Songklanakarin J. Sci. Technol.
38 (1), 83-90, Jan. - Feb. 2016

http://www.sjst.psu.ac.th

Original Article

Resistance test at early larval stage of blue swimming crab, *Portunus pelagicus*

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Received: 19 May 2015; Accepted: 28 August 2015

Abstract

The early larval stage is the crucial phase in the larviculture of blue swimming crab, *Portunus pelagicus*. The combination of stress resistance tests have been done to determine the maximum period of starvation and dissolved oxygen, the preferable range of temperature, salinity, pH and ammonia for *P. pelagicus* larvae survival at early larval stages. Larvae were exposed to various treatments as mentioned above to examine larvae competency against the stressor. In starvation test, at 12 hours, more than 50 percent of larvae were observed dead. While in the dissolved oxygen test, no larvae survived more than 6 hours without aeration treatment. The optimum temperature for larvae was 30°C due to high survival rate (43.18%) was found in the end of experiment. There were no larvae survival at salinity level 0 ppt, 10 ppt and 50 ppt respectively. The highest larvae survival was found at 30 ppt treatment (60.51%). The suitable pH for larval rearing condition was ranged from 8.0-8.2 (survival rate were 48.60% and 70.47%, respectively) owing to larval survival rate in others treatments were very low. In the last experiment, lower concentration of ammonia treatments (0.02, 0.2 ppm) showed higher survival rate (58.54% and 23.90%) whereas the larvae in 2 ppm and 20 ppm treatments died with 24 hours and 6 hours after spike.

Keywords: blue swimming crab, *Portunus pelagicus*, resistance test, larvae, zoea

1. Introduction

Crustacean is an important aquatic organism for human consumption. Many crustacean species are commercial species such as lobsters, shrimps and crabs. Blue swimming crab, *Portunus pelagicus* is interesting and commercial species worldwide due to it has a good taste, high nutritional value and affordable market price toward the consumers (Sahoo *et al.*, 2011, Ikhwanuddin *et al.*, 2012a). According to Andres *et al.* (2010), *P. pelagicus* productions have increased since early 1950s and in 2003 reached to 184,861 metric tons worldwide. The previous authors also referred to Otto and Jamieson (2003) that in present day the production of this species depends on fisheries only which is unreliable and seasonal. To sustain and meet market demand, aquaculture and research development of this species need to be encourage. Furthermore, this ubiquitous crab is potential candidate for aquaculture because of it fasts growing, high fecundity and relatively short larval duration (Romano and Zeng, 2008). *P. pelagicus* undergoes the same life cycle as other crustacean which mean starting with larval stage (four zoea and one megalopa stage) then metamorphose to benthic living life (Arshad *et al.*, 2006). The bottleneck for this portunid crab has been found during larval stages. Most of larvae suffered high mortality before metamorphosis to megalopa due to microbial infection, predation or failure to cope the stress in the changing environmental factors such as temperature, salinity or pH (Talpur and Ikhwanuddin, 2012). Stress resistance tests were applied in aquaculture because of its ability to reduce time requirements, equipment, apparatus,

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material and analytical resources (Samocha *et al.*, 1998). Environmental stress factors or abiotic factors have been known affecting immune response and resistance in crustaceans (Bryars and Havenhand, 2006). Water temperature may give direct effect on the metabolism, oxygen consumption, growth, molting and survival. During embryogenesis energetic and larval quality of the organism were also affected by salinity which may cause relatively little effect towards them. Mortality observed was possibly due to the imbalance in osmoregulatory mechanism. Excretion from cultured organisms was significantly causing the toxic levels ammonia to be accumulated and high level of ammonia will definitely give negative impacts towards the growth and development (Rosas *et al.*, 1999). Biotic factors such as accessibility of food, quality of food and predation factors also showed some effects on the metamorphosis rate on the larvae (Ravi and Manisseri, 2012).Thus objectives of the present study are; (i) to investigate the ability of *P. pelagicus* larvae to cope with different salinity, temperature levels, pH, ammonia concentration, period of starvation and dissolved oxygen during stress resistance tests and (ii) to determine the preferable range of salinity, temperature, pH, ammonia, dissolved oxygen and maximum period of starvation for optimum *P. pelagicus* larvae.

