www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 16: 669-677 (2016) DOI: 10.4194/1303-2712-v16_3_21

RESEARCH PAPER

Embryonic Development and Hatching Rate of Blue Swimming Crab, Portunus Pelagicus (Linnaeus, 1758) under Different Water Salinities

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Received 28 December 2015
Accepted 24 May 2016

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The effects of different salinity levels on egg size, spawning and hatching success of the blue swimming crab, Portunus pelagicus were studied. The morphology of embryo were observed and classified into 10 embryonic stages. This study is important to expand the knowledge on P. pelagicus embryo and it's hatching mechanism. Berried female incubated in 5 ppt did not survive thus any further study in this treatment was not conducted. Female incubated 15 ppt did not spawn and those incubated in 45 ppt had retarded development and eventually relaesed, thus the study on egg size was not conducted. The prehatch stage mean egg diameter that incubated in 25 ppt was largest compared to both incubated in 30 ppt and 35 ppt. Higher percentage increase in egg size occurred mostly at stage near hatching. Total incubation period for berried female incubated in 25, 30 and 35 ppt was 10 days.The morphological characteristic of P. pelagicus embryo was almost the same as other brachyuran crab in which, the appendage will form followed by eye formation, present of chromatophore, heartbeat and then ready to hatch. The results from the study could be used for further in vitro fertilization techniques of crustacean culture.

Keywords:Crustacean, reproductive biology, environmental factor, embryology study.

Introduction

Blue swimming crab, Portunus pelagicus (Linnaeus, 1758) is one of the most commercially important species (Azra and Ikhwanuddin, 2015; Ravi and Manisseri, 2013) and enjoys high market demand for their delicacy. New market chances and increasing price of *P. pelagicus* have contributed to higher number of *P. pelagicus* being harvested (Ikhwanuddin *et al*., 2009). The crab fishery and culture operations are expected to continue to grow in the future (Ikhwanuddin *et al*., 2012a). The study of embryonic development is important since this is the most sensitive stage to environmental changes (Garcia-Guerrero and Hendricks, 2006). Study of salinity affected the reproduction were mostly done in juveniles stages, and little on embryonic stages (Romano and Zeng, 2006; Romano and Zeng, 2010).

The study on embryology of Portunid crabs on embryonic development are such as on *Scylla spp.* (De Haan, 1833) (Churchill, 2003; Ates *et al.*, 2012), *P. pelagicus* (Ikhwanuddin *et al.*, 2012b), *Scylla olivacea* (Herbst, 1796) (Ikhwanuddin *et al.*, 2015), *Cyrtograptus angulatus* (Dana, 1851)and *Chasmagnathus granulata* (Dana, 1851) (Bas and Spivak, 2000), *Chasmagnathus granulata* (Dana, 1851) (Gimenez and Anger, 2001), *Ucides cordatus* (Linnaeus, 1763) (Pinheiro and Hattori, 2003), *Eurypanopeus canalensis* (Abele & Kim, 1989)and *Panopeus chilensis* (H. Milne Edwards & Lucas, 1843)(Garcia-Guerrero and Hendrickx, 2006) and *Perisesarma bidens* (De Haan, 1835) (Sarker *et al.*, 2009) have also been done. There is still, however lack of information and the study on the effect of salinity on embryonic development on *P. pelagicus*. Even though *P. pelagicus* is a marine organism, a study on the effect of different salinity regimes can traditionally explain the process of osmosis during embryonic development and its effect on hatching rate and mechanisms. This study is also important to expand the knowledge of embryology in *P. pelagicus*. From this study, the suitable salinity for rearing mated and berried female of *P. pelagicus* in hatchery can be determine so the maximum yield can be obtained.

