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Incubation and Hatching of *Tachypleus gigas* (Muller, 1785) Eggs in Sand and Water Media

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ABSTRACT

In order to develop suitable methods to successfully hatch *Tachypleus gigas* eggs, a study was carried out on different salinity and culture media. The main objective for this study was to determine the effect of watering frequency, salinity and media on the incubation period and hatching of *T. gigas* eggs. This research consisted of three experimental studies. In the first experiment, effect of water salinities (15, 20, 25 and 30 ppt) for watering or moistening sand was studied. For the second study, effect of watering frequencies (once in 1, 3 and 6 days) on the eggs incubated in sand were investigated. As for the last experiment, effect of incubation medium (water and sand) on eggs were compared. Data collected for these experiments were eggs diameter and hatching percentages. Embryonic developments were observed and photographed during the study period. Results from experiment 1, showed that at the end of the incubation period, watering with water salinity of 25-30 ppt produced significantly larger eggs diameter ($p<0.05$) while percentages of hatching was the highest with 30 ppt water. In experiment 2, it was found that percentages of hatching were significantly higher ($p<0.05$) when watered once a day and in three days. As for experiment 3, at the end of the incubation period, there was no significant different ($p>0.05$) in the eggs diameter and percentage of hatching between sand and water medium. In conclusion, the most suitable salinity and watering frequency were 25-30 ppt and once in 3 days, respectively. However, both sand and water are suitable media to successfully incubate *T. gigas* eggs. Overall, this study showed that *T. gigas* eggs can hatch as early as 40 days after fertilization.

Key words: Horseshoe crab, *Tachypleus gigas*, eggs, hatching, incubation, salinity

INTRODUCTION

Horseshoe crabs, horse foot or king crab are benthic bottom dwelling invertebrates found in both estuarine and continental shelf habitats. It is the close relative to trilobites which is still existence (Shuster, 1982). Therefore, they are often call "living fossil" (Iwanaga and Kawabata, 1998). Earth land masses have shifted dramatically, thousands of other species have gone extinct but horseshoe crabs remained. In the whole world, only four species remained. Those are *Limulus polyphemus* (in Atlantic coast), *Carcinoscorpius rotundicauda* (in Indo-Pacific) the mangrove horseshoe crab, *Tachypleus gigas* (in the tropical coast) and *Tachypleus tridentatus* a slightly temperate species (found in the coast of Japan, China and North Borneo). All these species

can be differentiated by their physical appearance such as size, color and shape of tail. The coastal horseshoe crab, *T. gigas* (diameter up to 25 cm) is grayish in color, with triangular and serrated telson (Sekiguchi, 1988). Horseshoe crabs begin life as embryos in unshelled, greenish and yellowish eggs. Eggs are laid in nests on the beach at a mean depth of about 15 cm beneath the sand (Rudloe, 1979). Each group of eggs contains between 2000 and 30000 eggs (Cohen and Brockman, 1983). After fertilization, each egg consist a central yolk, an embryo and a thick outer membrane. The embryo goes through 21 stages between two to four weeks of development (Sekiguchi *et al*., 1988).

The most studied species so far is *L. polyphemus*. It is being used to produce a commercial product called "Limulus Amebocyte Lysate" (LAL), a derivate from its blood. This LAL is being used by pharmaceutical and bio-medical industries to detect bacteria in drug and implantable devices before being used on patient (Botton, 1984). Ammonia was the one major problem in fish culture especially in fish ponds, recirculation systems and aquaria (Chezhian *et al*., 2012). Laboratory culture allows intensive study on the horseshoe crab larvae. However, suitable culture condition needs to be developed first. Thus, the goals of this study were to compare the incubation period and hatching percentage of *T. gigas* incubated in different culture media (sand and water), sand watered at different water salinities and sand watered at different frequencies.