2. Materials and Methods

2.1 Broodstock management

P. pelagicus broodstock (berried females) were collected from Gelang Patah (1° 22'60"N, 103° 37'60"E), Johor, Peninsular Malaysia (Figure 1). The broodstock were transported to marine hatchery, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu, Malaysia. Three berried females were placed in fiber tank and not fed during the incubation period. The temperature was maintained at ambient temperature (28°C) and salinity of seawater in the range of 30-34 ppt (Talpur and Ikhwanuddin, 2012). Waste materials were siphoned out daily in order to maintain water quality and hygiene until hatching.

2.2 Experimental design

Six stress tests included starvation, salinity, temperature, dissolved oxygen (DO), pH and ammonia stress were conducted with three replicates (100 larvae, Zoea 1 stage per replicate) in 4-liter aquarium containers. The summary of the test and treatments used in the present study was shown in Table 1. During experimental period, all larvae were fed with rotifer (*Branchionus* sp.) at stocking density 30-40 rotifers mL^{-1} except for the larvae in starvation test. In order to control temperature during experimental period, water bath water system was carried out except for two treatments, 10°C and 20°C; treatments use Water chiller (Resun CL650) to control temperature in temperature test. Four water quality parameters; temperature (29±1 °C), salinity (32±1 ppt), pH (7.5–8.5) and DO (>6 ppm) were monitored (depend on test) every day by using YSI 556 MPS multi probe meter (U.S.A). All tests were terminated as all the larvae died or metamorphosed to Zoea 2 stage.

2.3 Data collection and analysis

The survival rate (%) was determined as the number of larvae survived divided by the number of larvae from initial stocking in each replicate (Ikwanuddin *et al*., 2012b). One-way factorial Analysis of Variance (ANOVA) was used to process data for the stress test of starvation, salinity, temperature, oxygen, pH, ammonia. Survival rate (%) and time (min/hour) for larvae development at various levels of

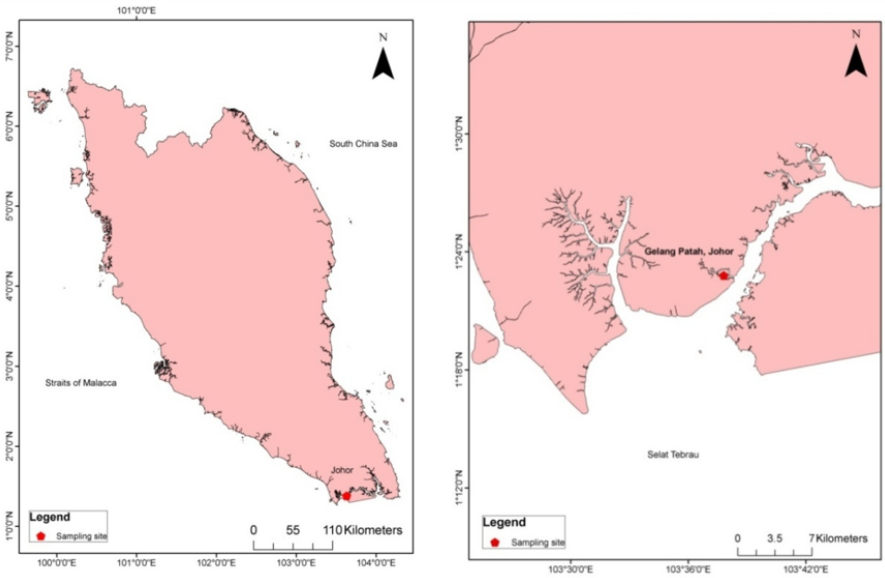

Figure 1. Sampling site of *P. pelagicus* berried females at Gelang Patah, Johor, Peninsular Malaysia.