The objectives of the study are i) to describe the embryonic development of *P. pelagicus* and ii) to determine the effect of different salinities (5, 15, 25, 30, 35 and 45 ppt) on the spawning success, egg sizes and hatching rate of *P. pelagicus.*

Materials and Methods

Broodstock Management

Liveberried females were collected from GelangPatah, Johor (1°22’60N, 103°37’60E) coastal water, Peninsular Malaysia and were transported to marine hatchery of the Institute of Tropical Aquaculture (AKUATROP), University Malaysia Terengganu (UMT), Malaysia. The egg mass of the samples collected were yellowish in colour. Filtered sea water and freshwater was used to prepare the different salinities Artificial marine salt was used to prepare salinity higher than seawater salt concentration. Salinity and temperature of the water was monitored using multi-parameter instrument YSI 63. The temperature ranged from 26 to 29 °C. In each tank, 0.8 g of oxytetracycline (OTC) was added once to 100 L of prepared water to inhibit pathogens. Sandy substrate placed in container was placed in each 100 L tank. The water stocks were aerated for one day before placing in the berried females. The newly arrived females were acclimatized in 30 ppt for one whole night. The berried females were slowly acclimatized in different salinities for an hour in each salinity before placing it in experimental salinity (5, 15, 25, 30, 35 and 45 ppt). Food was not given to berried females. Any present of faeces were siphoned out from time to time. Experiment start after spawning and ended when the berried females hatched which takes about two weeks depending on the water conditions. Berried females were stocked one berried female per tank and the identification of berried female is according to the study by Ikhwanuddin *et al.* (2012b). The value of salinity and temperature were in range between 25.6-28.7 ppt and 29.37-31.8°C.

Egg Observation

Minimum of 30 eggs were removed from the berried females and placed in urine bottle 100 mL containing seawater of respective tank. It was then pipetted using dropper and placed in excavated slide and observed under measuring microscope Nikon Motorized Multi-Purpose Zoom Microscope Multizoom (AZ100M). Pictures were taken and egg diameter was measured to the nearest to 0.01 µm. The mean egg diameter from two measurements was taken as the egg size of *P. pelagicus*. The morphology of *P. pelagicus* eggs was observed using AZ100M. The embryonic developments were described as 10 embryonic stages. The females where eggs had hatched were maintained at previous salinity for next batch of spawning. The spawned crabs were used in spawning success study and its eggs were used for early embryonic development observation. The period for spawning success study was two weeks for each female. For newly spawned female, observation was conducted every half hour to catch cell division until multicell stage. For later stages, the eggs were removed every morning for observation. The females maintained in 5 ppt (n = 3) did not survive, while female maintained in 15 ppt (n = 3) did not spawn. Female maintained in 45 ppt (n = 3) did spawn, but the embryo had retarded development at multicell stage and eventually released their egg mass. Thus, there were no further observations on egg size for females maintained in these salinities.

Hatching Rate

Another batch of berried females which has yellowish eggs was collected from sampling site and was used for hatching success. The same procedures were used to acclimatize the samples. In preliminary study, the females maintained in 5 ppt, did not survive, thus no further study was conducted on effect of this salinity on hatching rate of *P. pelagicus*. The berried females were weighted using portable weight balance. Minimum of 300 eggs were collected from the berried females. It was weighted and counted. After hatching occured, the berried females were weighted again. The fecundity for each female was calculated as follows;

$$\text{Fecundity}=(\text{Total egg mass})/(\text{Mass of individual egg})$$

The larvae that hatched were counted using volumetric sample analysis, where 10 replicates of 10 ml of 100 L water from different depths were collected using 10 ml of pipette. The numbers of larvae were counted and the mean was calculated as density in 10ml. The estimated number of larvae hatched in 100 L was calculated. The hatching rate was calculated using the formula;

$$(\text{No.of larvae hatched})/\text{Fecundity}\times 100\%$$

Statistical Analysis

The egg diameter was expressed as mean ± standard error of mean. Post hoc test was used to further analyze if there is significant difference ($P<0.05$) using SPSS version 20.0. Linear equation of mean egg diameter versus incubation period was plotted for each salinity. Spawning success was presented as percentage for each treatment. Hatching rate was stated as percentage of larvae hatched over fecundity for each sample in different salinity regimes.

