-cell	2.99±0.07[a]	2.90±0.09[a]	4.22±0.03[b]
Morulla	4.59±0.12[a]	3.84±0.13[b]	5.54±0.07[c]
Blastula	10.47±0.08[a]	10.19±0.04[b]	10.86±0.04[c]
Gastrula	21.46±0.17[a]	19.10±0.40[b]	23.28±0.16[c]
Early prism	27.23±0.17[a]	25.11±0.40[b]	28.24±0.06[c]
2-arm pluteus	38.79±0.24[a]	36.78±0.43[b]	40.41±0.16[c]
4-arm pluteus	52.46±0.42[a]	50.19±0.29[b]	58.11±0.17[c]

Mean values in the same row with the same superscript are not significantly different ($p>.05$).

Impacts of salinity on early larval growth are presented in Table 2, 3 and 4. As shown in Table 2, only three salinity levels (25, 30 and 35 PSU) had larvae that still alived and reached to prism stage after 24 h of insemination. The highest length and width of prism larvae at 30 PSU were 181.33 and 147.03 µm, while the lowest values of 173.64 and 141.93 µm were observed at 35 PSU. No significant differences ($p>.05$) were noted among these three salinity levels. Comparison of morphometric characters of 2-arm pluteus larvae at different salinities were also investigated (Table 3). In this stage, larvae attained both the highest larval length and body length of 261.16 µm and 202.31 µm at 30 PSU. The lowest larval and body length of 188.61 and 167.35 µm were observed in 2-arm pluteus at 35 PSU. The morphometric characters of 2-arm pluteus at differed salinity levels were also different significantly ($p<.05$). Table 3 showed the four morphometric characters of 4-arm pluteus larval of *S. sphaeroides*. The results demonstrated that 4-arm pluteus at 30 PSU attained the highest larval, body, post oral and anterolateral arm

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length of 332.44, 256.98 and 212.61 µm, respectively. Among the salinity treatments, 4-arm pleteus at 35 PSU possessed the lowest larval, body and anterolateral length of 311.51, 221.80 µm and 105.26 µm, respectively. Lowest post oral arm length of 178.29 µm was observed in 4-arm pluteus at 25 PSU. The body length and anterolateral arm length exhibited significant differences ($p<.05$) among the three salinity levels tested (Table 4).

Table 2: Comparison of two morphometric characters of the larvae of *S. sphaeroides* at prism stage. Thirty larvae were measured for each replicate in each treatment. All values represent mean ± SE in µm.

Morphometric characters	Salinity (PSU)		
	25	30	35
Larval length	178.09±2.80[a]	181.33±5.80[a]	173.64±1.86[a]
Larval width	141.09±4.36[a]	147.03±6.01[a]	141.93±2.82[a]

Mean values in the same row with the same superscript are not significantly different ($p>.05$).

Table 3: Comparison of three morphometric characters of the larvae of S. sphaeroides at 2-arm stage. Thirty larvae were measured for every replicate in each treatment. All values represent mean ± SE in µm.

Morphometric characters	Salinity (PSU)		
	25	30	35
Larval length	223.20±9.64ab	261.16±20.69b	188.61±5.77a
Post oral arm length	113.11±7.46b	150.48±12.68a	86.43±4.12c
Body length	177.33±12.52ab	202.31±7.45a	167.35±6.32a

Mean values in the same row with the same superscript are not significantly different ($p>.05$).

Table 4: Comparison of four morphometric characters of the larvae of S. sphaeroides at 4-arm stage. Thirty larvae were measured for each replicate in each treatment. All values are measured in µm and represent mean ± SE in µm.

Morphometric characters	Salinity (PSU)		
	25	30	35
Larval length	317.05±13.57[a]	332.44±10.28[a]	311.51±26.89[a]
Post oral arm length	178.29±10.58[a]	212.61±11.20[a]	202.91±15.62[a]
Body length	228.72±8.95[ab]	256.98±4.13[b]	221.80±15.03[a]
Anterolateral arm length	95.12±7.73[b]	105.26±3.91[a]	86.88±11.29[b]

Mean values in the same row with the same superscript are not significantly different ($p>.05$).

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Discussion

Several sea urchins have been studied to investigate the effects of salinity fluctuations on their embryonic and larval development. The results demonstrated that larvae of many sea urchin species are stenohaline and their survival and growth are greatly affected by salinity changes. (Bressen et al., 1995; Cowart et al., 2009; Allen and Pechenik, 2010). In this study, effects of salinity on fertilization, embryonic and larval development were investigated for the first time in tropical sea urchin, *Salmacis sphaeroides.* The results showed that embryos successfully survived and developed through fertilization within 25 to 35 PSU. There was abnormal development or no development occurred at salinities lower than 25 PSU or greater than 35 PSU. Extreme salinity may cause stress during embryogenesis and results in abnormal development (Roller and Sticker, 1993). In our present study, significantly lower development time was required to achieve each stage at 30 PSU compared to other two salinities tested. Lower salinity slowed the development rate of embryo (Metaxas, 1998; Cowart et. al.,2009), while higher salinity level increased the larval development rate (Bressan et. al., 1995). However, in this study, the higher salinity slowed the development rate by increasing the time taken for each stage to be attained. The sensitivity and reaction of *S. sphaeroides* larvae were unparalleled in the literature. This may be explained by the acclimation of gametes to fertilize and cleave well in their naturally adapted water salinity in which 30 PSU filtered seawater is more or less the same with the salinity level of 29 PSU at the collection site. Future studies can be worked on this conflict to investigate the effects of higher salinity on embryonic and larval development of *S. sphaeroides*. 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. Hence, gametes of some species are able to acclimate to the salinity alteration (Allen and Pechenik, 2010). This further explained the success of *S. sphaeroides* larvae to grow and develop at 25 and 35 PSU.

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 *S. sphaeroides* larvae were observed at 30 PSU. Highest morphometric measurements of 2- and 4-arm pluteus were exhibited by larvae at 30 PSU while the lowest values were found at 35 PSU. As referred to the study that had been done by Roller and Stickle (1993), slightly higher salinity resulted in abnormal development of *Lytechinus variegatus* larvae in later life. Therefore, our study suggest that the growth rate of morphometric characters of 2- and 4-arm pluteus was decreasing and slowing as they may come to abnormal development or no development up until next few stages before metamorphosis. Lowered salinity reduces the larval survival and development rate (Roller and Stickle, 1993; Cowart et al., 2009; Allen and Pechenik, 2010), nevertheless slightly decreased salinity within the tolerance range but not at salinity extreme may improve the growth of larval length as salinity shock

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induces the pluteus to grow further. To date, this is the first attempt to demonstrate the effects of salinity on larval morphometric development in tropical sea urchin,*S. sphaeroides*. The findings emerged from the designated study would greatly be helpful towards the development of induced breeding, larval rearing and seed production of this commercially important sea urchins for aquaculture industry. However, further research should be undertaken to determine the optimum salinity level within the range from 25 to 35 PSU for the best embryonic and larval development and growth of*S. sphaeroides* in captive rearing condition.

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