HORSESHOE CRAB AND ITS SPAWNING GROUND CONDITION IN JOHOR LAMA, JOHOR

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Received: 13th Apr 2021 Accepted: 2nd Aug 2021 Published: 28th Feb 2022
DOI: https://doi.org/10.22452/mjs.vol41no1.1

ABSTRACT This study was conducted at horseshoe crab's natural spawning ground in Johor Lama, Kota Tinggi, Johor, Malaysia (1°35'00"N 104°00'49"E). Six nests were excavated on 4 August 2020, four hours after the highest tide. Type of reclamation, and fishery activities at the spawning site were observed. Grain size analysis was conducted according to Blott and Pye procedure. Eggs hatching rate, larvae moulting rate, infection rate, and larvae abnormality rate tests were conducted. There are two types of reclamation observed in Johor Lama: (i) concrete wall and (ii) stack of boulders. Mangrove and muddy areas in Johor Lama are still preserved and in good condition. Sand at the horseshoe crab spawning beach in Johor Lama was coarser and poorly sorted ($\bar{x}_{\emptyset}$: 0.09± 0.01 cm; $\sigma_{\emptyset}$: 1.89 ± 0.03) as compared to those of Balok, Pahang (mid-tide mark, August 2012: $\bar{x}_{\emptyset}$: 2.38 ± 0.04 cm; $\sigma_{\emptyset}$: 0.86 ± 0.04). The quantity of the eggs inside each nest in Johor Lama was also in accordance with those of other previous studies (first nest n: 272, second nest n: 233, third nest n: 157, fourth nest n: 135, fifth nest n: 143, sixth nest n: 111). However, the hatching rates of each sample in this population were observed to be lower than those of the others previously studied (31.8% – 66.1%). Two types of larvae abnormalities were reported in this study: (i) genetic impairment that changed the basic Xiphosura's body plan, and (ii) external factor that caused by the impact of the substrate or predatory action that would distort the shape of the juvenile exoskeleton. Based on the observation, the natural ecosystem in Johor Lama is still in good preservation, since it is far from urbanisation and has less deforestation. However, the future of this spawning site is still in doubt due to many ports and industrial zones located on the opposite side of the river in Johor Bahru district that could lead to the worst water pollution.

Keywords: *Tachypleus gigas*, spawning site, horseshoe crab's eggs, sand grain size, abnormality.

1. INTRODUCTION

A surge in finding important natural resources in the medical field has increased the marketability of horseshoe crab (Naqvi et al., 2004; Fisher & Fisher, 2006; Gerhart, 2007). Researchers have acknowledged the capability of Amoebocyte Lysate compound in horseshoe crab's blueblood in detecting the presence of bacterial endotoxin i.e., the final sterility screening process of drugs and pharmaceutical apparatus (Naqvi et al., 2004; Gerhart, 2007; Gauvry, 2011; John, 2012; Botton et al., 2015). Currently, the production of Limulus-Amoebocyte Lysate (LAL) is dominated by the Atlantic species (*Limulus polyphemus*). While the market value of the Asian ones could also exceed multi-million dollars if this new economical field was implemented on the Asian species (Yan, 2008; Beekey et al., 2013). Nevertheless, the Asian species is facing a decline in their population due to anthropogenic pressure (Jackson et al., 2005; Ngy et al., 2007; Iwaoka & Okayama, 2009;

Hajeb et al., 2009a; Faurby et al., 2010; Cartwright-Taylor et al., 2011; Tan et al., 2012; Azwarfarid et al., 2013; Nelson et al., 2015). Hence, intensive study on this invaluable species is needed to maintain its sustainability.

Studies on horseshoe crab spawning and nursing grounds are crucial to evaluate the suitability of the area to support the development of the eggs and larvae (Nordstrom et al., 2006; Manca et al., 2016). Sungai Johor is one of the most strategic horseshoe crab spawning sites on the south coast of Peninsular Malaysia. This is because the area is protected from direct southwest and northeast monsoon currents. Besides that, there are many sandy beaches located along the riverbank of Sungai Johor stretching starting from the opening of the river up to 20 km inwards that is still under the influence of seawater, including Johor Lama. Based on the observation, the natural ecosystem in Johor Lama is still preserved and in good condition as it is far from the urban area that hence, led to less deforestation problem. According to Mazlan et al., (2005) mangrove deforestation would disturb the stability of the ecology of the nursing area of horseshoe crabs' larvae and juveniles. Among the anthropic activities that could influence the condition of horseshoe crab spawning site and nursing ground include (i) reclamation (Shinohara, 1989; Chen et al., 2004; Fairuz-Fozi et al., 2018), (ii) habitat destruction (Itow et al., 1991), (iii) overexploitation (Jackson et al., 2005; Ngy et al., 2007; Faurby et al., 2010; Nelson et al., 2015) and (iv) pollution (Itow, 1998; Tan et al., 2012; Azwarfarid et al., 2013).

