

Egg Hatching Success in an Asian Horseshoe Crab, *Tachypleus gigas* Incubated in Different Sediment as Substrate

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Abstract

Tachypleus gigas is one of the Asian horseshoe crabs facing threats from human exploitation and habitat degradation. This study aimed to determine the effect of using sediment as substrate in the egg incubation procedure. Freshly laid eggs clutches were collected from a nesting beach during the low tide. Eggs were incubated under the treatment of seawater without sediment (T1), with fine sand (T2), coarse sand (T3), and muddy sand (T4) in the laboratory condition for seven weeks to observe the size, colour changes, and hatching success. Sediment was moderately sorted and poorly sorted for T2 and T3, and T4 respectively, which could be caused to the failure of egg development and hatching. Eggs in T1 (without sediment) developed as shown by the significant increase in size ($p < 0.05$) according to week and hatching success was at 27.8%. The egg's colour was normal for T1, but eggs turned black and rotten for T2, T3, and T4 despite the good maintenance of water parameters (temperature $29.18 \pm 0.57 - 29.56 \pm 0.7$ °C; salinity $29.4 \pm 0.5 - 29.8 \pm 0.8$ ppt; dissolved oxygen $6.16 \pm 0.90 - 6.64 \pm 0.90$ mg/L; pH $7.7 \pm 0.7 - 7.9 \pm 0.4$) during the incubation period. Further study is needed to verify the association of sediment grains and microenvironment thus improving the incubation protocol in the conservation initiative of this important living fossil.

Keywords: Horseshoe crab's egg, Sediment, Hatching

1. Introduction

Horseshoe crabs are interesting creatures from the phylum of Arthropoda which have maintained their features since 445 million years ago. The populations are reported to have been declining worldwide due to many reasons such as human exploitation and habitat degradation. At the same time, there is demand for the biomedical and pharmaceutical industry (Li et al., 2021), along with its use as a delicacy in some countries such as Japan, Hong Kong, and Malaysia. While *Tachypleus tridentatus* is a delicacy in Japan and Hong Kong,

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Tachypleus gigas is the most targeted species in Malaysia. *T. gigas* was reported to have high protein content and energy (Iszhan et al., 2021).

Horseshoe crabs need to land on a beach for breeding but there are many obstacles that need to be overcome before they successfully breed. They selected their preferred beach and tide level to increase the hatching success. For example, *Limulus polyphemus* was reported to choose the highest tide level at the beach with good temperature and avoid hypoxia area to lay eggs (Vasquez et al., 2015). Differences in beach geomorphology over short distances could be detected by horseshoe crabs (Botton et al., 2018). Additionally, fishing gear deployment would affect the landing effort as well (Wang et al., 2021).

T. tridentatus population size was estimated to range from 182 to 1095 on Sabah Beach (Manca et al., 2017). In Sarawak, the highest landing population was distributed at 1.72 individuals per hectare (Jawahir et al., 2017). The number of landings showed comparatively lower than those horseshoe crabs caught by fishermen for commercial purposes. Estes et al. (2021) suggested small-scale variation in habitat quality can affect the occurrence of horseshoe crabs in sparse populations where density is not a limiting factor. Manca et al. (2016) emphasized the role of sediment composition on the breeding success of the animal in the wild. Earlier, Jackson et al. (2005) reported on how sediment texture influenced egg viability and development. Medium-sized sand grains could be the best character of sediment preferred for nesting (Kwan et al., 2022). Furthermore, predation also affects hatching in the wild (Beekey et al., 2013), thus it is necessary to establish a hatchery that can be dependable for the survival of horseshoe crab eggs and larvae to maintain their population in the sea. This study aimed to determine the effect of different sediment types on the development and hatching of eggs in a laboratory. It will lead to the establishment of a possible protocol for hatching and nursing activities for horseshoe crabs to support any conservation initiative for this animal.

2. Materials and Methods

2.1 Field sampling

The eggs of *T. gigas* were sampled from the beach at Tanjung Gosong, Pekan Pahang (Lat.3°36.181' N, long.103°23.946' E), on the east coast of Peninsular Malaysia. Freshly laid eggs clutches were collected during the low tide at approximately 9-20 cm depth of nest. The eggs were then placed in a sealed plastic bag and brought back to the laboratory for further treatment.

2.2 Sediment preparation

Sediment samples collected during the field sampling were first oven dried at 70°C for four hours. This was followed by sedimentary analysis which was determined based on the characteristic shown by the sediment. Sediment samples with high content of fine particles were analysed through Particle Size Analyzer equipment while coarse sediment went through a dry sieving method (Forde *et al.*, 2012; Romano *et al.*, 2017). The sampled sediment of different class types was then prepared as the substrate for egg incubation.