Results

Morphological Characteristic of *Portunus Pelagicus* Embryo

The structures observed were appendage bud, abdomen, eye, heart, telson, chromatophore and cephalothorax. The proportion of yolk was used to distinguish the eye formation stage from thoracico abdominal stage and heartbeat stage from prehatch stage. Cell division occurred between 1500 to 1700 hours. Unsynchronised cell division between different eggs was observed. The time taken for two cell division to four cell division was short. Unsynchronised development was also observed during gastrula and naupliar stage. The embryo started to move during Prehatch stage when most of the characteristics of zoea can be observed. Table 1 shows the description of embryonic development and Zoea 1 stage of *P. pelagicus*, while Figure 1 and 2 shows the embryonic development and Zoea 1 stage of *P. pelagicus* respectively.

Effect of Different Salinity Regimes on Spawning Success of *Portunus Pelagicus*

Three replicates were used for each salinity treatment and two week period for spawning observation was conducted for each sample. *P. pelagicus* showed greatest spawning success at 30 and 35 ppt. No spawning was observed for females placed in 15 ppt, although the females did survived. Further observation was conducted by removing the carapace to see the ovarian development which was light orange in colour. The female did not survive at 5 ppt. Table 2 shows the spawning success of *P. pelagicus* in different salinity regimes with the mean carapace width ± SD of females used in the present study.

Effect of Different Salinity Regimes on Egg Sizes of *Portunus Pelagicus*

Mean egg diameter of *P. pelagicus* incubated in 25 ppt ranged from 301.60 to 380.24μm which was the largest egg. While egg diameter of *P. pelagicus* incubated in 30 and 35 ppt ranged from 294.76 to 365.23μm and 295.23 to 357.34μm respectively (Table 3). Precleavage, Naupliar, Eye formation and Prehatch stage. Gradual increase in egg size was observed for the three treatments except for certain stages. Ones incubated 35 ppt showed the largest decrease in mean egg diameter from cell division stage to multicell stage with percentage of 5.55%. The eggs incubate in 35 ppt also shows largest increase at eye formation stage to thoracico abdominal stage with percentage of 7.45% (Table 4). The water salinity treatment of 25 ppt is the most suitable salinity regime for the embryonic developmental stages because it produced final 23.55% of the embryonic stage to developed into the next stages as compared to the only 21.92% and 14.97% for 30 ppt and 35 ppt treatment respectively (Table 4). The size development of egg of P. pelagicus throughout the incubation period in three different salinities was in a linear equation with $y= 8.345x+290.9$ and R2=0.763 for 25ppt, $y= 7.964x + 285.6$ and $R^2=0.789$ for 30 ppt and $y= 8.216x + 280.8R^2=0.662$ for 35ppt salinity regimes respectively. As the incubation period increase, the egg diameter increase gradually. Total incubation period for berried female incubated in 25, 30and 35 ppt was 10 days. Berried females incubated in 25 ppt had shorter Prehatch stage (1 day) compare

Table 1. The description of embryonic development and zoea 1 stage of *P. pelagicus*