MATERIALS AND METHODS

Eggs of *Tachypleus gigas* and sand were collected from natural spawning, Sitiawan, Perak, Malaysia. Experimental studies were carried out at Endocrinology Laboratory, Department of Aquaculture, Faculty of Agriculture and Marine Laboratory at MTDC, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. This study consisted of 3 experiments. Experiment 1 was conducted to determine the best water salinity for watering or moistening sand where eggs were incubated. Experiment 2 was to determine the best watering frequency for eggs incubated in sand bed. While in experiment 3, the used of sand and water as culture medium for the incubation of eggs were compared.

Preparation of sand and trays: Sand used in this study was autoclaved at 121°C for 15-20 min. The size of trays used in this study were 44×33×10 cm. Eggs samples (50 eggs/basket) were placed in baskets (10×15 cm) made from plastic net and buried in sand bed at 8 cm depth. Baskets were prepared in triplicates for all treatments. During the watering or moistening of the sand bed, seawater was poured until it drained through the outlet at the bottom of the tray. All experiments were carried out at room temperature.

Conditioning of *T. gigas* eggs: In experiment 1 and 3, one-week old eggs were conditioned in sea water in laboratory for five days prior to the initiation of the experiments. These eggs were basically 12 days old. While for experiment 2, the eggs used were three weeks old. After 5 days conditioning, eggs were 26 days old when used for the experiment.

Experimental design: In the first experiment, water salinities of 15, 20, 25 and 30 ppt were used to water or moisten sand bed containing baskets of *T. gigas* eggs. For the second experiment, watering frequencies of once in 1, 3 and 6 days were applied on sand bed containing baskets of *T. gigas* eggs. A control treatment was added, whereby eggs were incubated in water with daily water change throughout the experimental period. As for the last experiment, incubation medium

using water and sand were investigated and compared. In this experiment 30 ppt seawater was used. Sand medium was watered once in 2 days while for water medium, water changed was carried out once a day.

Data collection: Data collected for these experiments were eggs diameter and hatching percentages. Eggs diameter were measured using a Digital caliper (DigiMax). Eggs samples in each net baskets were observed using dissecting microscope (LEICA EZ4) once a week. Embryonic developments were observed and photographed during the study period. Hatched eggs were also counted and percentage of hatching calculated. All data from these experiment were compiled and stored using Microsoft Office Excel. ANOVA was used to analyzed data for eggs diameter and percentages of hatching from experiment 1 and 2. As for experiment 3, student t-test was used to determine the significant difference between the incubation media (sand and water).

RESULTS AND DISCUSSION

Hatching and development of *T. gigas* eggs incubated in sand at different salinities: During the first week of this experiment, the recorded mean diameter of horseshoe crab eggs was 3.65 mm and kept on increasing weekly. Maximum mean size attained by the eggs at salinity 25 ppt was 6.80±0.04 mm. Result showed that 25 and 30 ppt produced eggs with significantly larger ($p<0.05$) diameter (Table 1). This is due to the fact that 25-30 ppt is the same salinity as it is in natural habitat. Salinity fluctuations usually occurs at coastal water near river mouth and that affects the growth and survival of marine organisms including horseshoe crab (Botton and Itow, 2009). This study showed that differences in egg diameter is possibly due to the different salinity used to water the eggs.

Earlier study indicated the hatching of *T. gigas* occurs at 20-32 ppt (Christianus and Saad, 2010). However, this study showed that there was no significantly different ($p>0.05$) on the percentage of hatching between 25 and 30 ppt. This finding has narrowed the salinity margin to 25-30 ppt. Eggs started to hatch at week 5 and the subsequent, week 6, showed the highest percentage of eggs hatched for every treatment (Fig. 1). At lower salinity, eggs development were rather slow but the increased in the water content (perivitelline fluid) of the eggs may have accelerated the hatching process of horseshoe crab eggs (Saigusa, 1996). Ehlinger and Tankersley (2003) showed that eggs of *L. polyphemus* successfully can hatch at salinity 10-70 ppt. However,

Table 1: Means diameter (mm) of *T. gigas* eggs incubated in sand and watered with seawater of different salinities during the eight weeks period