Table 1. Stress resistance tests and treatments on *P. pelagicus* larvae used in the present study

Tests	Treatments
Starvation	Two treatments were assessed namely; starve treatment and feed treatment (control). The larvae were checked every three hour until they died or metamorphosed into Zoea 2.
DO	During the tests, no aeration and the control with aeration were conducted. Each beaker was wrapped with aluminium foil in order to reduce evaporation and oxygen from atmosphere. The duration time of this test was 5 hours.
Temperature	In temperature stress test, larvae were tested to 10°C, 20°C, 30°C and 40°C. The larvae were observed every three hour within 24 hours duration time. The control of temperature used was the ambient temperature (28-30°C).
Salinity	In salinity stress test, larvae were monitored to different salinity levels included 0 ppt, 10 ppt, 20 ppt, 30 ppt, 40 ppt and 50 ppt. They were observed for every three hour in 24 hours duration time. The control for salinity was 30 ppt.
pH	Different pH levels of 6.0, 6.5, 7.0, 7.5 and 8.0 were conducted. To decrease pH in sea water become acidic, hydrochloric acid (HCL) was used to reduce acidic condition while sodium carbonate ($NaCO_3$) was used to increase pH values. The test was set for 24 hours and the reading was recorded using pH meter (pH 2700) EUTECH Instruments
Ammonia	During the tests, all treatment and control were placed in the water bath. Pure ammonium chloride (NH_4Cl) was used and prepared into different concentration of 0.02mg/L, 0.2mg/L, 2mg/L and 20mg/L. The test was conducted for 24 hours

salinity, temperature, oxygen, pH, ammonia and survival rate (%) and time (min/hour) for larvae development without provided of food in these experiments were subjected to Analysis of Variance (ANOVA). MINITAB 16 was used for ANOVA analysis. ANOVA was used to compare significant different exist between the parameter tested or in the other way round (Ravi and Manisseri, 2012). Turkey's multiple comparison tests were then used to classify the significantly different mean values ($P<0.05$).

3. Results

3.1 Starvation test

The results showed the survival rate of starved and control treatment was statistically significant difference ($p< 0.05$) since the first 3 hours of the experiment (Figure 2). This experiment has been conducted for 48 hours, there were no zoea 1 stage survived in the starvation treatment but in the control treatment the zoea survived 4.53±0.66% in the end of experiment. First 24 hours the zoea survived in control treatment 48.65±8.85% and 6.23±1.78% in starved treatment. At 45 hours of experiment all the zoea in starved treatment died while the survival for control treatment was 6.78±0.63%.

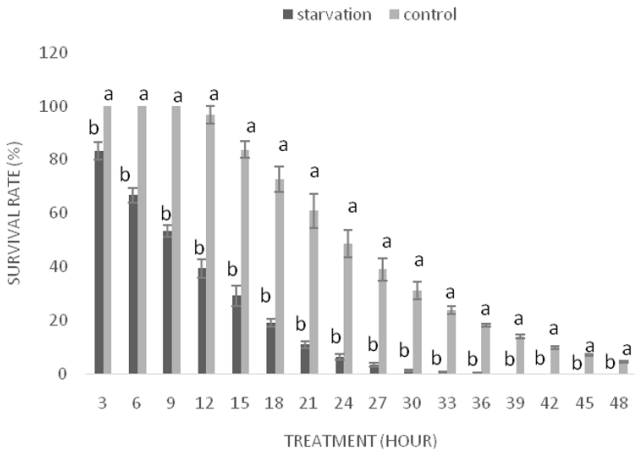

Figure 2. Survival rate (%) of newly-hatched Zoea1 stage (Z1) of *P. pelagicus* in starvation test. Different alpha shows that there is significant difference between treatments.

3.2 Dissolved oxygen (DO) test

The experiment has been monitored for 6 hours due to there were no zoea survived after that in without aeration treatment where the survival rate of control treatment (supplied with aeration) was 69.25±5.15%.The first 4 hours there was no significant difference of survival in both treatments (Table 2). At the hour 5, the survival rate of control treatment (84.63±5.73%) showed the significant difference than another treatment (10.66+10.97%). In the end of experiment, mean of dissolved oxygen was measured in without aeration treatment and control beakers and the dissolved oxygen reading was 0.65 mg/L and 6.44 mg/L, respectively.

3.3 Temperature test

The temperature test was conducted for 24 h, no Zoea 1 survived in 40°C temperature group since the first 3 hours of experiment. In the temperature group of 10°C, the mean of larvae that was survived were 0.11 ± 0.09%. The larvae

Table 2. Survival rate (%) of newly-hatched zoea1 stage (Z1) of *P. pelagicus* in dissolved oxygen (DO) test. Difference alpha shows that there is significant difference between treatments.