Stage	Description
Embryo	
Precleavage	The egg filled with yolk. Fertilization membrane can be observed (Fig. 1a). The egg is spherical in shape. 1^{st} polar body and 2^{nd} polar body appear right before cell division (Fig. 1b).
Cleavage	Cleavage furrow form (Fig. 1c, 1d). Certain have equal cleavage at two-cell stage while others not. The egg is spherical to ovoid shape. The cells continue to divide until multicell.
Multicell	Egg filled with divided cells (Fig. 1e). Formation of one to two patch of presumptive primordial cell known as tissue cap (Fig. 1f). Egg is mostly ovoid.
Intermediate multicell-gastrula	One or two yolk free portion formed (Fig. 1g). Egg is spherical in shape.
Gastrula	Formation of U-shape band that is the germinal disc of embryo. Formation of tissue (appendage bud) is clear at yolk free portion. Optic lobe can be differentiated (Fig. 1h). Egg is spherical in shape.
Naupliar	Appendage bud elongated (Fig. 1i). Yolk free portion increase in size.
Eye formation	Present of short, slightly curve and thin strip of eye at optic lobe (Fig. 1j). Egg spherical in shape.
Thoracico-abdominal	Thorax and abdominal regions was differentiated. Eye enlarge to ovoid sharpen at one end (Fig. 1k). Yolk is bilobed structure (Fig. 1l). Light lining of chromatophore present at vascular system.
Heartbeat	Heartbeats present (Fig. 1m). Eye enlarge and darker. Chromatophore obvious at abdomen part. Formation of telson at the abdomen which curved near the optic lobe (Fig. 1n). Slight movement of embryo.
Prehatch	Zoea clearer (Fig. 1o). Cephalotorax more defined (Fig. 1r). Cornea developed; compound eye observed; eye completely differentiated (Fig. 1p). Chromatophore clearer at abdomen and cephalotorax (Fig. 1q). Regular movement of embryo.
Zoea	
Zoea 1	Zoea has five abdomens including the telson. Chromatophore clearly separate the abdomen. Telson is fork shaped. Eyes is sessile (Fig. 2)

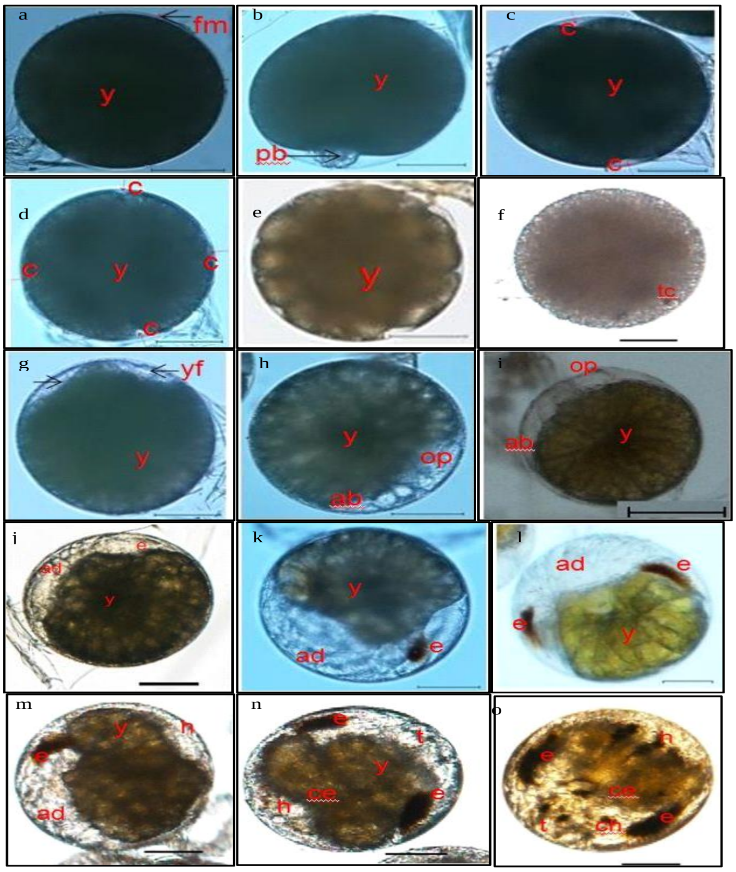

Figure 1. The embryonic development of *P. pelagicus* under 35 ppt salinity regime; (a-b) Precleavage, (c-d) Cleavage, (e-f) Multicell, (g) Intermediate multicell-gastrula, (h) Gastrula, (i) Naupliar, (j) Eye formation, (k-l) Thoracico-abdominal, (m-n) Heartbeat, (o-r) Prehatch; ab, appendage bud; ad; abdomen; c, cleavage; ce, cephalothoraxes; ch, chromatophore; co, cornea; e, eye; fm, fertilization membrane; h, heart; op, optical; pb, polar body; t, telson; y, yolk; yf, yolk free; scale bar in each Fig. indicate 100µm.