		Eggs diameter (mm)							
		Weekly sampling							
Salinity (ppt)	N	1	2	3	4	5	6	7	8
15	3	3.65	3.91±0.26^{a}	4.51±0.48^{a}	4.86±0.51^{a}	4.97±0.24^{a}	5.77±0.70^{a}	5.86±0.77^{a}	6.11±0.81^{a}
20	3	3.65	3.87±0.26^{a}	4.63±0.73^{a}	5.08±0.50^{a}	5.69±0.20^{b}	6.30±0.32^{a}	6.34±0.32^{a}	6.53±0.25^{a}
25	3	3.65	4.39±0.42^{b}	4.98±0.67^{a}	5.40±0.72^{a}	5.95±0.09^{c}	6.71±0.07^{b}	6.75±0.08^{b}	6.80±0.04^{b}
30	3	3.65	4.32±0.40^{b}	4.99±0.94^{a}	5.44±0.63^{a}	5.92±0.66^{c}	6.54±0.31^{b}	6.66±0.24^{b}	6.73±0.19^{b}

Values with same superscripts within a column are not significantly different at $p>0.05$, Eggs were 12 days old at the initiation of the experiment

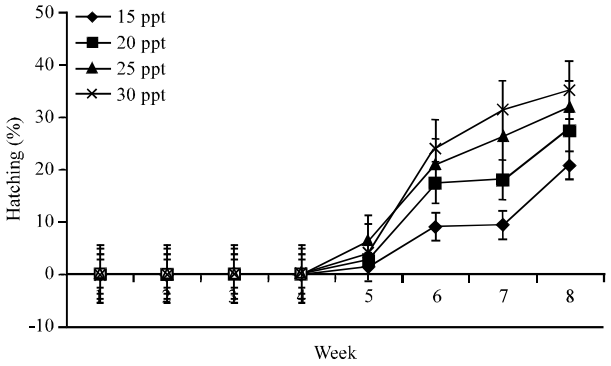

Fig. 1: Hatching percentage of *T. gigas* eggs incubated in sand and watered with seawater of different salinities during the eight weeks period, Eggs were 12 days old at the initiation of the experiment

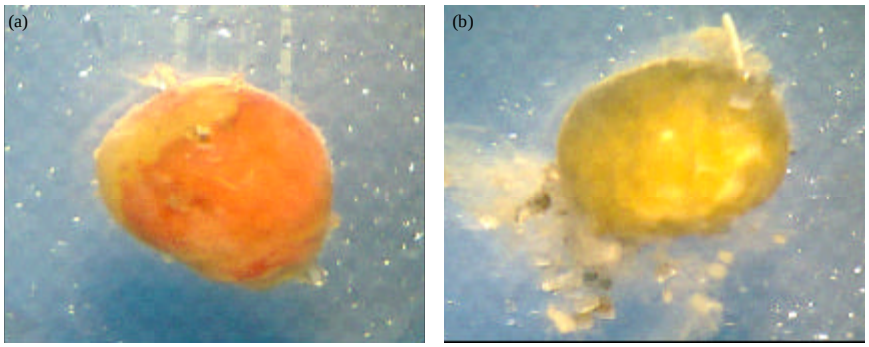

Fig. 2(a-b): Fungal infection on *T. gigas* eggs

the results of this experiment indicated negative effect on percentage of eggs hatching when incubated at salinity below 25 ppt. Study by Zaleha *et al.* (2011) on *T. gigas* is in agreement with this study.

In this study, it was observed that eggs incubated with salinity of 15-20 ppt were prone to fungal infection (Fig. 2). This kind of infection was also observed on horseshoe crab larvae (Faizul *et al.*, 2011). Undeveloped eggs were distinguishable since it will turned black or red and start to decompose.