Hour (s)	Control (with aeration)	Without aeration
1	100.00±0.00[a]	76.67±11.55[a]
2	100.00±0.00[a]	57.33±20.79[a]
3	96.67±7.07[a]	44.13±25.32[a]
4	90.33±6.36[a]	31.34±23.52[a]
5	84.63±5.73[a]	10.66±10.97[b]
6	69.25±5.15[a]	0.00±0.00[b]

subjected to 20°C condition, showed that most of them were still survived with the final mean of the larvae could only survive 0.65±0.56%. There was statistically significant ($p< 0.05$) recorded in the temperature group of 30°C which showed the highest mean rate of 43.18±7.32%. However, there was no significant different achieved between the temperature group of 10°C, 20°C and 40°C (Figure 3).

3.4 Salinity test

In the salinity test, all Zoea 1 die at salinity level 0 ppt, 10 ppt and 50 ppt within 3 hours. Low survival was observed in the salinity of 40 ppt, but they were able to survive only for 12 hours with the mean survival of 2.16±2.08%. After 12 hours, all of the larvae in that particular salinity level died. In addition, salinity 20 ppt did produce a very low mean survival of 0.02±0.02%. Zoea 1 was observed with the mean of 60.51±4.43% survived in the controls (30 ppt). There was statistically significant ($p<0.05$) observed between controls and the other range of salinities (Figure 4).

3.5 pH test

The results show that pH 6.0, 6.5, 7.0, and 7.5 found a low survival rate of the Zoea 1 with mean survival rate recorded were 0.91±0.21%, 2.13±1.13%, 4.93±0.85% and 6.88±0.75% respectively. Survival of larvae in the treatment subjected to pH 8.0 was 48.60±3.41%. Control (pH 8.2) showed statistically significant ($p<0.05$) and had recorded the highest survival rate (70.47±9.13%) in this experiment (Figure 5).

3.6 Ammonia test

No survival of Zoea 1 was observed after 24 hours in the treatment of 2mg/L and 20mg/L of ammonia respectively. Zoeas 1 were only survived for 6 hours when subjected to 2mg/L of ammonia and for just 3 hours when subjected to 20mg/L of ammonia. Thus, the highest survival of larvae was observed in the treatment of 0.02 mg/L with the mean survival of 58.54±6.16%. The second highest of mean survival was 23.90±3.49% in 0.2 mg/L ammonia treatment. There was statistically significant ($p<0.05$) between the challenge groups (Figure 6).

4. Discussion

4.1 Starvation test

Starvation plays a significant role in determining the survival and growth of the larvae. Because food availability

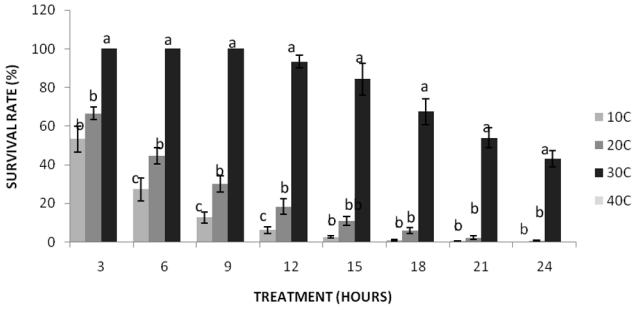

Figure 3. Survival rate (%) of newly-hatched Zoea 1 stage (Z1) of *P. pelagicus* in temperature test. Different alpha shows that there is significant difference between treatments.

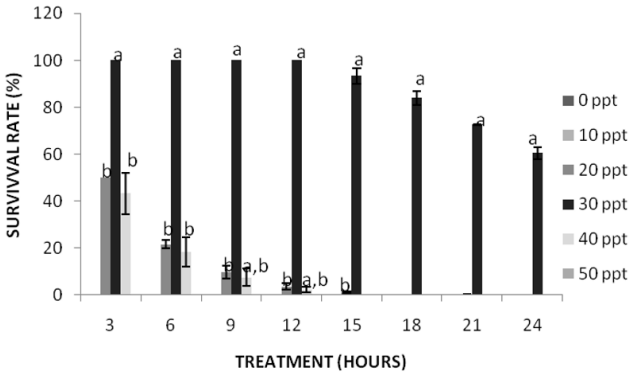

Figure 4. Survival rate (%) of newly-hatched Zoea1 stage (Z1) of *P. pelagicus* in salinity test. Different alpha shows that there is significant difference between treatments.