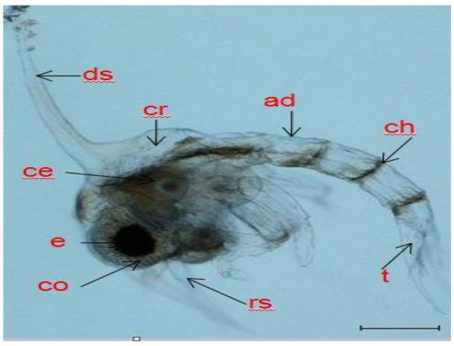

Figure 2. First zoea of *P. pelagicus*; ad, abdomen; ce, cephalothoraxes; co, cornea; cr, carapace; ds, dorsal spine; e, eye; rs, rostral spine; t, telson; scale bar in Fig. indicate 100µm.

to berried female incubated in 30 ppt (2 days) and 35 ppt (3 days) (Table 5).

Effect of Different Salinity Regimes on Hatching Rate of *Portunus Pelagicus*

For treatments 25 and 30 ppt, only one replicate showed synchronized hatching with hatching percentage of 38.92% and 83.97% respectively, while two replicates from treatment 5 and 45 ppt shows synchronized hatching. Berried females incubated in 15 ppt released their eggs after reaching Prehatch stage (Table 6).Unsynchronized hatching occurred in all treatments for at least one replicate except in 15 ppt where hatching does not occurs for all the replicate.

Table 2. Spawning success rate of *P. pelagicus* in different salinity regimes

Salinity (ppt)	Spawning success (%) (n=3)	Mean carapace width ± SD (cm)
5	0	10.372 ± 0.782
15	0	11.288 ± 0.905
25	33.33	10.045 ± 0.546
30	100.00	11.542 ± 0.932
35	100.00	11.301 ± 0.488
45	66.66	10.974 ± 0.645

Table 3. Mean egg diameter with different salinity regimes 25, 30 and 35ppt according to different stages of embryonic development. Different letter in each colums showed significant different (P<0.05) between salinity regimes of the present study

	Salinity (ppt)					
	25		30		35	
Stage	No. of eggs	Mean egg diameter±SD	No. of eggs	Mean egg diameter±SD	No. of eggs	Mean egg diameter±SD
Precleavage	79	301.60±3.6855[b]	39	294.76±2.9662[a]	73	309.49±5.1548[c]
Cleavage	65	307.78±2.7054[a]	47	308.71±4.2724[a]	17	312.58±3.9499[b]
Multicell	17	306.93±1.7054[b]	64	308.85±1.1633[b]	62	295.23±3.2672[a]
Intermediate multicell-gastrula	41	312.87±3.6848[b]	49	310.52±2.3591[b]	84	302.06±3.3040[a]
Gastrula	109	322.17±4.9570[b]	97	304.72±2.0439[a]	97	304.01±4.8144[a]
Naupliar	176	326.76±4.1745[c]	61	317.00±2.9881[b]	156	307.19±3.7514[a]
Eye formation	102	338.37±2.1993[c]	96	324.47±3.7732[b]	160	310.98±5.4385[a]
Thoracico-abdominal	90	350.93±4.5953[b]	80	335.66±3.8777[a]	40	334.15±4.1854[a]
Heartbeat	84	364.50±2.6821[b]	130	351.30±4.1075[a]	98	349.16±3.8899[a]
Prehatch	46	380.24±3.7757[c]	234	365.23±5.6390[b]	76	357.34±2.0412[a]