2.3 Laboratory experimental design

There was a total of 420 eggs secured from the sampling. The entire 420 eggs were divided into 4 groups (T1, T2, T3, and T4) hence all the groups have 3 replicates with 30 eggs in each replicate. Eggs were cleaned by rinsing them using sterile seawater for 30 seconds to remove the fouling dirt and sand. Then they were split evenly into 4 groups and each group was in triplicate (Table 1). A total of 12 units of 500 ml glass beakers were prepared according to treatment (T1, T2, T3, T4). For each treatment, a three cm sediment was placed in the beaker and added with 150 ml sterilized seawater. 30 eggs were buried two cm depth in each beaker and supplied with soft aeration.

Table 1 Summary of treatment for *T. gigas* eggs

| Treatment | Incubation condition |
|--------------|----------------------------------|
| T1 (control) | No substrate in sterile seawater |
| T2 | Fine sand in sterile seawater |
| T3 | Coarse sand in sterile seawater |
| T4 | Muddy sand in sterile seawater |

2.4 Water parameters monitoring

Water parameters including temperature, salinity, dissolved oxygen, and pH were maintained accordingly. Temperature, pH, and dissolved oxygen were measured using YSI multiprobe (Model 556 MPS) and salinity using a refractometer twice a day. Water exchanges were carried out once every 2-3 days during the trial. The temperature was controlled by locating the beakers in the water bath.

2.5 Data collection and statistical analysis

The experiment was carried out for about 70 days whereby measurement and observation were carried out weekly. It was expected that the eggs would hatch within the incubation period as reported by the previous study (Faizul et al., 2013; Zaleha et al., 2011). The diameter of the egg was measured once a week using a digital caliper in 0.01mm units. Five out of 30 eggs for each replicate were measured randomly. The eggs' colour was observed once a week. The color changes from the initial were observed until hatching. The hatching period and successful hatching according to treatment were recorded daily. Hatching is measured by counting the number of trilobites hatched from the total buried eggs. Significant difference effects among treatments were determined using One-Way ANOVA. Post hoc analysis, primarily Duncan and LSD were applied to investigate the treatment that gives the most significant performance. Data were analysed using SPSS version 22.0.

3. Results

3.1 Sediment substrate

Sediment substrates used as a treatment in this experiment have a range of mean sizes from mud to coarse sand characters. Only T2 had a well-sorted sediment, while T3 and T4 had a poorly sorted condition as shown in Table 2.

Table 2 Characteristics of sediment substrate

| | T2 (Fine sand) | T3 (Coarse sand) | T4 (Muddy sand) |
|-------------------|----------------|------------------|-----------------|
| Mean (phi) | 2.91± 0.01 | 0.83± 0.67 | 6.85±0.13 |
| Sorting | 0.43± 0.03 | 1.10± 0.89 | 1.19±0.11 |
| Sorting condition | Well-sorted | Poorly-sorted | Poorly sorted |
| Skewness | -1.63±0.12 | 0.23± 0.01 | -0.79±0.19 |
| Kurtosis | 12.61± 1.03 | 1.90± 0.91 | 3.78±0.11 |

3.2 Water parameters

During the incubation period, all treatments (T2, T3, T4) and control (T1) showed warm water conditions and had a comparable mean value of salinity, dissolved oxygen, and pH (Table 3).

Table 3 Mean of water parameters ± (Standard Deviation, SD) during the incubation period of week 1 to week 7

| Treatment | Temperature (°C) | Salinity (ppt) | Dissolved oxygen (mg/L) | pH |
|-----------|------------------|----------------|-------------------------|-----------|
| T1 | 29.56 ± 0.7 | 29.4 ± 0.7 | 6.44 ± 0.86 | 7.8 ± 0.4 |
| T2 | 29.38 ± 0.73 | 29.8 ± 0.8 | 6.32 ± 0.89 | 7.9 ± 0.4 |
| T3 | 29.41 ± 0.75 | 29.4 ± 0.5 | 6.16 ± 0.90 | 7.9 ± 0.4 |
| T4 | 29.18 ± 0.57 | 29.4 ± 0.6 | 6.64 ± 0.9 | 7.7 ± 0.7 |

3.3 Colour of eggs

All eggs were greenish in color when first collected from the beach, but some changed differently towards the end of the experiment. Table 4 shows the percentage of color in each treatment from week 1 until week 7. In T1, the color of the egg was the same for the first three weeks before it changed into dark green and finally yellowish and became translucent. At week 6 and week 7, 18 and 7 of the eggs hatched into trilobite larvae respectively, thus the percentage of yellow eggs reduced. The larvae were greenish in colour as normal larvae of *T. gigas*.