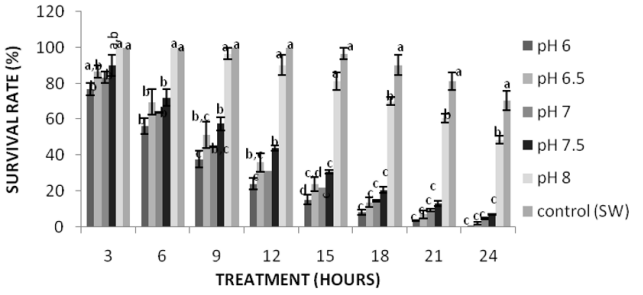

Figure 5. Survival rate (%) of newly-hatched Zoea1 stage (Z1) of *P. pelagicus* in pH test. Different alpha shows that there is significant difference between treatments.

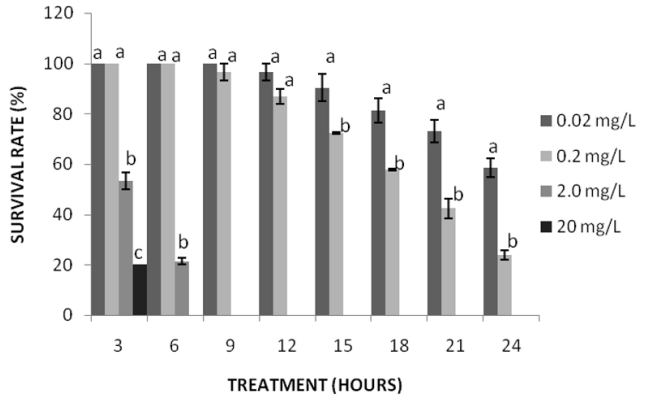

Figure 6. Survival rate (%) of newly-hatched zoea 1 stage (Z1) of *P. pelagicus* in Ammonia test. Different alpha shows that there is significant difference between treatments.

is effective influence to larval ecology and starvation could cause nutritional stress then decrease or cease larval development (Desai and Anil, 2002). The results of present study showed that after 24 hours, the mean survival was 6.23±1.79% in starved treatment and there was no more larvae survived at 45 hours after hatching. The main reason of the high mass mortality of larvae was due to lack of nutritional reserves in the yolk egg from the hatched females (Schuh & Diesel, 1995). Zheng *et al.* (2005) observed that starvation or temporary starvation in early larval stage could greatly reduce growth rate, development and metamorphosis to next stage. The authors indicated further that the larvae could not complete metamorphose in the completely absence of food resources condition. The present study showed that the crab larvae were sensitive and intolerant to the starvation condition if compared to the other marine invertebrate larvae due to they could survive only 45 hours after hatching. Veliger larvae of gastropod (*Babylonia formosae habei*) would starve for 3 day where polychate larvae (*Hydroides elegans*) could survive without feeding for 4-5 days (McEdward and Qian, 2001). According to Talpur and Ikhwanuddin (2012), the number of predations would increase if the larvae starved for hours. Then the larvae weakened due to predation caused them injured and pathogen attack which increase mortality. Starvation could not only kill the larvae, it prolonged larval stages and extent metamorphosis period. Desai and Anil (2004) and Zheng *et al.* (2005) explained that after feed larvae in starved treatment larvae grow rapidly but still could not keep up with non-starved one due to starved larvae needed to store energy in order to molt and survive to the next stage. This experiment support that the *P. Pelagicus* larvae should be fed immediately after hatching because the larvae could not tolerate with food starvation. In the other hand, low survival rate observed in control treatment may also due to unhealthy hatched females which may be stressed due to the new environment adaptation during their transferred from Gelang Patah, Johor to Universiti Malaysia Terengganu hatchery.