Table 4. Stage wise change in egg diameter (%) in different salinity regimes

Stage		Percentage changes from A to B (%)		
A	B	25 ppt	30 ppt	35 ppt
Precleavage	Cleavage	2.05	4.73	1
Cleavage	Multicell	-0.28	0.05	-5.55
Multicell	Intermediate-multicell gastrula	1.94	0.54	2.31
Intermediate-multicell gastrula	Gastrula	2.97	-1.87	0.65
Gastrula	Naupliar	1.42	4.03	1.05
Naupliar	Eye formation	3.55	2.36	1.23
Eye formation	Thoracico-abdominal	3.71	3.45	7.45
Thoracico-abdominal	Heartbeat	3.87	4.66	4.49
Heartbeat	Prehatch	4.32	3.97	2.34
Final percentage from A to B		23.55	21.92	14.97

Discussion

In this study, the embryonic development of *P. pelagicus* were described into 10 embryonic stages based on the morphological changes and yolk consumption. Study on embryonic development of *P. pelagicus* by Soundarapandian and Tamizhazhagan (2009) used five stages that are blastula, gastrula, eye placode, pigment and heartbeat. While study done by Arshad *et al.*(2006) classified the embryonic development based on the macroscopic observation in the same time described the embryo development into eight stages. Ikhwanuddin *et al.* (2015) classified the embryonic development based on the macroscopic observation of the egg colour. Despite the different stages used, the embryonic development in this study matched up with other studies. The polar body will form the ectoderm (Browne *et al.*, 2005) which is the outermost among the three primary germ layers.

The formation of U-shaped band at Gastrula stage indicates the first embryonic morphology in mostbrachyurans (Furota, 1996). The onset of heartbeat indicates the living embryo (Nagao, 1999).In this study, the females used for spawning success is not the first time spawners. The study used the one that had hatched their egg mass before since the brachyuran female can produce at least two batches of egg in a season (Kumar *et al.*, 2003). Females placed in 15 ppt did not spawn, yet ovary was light orange in colour indicating it was at late

Table 5. Incubation period (days) of berried Portunus pelagicus incubated in three different salinities. (PC) Precleavge, (C) Cleavage, (M) Multicell, (MG) Intermediate multicell-gastrula, (G) Gastrula, (N) Naupliar, (EF) Eye formation, (TA) Thoracico abdominal, (HB) Heartbeat, (PH) Prehatch. Different letter in each rows showed significantly different (P<0.05) between salinity regimes in each embryonic development