Eggs exposed to T2 and T3 changed their colour from greenish to blackish green towards the end of the experiment. A small percentage of development was shown under T3 treatment by the yellow colour changes but did not progress. The eggs were black with a rotten smell in T3 and T4. These black eggs were found to be covered by *Vorticella* sp.

Table 4 Effect of sediment type on colour changes of eggs from week 1 to week 7

| Weeks | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|-------|-------------|------|------|------|-----|------|------|------|
| T1 | Green | 100% | 100% | 49% | 19% | 8% | 8% | 8% |
| | Dark-green | - | - | 39% | 33% | 29% | 22% | 17% |
| | Yellow | - | - | 12% | 48% | 63% | 70% | 48% |
| | Black | - | - | - | - | - | - | - |
| T2 | Green | 100% | 100% | - | - | - | - | - |
| | Dark-green | - | - | 100% | 81% | 78% | 27% | 11% |
| | Yellow | - | - | - | - | - | - | - |
| | Black | - | - | - | 19% | 22% | 73% | 89% |
| T3 | Green | 100% | 100% | - | - | - | - | - |
| | Dark-green | - | - | 88% | 79% | 78% | 62% | 61% |
| | Yellow | - | - | 7% | 7% | 6% | 6% | 6% |
| | Black | - | - | 6% | 14% | 17% | 32% | 33% |
| T4 | Green | 100% | 100% | - | - | - | - | - |
| | Dark- green | - | - | 67% | 52% | - | - | - |
| | Yellow | - | - | - | - | - | - | - |
| | Black | - | - | 33% | 48% | 100% | 100% | 100% |

3.4 Saiz of eggs and hatching success

Fig. 1 shows the size changes in eggs when exposed to different treatments, T1, T2, T3, and T4. The One-Way ANOVA statistic recorded that the size of the egg was not significantly different ($F=99.7$, P value = 0.00) at least between three treatments. There was also no significant difference at least between 6 weeks for control ($F=28.8$, P value = 0.00), fine sand ($F=4.15$, P value = 0.001), coarse sand ($F=7.86$, P value=0.00), and muddy sand ($F=3.30$, P value = 0.005). Duncan's result shows that T2, T3, and T4 had no significant difference between them, meanwhile, T1 shows a significant difference between weeks. A significant increase in size for T1 was observed between week 4 and week 7. Following the increase in the size of the eggs in T1, some of the eggs hatched in week 6 and week 7 (Table 5). The success rate was 27.8%. The change in size and condition of eggs under T1 is represented by Fig. 2. No egg successfully hatched under T2, T3, and T4.

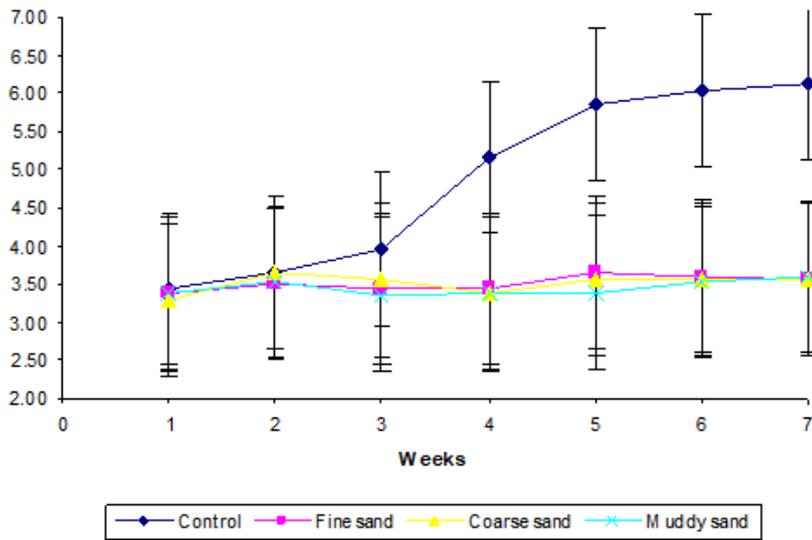


Fig. 1 Size changes in eggs of *T. gigas* incubated in different treatments (T1, T2, T3, T4) between week 1 and week 7