4.2 Dissolved Oxygen (DO) test

Anaerobic condition occurs when there was a low level of oxygen exists (hypoxia). This condition might cause nitrate to turn into toxic ammonia and increase the pH (Talpur and Ikhwanuddin, 2012). Aquatic organism not only requires dissolved oxygen for respiration but also in maintaining well rearing chemicals and minerals in water and hygienic environment. Metabolic performance and growth process will be affected by low oxygen content which may directly cause mortality (Cheng *et al.*, 2004). Moreover, it also weakens the immune system of the organism and become very susceptible to infectious disease. During present study, no oxygen was provided for treated group and continuous aeration was supplied for control groups. The larvae were able to survive only for 5 hours and were observed dead after 6 hours without oxygen. Crustacean are one of aquatic animal with less tolerate to hypoxia and juvenile stage is sensitive than adult (Alberto Geihs *et al.*, 2013). The latest authors referred that the younger stage needed to moult for growth, and during molting, respiration is profound demanding. It could be reason why the larvae in this study all died within 6 hours. Rosas *et al*. (1999) indicated that low dissolved oxygen concentration condition (< 2 mg/L) affected the growth rate of *Penaeus monodon* even at 4-4.5 mg/L seem to be critical level for this species. The results obtained suggest that during larval rearing oxygen should be provided continuously due to the larvae could not tolerate for low dissolved oxygen condition.

4.3 Temperature test

One of the most essential environmental factors is water temperature for larval rearing, as it has been stated to affect larvae in terms of morphology, metabolism, oxygen consumption, feeding rate, growth and development, survival and biochemical composition during their early stage (Dahlhoff, 2004; Pepin, 1991; Portner *et al.*, 2005; Shirley *et al.*, 1987). In the present study, the highest survival rate was found in control of the temperature of 30°C. At 10°C and 20°C, there was a very low survival percentage and no zoea survived at 40°C. This study obtained the result the same direction as Bryars and Havenhand (2006) where the *P. pelagicus* larvae found the highest survival rate at 25°C and highest mortality at 17°C. The latest researcher emphasized that temperature is an abiotic factor profoundly influenced the larval rearing of *P. pelagicus.* Temperature not only influenced the survival rate, growth rate, metamorphosis rate and period also deteriorated due to temperature changes adversely affected lysosomal membrane development (Lima and Pechenik, 1984; Deschaseaux *et al.*, 2011). Besides, Moullac (2000) reported that number of circulating hemocytes and capability of their phagocytic will be reduced if restricted in lower temperature while the number of circulating hemocytes and plasmatic protein will be increased if subjected to increase temperature but causing a reduction in

total hemocytic prophenoloxidase. It might be conclude that 40°C was the extreme temperature for the larvae. Optimum temperature will be range 25- 30°C for better survival.

4.4 Salinity test

The result obtained in this study suggested that *P. pelagicus* larvae should be exposed at salinity level 30 ppt owing to the larvae in the rest treatments produced low survival rates (20 ppt) and in certain treatments the larvae die within 3 hours (0, 10 and 50 ppt). According to Ruscoe *et al.* (2004), the salinity not affected only on frequency of molting but certainly on survival due to the salinity increased additional load of metabolic required for all aquatic animals. The authors conducted experiment on mud crab, *Scylla serrata* juvenile with different salinities, the result showed that there was no juvenile survive at 0 ppt within 6 hours compatible with the present study. Salinity requirement for larval rearing is different in each marine organism or even in crab species. Parado-estepa and Quinitio (2011) indicated that *S. serrata* larvae cultured in lower salinity (12-26 ppt) attained maximum growth and survival rates compared with those larvae in high salinity (32-40 ppt). it could be explained the reason of rapid mortality on larvae at unsuitable salinity that low salinities level or fluctuation of salinity levels attributed to slow growth and development process that probably caused increasing rate of excretion. On top of that blood and body fluids salt concentration of marine organism was maintained by osmoregulation process which caused prolonged molting occurred as a result of difficulties in casting old cuticle was related to high mortality of larvae in low salinities (Samuel and Soundarapandian, 2010; Ravi and Manisseri, 2012). The study of Pechenik (1982) emphasized the effect of low salinity level on marine organism in younger stage. The author narrated that low salinity level deteriorated the osmatic concentration rate of the fluid surrounding the embryos then caused the development and hatching period of veliger larvae delay.