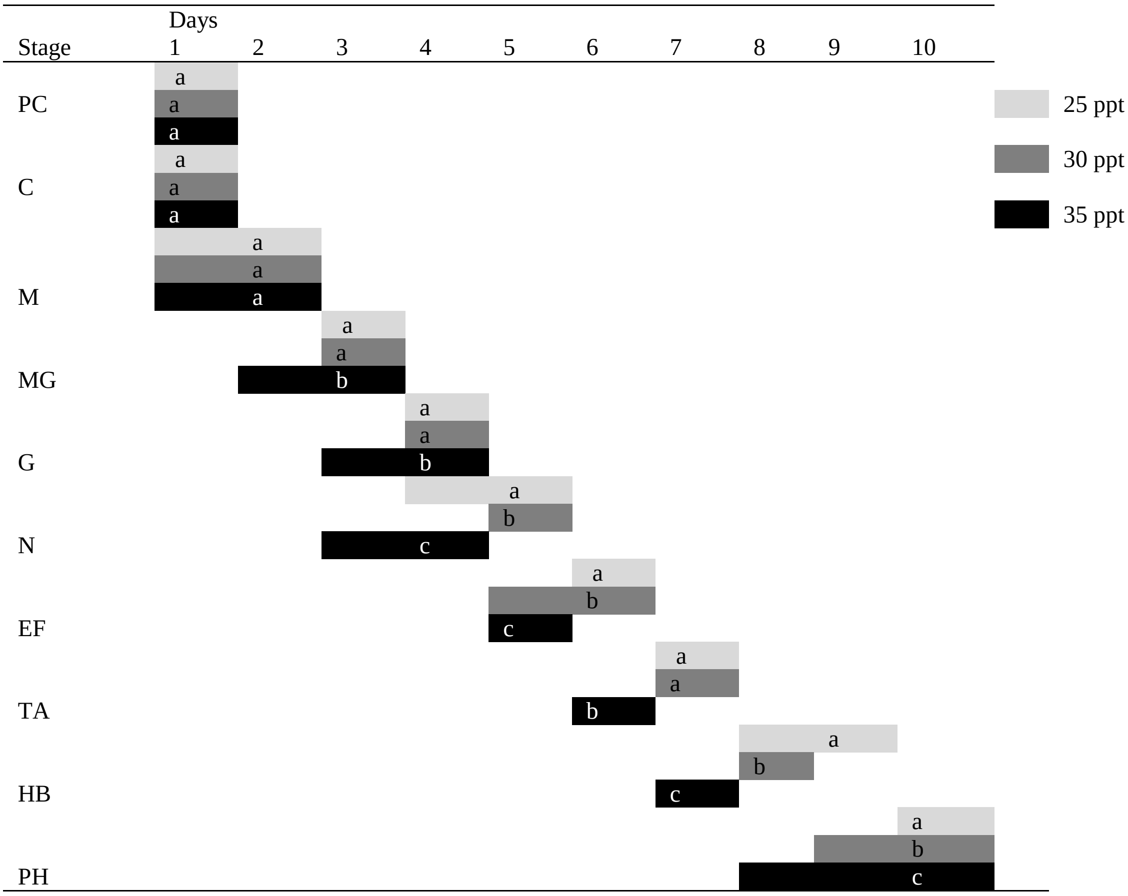

Table 6. Hatching rate (%) of berried female of *P. pelagicus* at different salinity regimes

Salinity (ppt)	Hatching rate (%)		
	Replicate 1	Replicate R2	Replicate R3
15	0	0	0
25	38.92	Unsynchronized	Unsynchronized
30	83.97	Unsynchronized	Unsynchronized
35	87.96	90.47	Unsynchronized
45	44.58	38.57	Unsynchronized

maturity stage (Ikhwanuddin *et al.*,2015). In this study, 100% spawning occurred for female placed in 30 and 35 ppt while 66.66% spawning occurred for female placed in 45ppt; yet the development of embryo was arrested at multicell stage. In natural habitat, berried females *P. pelagicus* were believed to migrate to ocean at deeper water (de Lestang *et al.*, 2003) which has salinity range 30 to 35 ppt. Low spawning success was observed for females placed in 25 ppt. This probably indicated that the females of *P. pelagicus* did not tolerate wide fluctuation of salinity for spawning in hatchery.

This study shows that the egg that incubated in 35 ppt has larger egg diameter compare to ones incubated in 30 and 25 ppt during the precleavage and cleavage stage, while embryo incubated in 30 ppt has larger egg diameter than ones incubated in 35 and 25 ppt during multicell stage. This can be explain through the morphology of outer membrane of embryo which are thicker during early developments compared to when near hatch (Lee, 2011). The thicker membrane may act as barrier to reduce the water uptake. Genetic, female age and other factors may be responsible for different egg size produce (Gimenez and Anger, 2001). Later stage shows embryo in low salinity has mean egg diameter larger compare to one incubate in higher salinity. Higher percentage of increment in egg size occurs at stage near to hatch for the three different treatments. This was almost parallel to study done by Ates *et al.*(2012). The increase in egg diameter at later stage can be explained from the uptake of water which occurs mainly at heartbeat stage of anomura and brachyura (Ates *et al*., 2012).