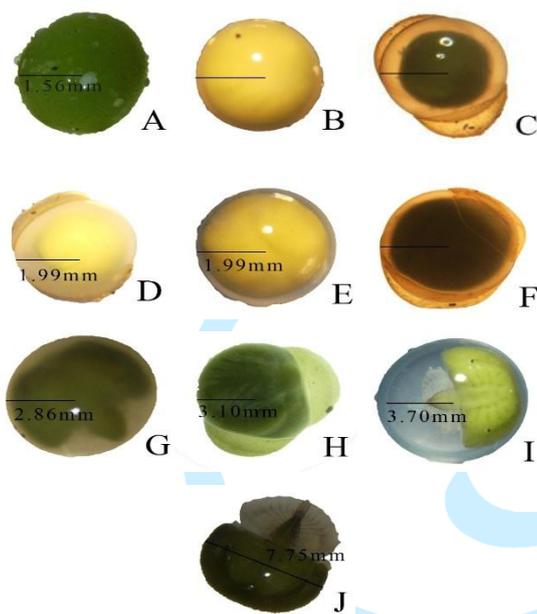


Fig. 2 The stages of horseshoe crab eggs development: (A&B) Stage 1-10, Fertilised egg undergo cleavage, blastula and gastrula stage; (C) Stage 17, first embryonic moulting, (D) Stage 18, second embryonic moulting; (E) Stage 19, third embryonic moulting, horseshoe crab larvae appendages develop; (F) dorsal view of horseshoe crab embryo; (G-I) After fourth embryonic moulting, larvae flattens considerably and is ready to hatch; (J) A dorsal side of hatched trilobite larvae

Table 5 Hatching duration of *T. gigas* eggs within 7 weeks of the experiment and hatching success (%)

| Treatment | Hatching duration of <i>T. gigas</i> eggs | | | | | | | Success (%) |
|------------------|---|--------|--------|--------|--------|--------|--------|-------------|
| | Week 1 | Week 2 | Week 3 | Week 4 | Week 5 | Week 6 | Week 7 | |
| T1 (Control) | 0 | 0 | 0 | 0 | 0 | 18 | 7 | 27.8 |
| T2 (Fine sand) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T3 (Coarse sand) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| T4 (Muddy sand) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

4. Discussion

T. gigas eggs were reported to be able to hatch when incubated in either water or sediment of their suitable environmental condition (Faizul et al., 2013). As reported by Biswal et al. (2016) eggs could be hatched between 35 to 42 days of incubation in seawater and a sediment substrate. Nonetheless, the type of sediment used was not further detailed for reference. In the present study, it was recorded that all sediment types used which were muddy, fine sand, and coarse sand did not support success in egg hatching during the incubation period.

Natural and cleaned sediment was reported to give different effects on the growth of horseshoe crab larvae (Hieb et al., 2015). Similarly, in nature, sediment from beaches and salt marshes does support the

development of the egg but up to a certain stage only (Kingsley-Smith *et al.*, 2016). As reported by Kwan *et al.* (2022) the nest which was characterized by medium-sized sediment grains (0.5 – 0.9 mm) would support the hatching success in combination with other favorable water parameters.

Biswal *et al.* (2016) used a cooler temperature for the incubation and found that it helped the laboratory incubation process. As mentioned by Vasquez *et al.* (2015), the concerted effect of temperature, salinity, dissolved oxygen, and pH resulted in the hatching success of eggs. Smith *et al.* (2017) reminded us of the disturbance risk found in the natural nest which would change favorable water parameters. An unexpected change in colour (red, grey, black) of the eggs could indicate a bacterial and fungal infestation (Faizul *et al.*, 2015). The same situation was reported in the work of Razak *et al.* (2022). Vorticella, as an ectoparasite protozoan was reported to infest the eggs of cultured lobsters (Nur & Yusnaini 2016).

Hatching was successful only after 35 days in this study, indicating some environmental pressure occurred in the incubator. The absence of sediment could help in avoiding disease risk, but possibly not other environmental stressors such as pollutants from the nest site. Vasquez *et al.* (2015) showed how environmental multiple-stressor could affect embryo development in horseshoe crab eggs. Previously, Zaleha *et al.* (2011) reported on the high metal concentrations in the nesting ground on the coast of Pahang. The seabed of Kuala Pahang River in front of the Pekan nesting area for the present study was also reported as a sink area for marine debris (Hamizah *et al.*, 2022). There could be a possible interference of the pollutants in the nest area and affect the quality of the eggs obtained for the present study. The origin of the eggs collected and used for the incubation should now be considered to determine the hatching success.

5. Conclusions

T. gigas, the Asian horseshoe crab, which is commonly found in the coastal water, is exposed to climatic changes effects as well as human activities. All sediment-type tests which include fine, coarse, and muddy sand with poorly sorted or moderately sorted conditions did not support hatching but could trigger bacterial and fungal infection leading to unsuccessful hatching until the end of the incubation in week 7. Incubation without sediment gave a better effect on the hatching success. Further study is needed to verify the association of sediment grains and microenvironment with the embryogenesis activity in the eggs during incubation. This will contribute to the improvement of incubation protocol in the conservation initiative of this important living fossil.

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Declaration of Conflict

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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