4.5 pH test

The pH also plays vital role in culture system which can directly affect the organism's well- being in terms of metabolism and physiological processes. A changing of pH would give negative effects on that particular organism because the changes often take place due to residual feed and excretion product of organisms (Talpur and Ikhwanuddin, 2012). The present study gained the optimal pH range result as Kannupandi *et al.* (2002). The authors suggested that crustacean larvae should be reared in pH range of 8.2-8.5. In this study, *P. pelagicus* larvae in lower pH range from 6-7.5 contributed to a very low survival. According to Yu *et al.* (2009), found that high mortality (92%) of *Physocypria kraepelini* (Crustacean; Ostracod) was observed in acidic pH treatment conducted for 72 hours which went the same way as the result in present study. The latest authors indicated further that pH value of water could be fluctuated in natural water because of environmental factors (eutrophication and typhoon) or human activities (sewage discharge and urbanization). If the fluctuation occurred shortly, the aquatic animals could only stress, whereas if the fluctuation is long period, great mortality will be found. Interestingly, high-tolerated crustacean such as rotifer (*Branchionus urceolaris*) and *Daphnia magna* development also degenerated because pH changing. El-Deeb Ghazy *et al.* (2011) reported that the fecundity of *B. urceolaris* decrease or high mortality of eggs were found if the broodstock exposed to pH< 6 or >10. The latest authors indicated further that the suitable pH for *D. magna* was 8.33due to the growth rate of *D. magna* reduced in pH range 4.44-4.66 and 10.13-10.55.

4.6 Ammonia test

Ammonia is well-known as a common toxicant in culture system because it is very poisonous to crustaceans and other aquatic organisms. Basically, unconsumed feed and feces contributed to an increasing of ammonia level. Severe problem related to the high level of ammonia concentration will be loss of balance, hyper excitability, paroxysms and serious fatality (Chih-Hung *et al.*, 2003). Results of present study demonstrated that survival rate decreased when concentration of ammonia increased. Larvae survived more than 50% when subjected to 0.02mg/L and 0.2 mg/L. However, larvae in higher ammonia concentration (2mg/L and 20mg/L) encountered high mortality within a few hours. The results indicated that *P. pelagicus* larvae could survive at 0.2 mg/L which was higher than permitted maximum ammonia concentration in aquaculture; 0.1 mg N/L (Yu *et al.*, 2009). It seems to be an ammonia elevation in crustacean associated with other stress factor where the animals encounter in the wild. Low salinity level would weaken decapods ability to tolerate the increasing of ammonia which that stress intervened the osmoregulatory system later (Romano and Zheng, 2012). Moullac (2000) conducted experiment on shrimp had proved that activity of hematopoietic tissue, oxidative metabolism of hemocytes and genes expression, which has been found implicated in the shrimp defense system will be diminished by high ammonia levels, which means that most of the crustacean are sensitive to elevation of ammonia. Actually, *P. pelagicus* is able to state as indicator organism owing to its low-tolerated to ammonia elevation and prefer to embed in the sand or bottom of sea bed most of the time which could indicate that the particular area is polluted or not.

5. Conclusions

Overall, the present study indicate that *P. pelagicus* larvae of Zoea 1 stage was able to survive if they was compromised at the certain level of stressor but the survivorship of larvae will be reduced when there was elevated stressors. This study clearly demonstrates the larvae were unable to survive when they were being starved for a long period (45

hours). Maximum period of starvation for *P. pelagicus* larvae was less than 12 hours. Dissolved oxygen on larval rearing should be more than 6 mg/L. The preferable range or selected conditions of optimum larvae survival will be at 28-30°C temperature, salinity of 30-34 ppt, pH range of 8.2-8.5 and ammonia concentration < 0.02 mg/L. Wide fluctuation in temperature and salinity will causes profound effect on larval survival. Stable pH was required for better larval development and low ammonia level will not let to serious problem towards the larvae survival. In addition, different berried female of *P. pelagicus* used in this experiment caused different trend of survival rate result. Namely, unhealthy berried females (stress) provided weak larvae then implied low survival rate in certain experiment. The experiment of interaction between environmental factors such as salinity and ammonia, temperature and dissolved oxygen could be conducted in further study in order to increase basic information of *P. pelagicus* larvae and develop understanding of environmental factors toward the larvae. Finally, *P. pelagicus* could be named as indicator organism due to it is sensitive to high concentration of ammonia, low salinity level and pH ranges.

Acknowledgements

The research was supported by a grant from the Ministry of Higher Education under Knowledge Transfer Program (KTP) grant, Government of Malaysia under grant vote No.53126. Appreciation also goes to the staff of Institute of Tropical Aquaculture (AKUATROP) and the hatcheries staff for their technical support.

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