Hatching of *T. gigas* eggs incubated in sand and watered at different frequencies: Results of experiment 2 showed the percentage of hatching of *T. gigas* eggs incubated in sand and watered at different frequencies during the six weeks period (Table 2). Eggs incubated in sand and watered daily (T_1) and once in 3 days (T_2) started to hatch on week 2 with hatching 90 and 80%, respectively were observed at week 4. Eggs incubated in sand and watered once in 6 days (T_3) and control (T_4) started hatching only at week 3 and 4, respectively. There was no significant differences ($p>0.05$) in the hatching percentage between T_1 and T_2 from week 1-3. However, T_1 produced the most significant ($p<0.05$) hatching percentage on week 4 as compared to all the other treatments. This experiment showed that the incubation period is shorter when eggs are incubated in sand with watering frequency of once in 1-3 day times. In this study, it was observed that *T. gigas* eggs

started to hatch at week 2 (in treatment 1 and 2 of experiment 2). Eggs were 26 days old at the initiation of the experiment, therefore at week 2 of this experiment, eggs were 40 days old. This result falls within the 40-45 days range reported by Chatterji *et al.* (2004). Comparatively, this incubation period for *T. gigas* eggs is much longer than the 28 days of *L. polyphemus* eggs as reported by Penn and Brockmann (1994). Recorded temperature throughout this study was between 29-34°C. According to Karl (2004), horseshoe crabs are able to tolerate a wide range of temperatures and low oxygen environments. In this study, some eggs failed to hatch even with extended time. Morton and Blackmore (2001) stated that eggs may not hatch due to unsuitable environment and chemical processes in the sand of its spawning site. The abundance of aquatic organism is very much affected by development, industrialization and agriculture activities (Sasi, 2011) at the surrounding areas.

Hatching and development of *T. gigas* eggs incubated in sand and water media: In this experiment, water medium gave the higher hatching percentage at week 5 and 6. However, from week 7-8, water and sand media were not significantly different (Table 3). Egg diameter increased drastically from week 2-6. After week 6, the diameter increased at slower pace due to the fact that the eggs are near hatching. This study showed that there is no significant different in the percentage of *T. gigas* eggs hatched in both water and sand medium. In this experiment, eggs of *T. gigas* started to hatch at week 5 with a diameter of 5.90 and 5.92 mm in water and sand medium, respectively. However these eggs can increase to a maximum diameter of 7.06 and

Table 2: Hatching of *T. gigas* eggs incubated in sand and watered at different intervals during the six weeks period

	Hatching (%)					
	Incubation time (week)					
Treatment	1	2	3	4	5	6
T_1: Sand+watered daily	0.00±0.00^a	40.50±0.30^a	70.00±0.40^a	90.00±0.40^a	0.00±0.00^a	0.00±0.00^a
T_2: Sand+watered once in 3 days	0.00±0.00^a	34.75±0.25^a	65.25±0.25^a	80.00±0.40^b	0.00±0.00^a	0.00±0.00^a
T_3: Sand+watered once in 6 days	0.00±0.00^a	0.00±0.00^b	11.75±0.25^b	60.25±0.25^c	73.75±0.25^b	80.00±0.40^b
T_4: Water+change daily (control)	0.00±0.00^a	0.00±0.00^b	0.00±0.00^c	34.75±0.25^d	54.75±0.25^c	70.25±0.25^c

Values with same superscripts within a column are not significantly different at p>0.05, Eggs were 26 days old at the initiation of the experiment

Table 3: Means diameter and hatching of *T. gigas* eggs incubated using different media during the eight weeks period

	Diameter (mm)			Hatching (%)		
Week	Water	Sand	Significance	Water	Sand	Significance
1	3.65±0.00	3.65±0.00	NS	0.00	0.00	NS
2	3.99±0.16	4.32±0.40	NS	0.00	0.00	NS
3	4.54±0.30	4.99±0.94	NS	0.00	0.00	NS
4	5.10±0.41	5.44±0.63	NS	0.00	0.00	NS
5	5.90±0.29	5.92±0.66	NS	14.67±2.31	8.00±0.00	S
6	6.69±0.38	6.54±0.31	NS	59.33±8.08	44.67±6.11	S
7	6.87±0.27	6.66±0.24	NS	66.00±6.91	59.33±7.02	NS
8	7.06±0.33	6.73±0.19	NS	70.67±9.48	66.67±6.11	NS

NS: Nousignificant at p>0.05, S: Significant at p<0.05, Eggs were 12 days old at the initiation of the experiment