The incubation period of P. pelagicus embryo for the three treatments was 10 days. Arshad *et al.* (2006) and Soundarapandian and Tamizhazhagan (2009) recorded the incubation period of *P. pelagicus* wassix to seven days. Embryo incubated in 35ppt reach stage thoracico-abdominal earlier than ones incubated in 25 and 30ppt. This stage and near hatching was when the yolk was used rapidly. This could be associated with faster metabolic rate and due to its small size. Taylor and Leelapiyanart (2001) study on big hand crab, Heterozius rotundifrons (A. Milne Edwards, 1867)embryos showed that smaller eggs has faster metabolic rate resulting in short incubation period. Retarded development of embryo can occurs due to fungal infection or abnormal morphology of the embryo (Ates *et al.*, 2012). In this study, retarded development of embryo occurs in berried females incubated in 45 ppt during the Multicell stage. Since OTC was used to inhibit pathogen, possible cause for this phenomenon was the abnormal morphology of the embryo due to extreme salinity.

Unsynchronized hatching may occurs due to stress exhibit by the berried female during journey from Johor to Terengganu. It can also occur due to unsynchronized fertilization. Higher percentage of hatching rate occurs for berried female incubated in 30 and 35 ppt which are the range of salinity in natural habitat. Churchill (2003) reported that optimal water quality and maintenance in environmental condition can produce high hatching rate. The embryo may take in water that causes the egg to swell and burst the membrane. In this study the opposite occurs where berried females incubated in 15ppt did not hatch. The eggs were released and deposited at the bottom of the tank. This may cause from weak attachment of funiculus which is the connection between the mother and the egg mass. Saigusa (2000) proposed that embryo can detach from mother body due to continuous light, salinity and low temperature. He concludes that hatching of Sesarma haematocheir (De Haan, 1833)embryo cannot be fully explained through osmotic effect instead unknown stimulus may be responsible in hatching among embryo. Brood loss does occurs and common among crustacean. Brood loss reduces the percentage of hatching rate. Factors such as scratching of egg mass to the substrate, reduce available size for attachment due to increase volume (Figueiredo *et al*., 2008), weak attachment, ciliates infection (Quinito *et al*., 2001), embryo abnormalities, infection of microbial (Ates *et al*., 2012) and mechanical stress (Oh and Hartnoll, 1999). The water salinity and temperature recorded in the present study are in level from the previous study by Othman *et al.* (2015) at Tebrau Strait, Malaysia.

Conclusion

In conclusion, the embryonic development of *P. pelagicus* follow the basic morphology of Brachyuran embryo in which appendage was first observed, followed by eye formation, chromatophore appearance, heartbeat and lastly the embryo looked like zoea and ready to hatch. The egg increase in size. Salinity does shows influence on egg size after Intermediate multicell-gastrula stage, in which eggs incubated in 25ppt have largest egg diameter compare to ones incubated in 30 and 35ppt. The incubation period for the three salinity treatment was 10 days. Female *P. pelagicus* did not spawn at 15ppt. It shows high percentage of spawning in 30 and 35ppt which is the salinity range of natural habitat. Highest percentage of hatching occurred at in 35ppt. Further study on effect of salinity on embryonic development and larvae should be conducted to determine the suitable salinity to produce good seeds in hatchery production. In addition, all the water salinity treatments used in the present study were in a salinity range of marine conditions. One of the possible reason why the 25ppt treatment produced the best results is because the matured females used in the study were sampled from the Straits of Tebrau, Gelang Patah, Johor, which experienced a lower water salinity of 25±3ppt almost through the year where freshwater discharge from the main river surrounding the district of Gelang Patah.

Acknowledgments

This study was supported by research grant from Malaysian Ministry of Higher Educationunder grant Vote No. 53126. Authors wish to thanks to all staff at Institute of Tropical Aquaculture and School of Marine Science and Environment and Marine Hatchery of School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu for technical assistance throughout research.

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