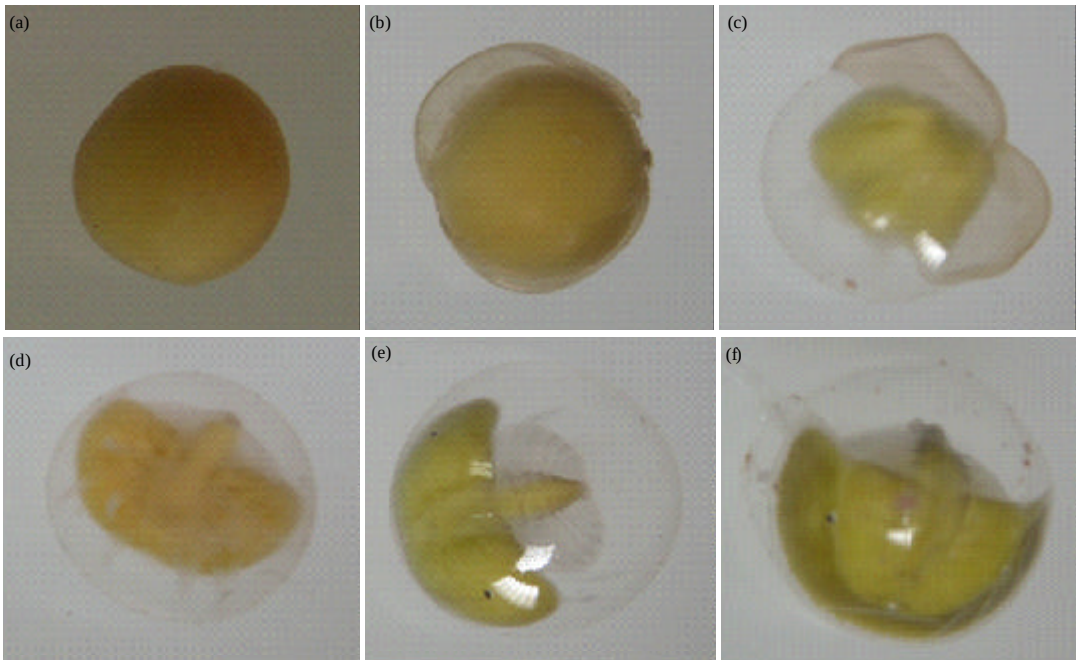

Fig. 3(a-f): Embryonic development of *T. gigas* eggs after fertilization, (a) One week old, olive green colored eggs, (b) Three weeks old, shedding of the elastic green colored chorion membrane, (c) Four weeks old, undifferentiated small sized embryo visible though the transparent membrane, (d) Five weeks old, embryo with appendages is visible, (e) Six weeks old, morphology of embryo becomes more prominent, a lateral eyes (black dots) on the side can be seen and rotating vigorously in the perivitelline fluid and (f) Seven weeks old, embryo with prominent morphology ready to hatch

6.73 mm in water and sand medium, respectively. The eggs at the initiation of the experiment were 12 days old, therefore at 5th week, eggs were actually 47 days old. Thus, this period is a bit longer when compared to the 40 days observed in experiment 2 (of the current study) and also the 40-45 days as reported by Chatterji *et al.* (2004). Observation on the embryonic development of *T. gigas* from 1-7 weeks after fertilization is as shown in Fig. 3. Some eggs start to shed its elastic olive colored chorion membrane on week 3, thereby making the embryo visible. From week 4 onwards, the appendages of the embryo in the perivitelline fluid was visible through the transparent membrane. Embryo continues to grow and it changes color from yellowish green to olive green. During the last 2 weeks (week 6 and 7) of development, morphology of the embryo becomes more prominent with visible pair of lateral eyes and rotating vigorously in the perivitelline fluid. At this time the embryo is ready to hatch.

CONCLUSION

In conclusion, the most suitable salinity and watering frequency were 25-30 ppt and once in 3 days, respectively. Both media, water (liquid medium) and sand (solid medium) are equally suitable to be used as incubation media for *T. gigas* eggs. Overall, this study showed that *T. gigas* eggs can hatch as early as 40 days after fertilization depending on its incubation conditions.

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