These activities would deteriorate the condition of the spawning ground be it directly or indirectly. Land reclamation would decrease the size of exposed sandy beaches as well as increase the size of the grain sand in the perturbed area. According to the previous studies, horseshoe crabs prefer to lay their eggs at the beach that has higher fine grain sand composition. Additionally, overexploitation would, in turn, decrease the percentage of the successful spawning ritual by adult horseshoe crabs as they fail to reach the spawning site (Rahman, 2019). The percentage of the horseshoe crabs' carcasses and exuviae along the spawning beach could be an indicator of horseshoe crab exploitation. The effect of heavy metal pollution in the spawning ground on the horseshoe crabs' larvae during the early stage of development was previously reported by Itow et al., (1998). Higher numbers of deformed horseshoe crabs' larvae during their early development stage in particular spawning sites could be an indicator of pollution. Liang et al., (2020) study has reported that heavy metal concentrations in Johor River exceeded the allowable limit. Nevertheless, no data on the horseshoe crab's development during the early stage in Johor Lama was reported by the researcher.

As the stabilomorph animals, most of the horseshoe crab's conditions and behaviours such as, hatching rate, ecdysis, type of infection, and larvae movement under the optimum salinity and temperature might have no significant difference between populations. However, an observation is needed since there is no report on the adaptation of horseshoe crabs' larvae to their nursing grounds in Johor lama. There are many horseshoe crab spawning grounds located throughout the Malaysian peninsular coastal areas, namely; Balok (Tan et al., 2012; John et al., 2013; Nelson et al., 2016), Cherok Paloh (Mohd Razali & Zaleha, 2017; Razak et al., 2017; Razak & Kassim, 2018a,b,c), Pekan (Kamaruzzaman et al., 2011; John et al., 2012; Nelson et al., 2015), Mersing (Muda et al., 2010), Sedili (Jaffar, 2013; Sulaiman et al., 2019), Pendas (Fairuz-Fozi et al., 2018), Tanjung Piai (Ang, 2016), Port Dickson (Hajeb et al., 2009a; Liew et al., 2015), Pulau Lumut (Hajeb et al., 2009b; Shakiba Zadeh et al., 2009), Teluk Senangin (Yung, 2015), Tanjung Dawai (Ismail et al., 2011; Ismail & Sarijan, 2011) and Kuala Perlis (Yap et al., 2019). However, there is no reliable study on Johor Lama to date. This study was conducted to review the conditions of the horseshoe crab spawning sites in Johor Lama, specifically on the conditions of the spawning grounds (type of reclamation, size of the exposed sandy beach and sand composition), the properties of horseshoe crab eggs (quantity, hatching rate, and infection rate) and the development of the larvae (ecdysis and abnormalities).

2. METHODOLOGY

2.1 Site Survey

A survey was conducted at a horseshoe crab natural spawning ground in Johor Lama, Kota Tinggi, Johor, Malaysia (1°35'00''N 104°00'49''E), (Figure 1). The conditions of the sampling area such as the type of reclamation, the area of the exposed sandy beach at the horseshoe crabs' nest and deforestation as well as fishery activities such as the number of ports and raft houses were observed and recorded to plan further procedure. The presence of horseshoe crab carcasses and exuviae along the beach were collected for identification. Sand from the top until the level of the deposited eggs at each nest was collected for grain sand size analysis.

2.2 Horseshoe Crab Nests Sampling

Six nests were excavated after four hours of the highest tide on 4 August 2020. The depth of the nest (mm) and the width of the beach (m) were measured and recorded. There were three nests contained with freshly laid eggs (first nest n: 272; second nest n: 233; third nest n: 157), one nest with embryonic eggs (fourth nest n: 135), and 2 nests with hatched first instar larvae (fifth nest n: 143; sixth nest n: 111), (Figure 2). All nests were located side by side with the same distance from the water line (Figure 3). The eggs were collected and placed separately according to their nests in a container filled with some sand from around them to ensure the eggs will be fully fertilized by the remaining sperm in the sand (Zaleha et al., 2011). Each container was also filled with filtered seawater. The eggs were acclimated at room temperature for 30 minutes before washing to discard any excessive sand around them (Schreibman & Zarnoch, 2009). The collected larvae were washed immediately by the filtered seawater. During transportation to the laboratory, the eggs and the larvae were kept in a cold polystyrene box with the temperature controlled around 25ºC to avoid desiccation and overheating. The samples were brought to the laboratory for incubation, rearing, and observation.

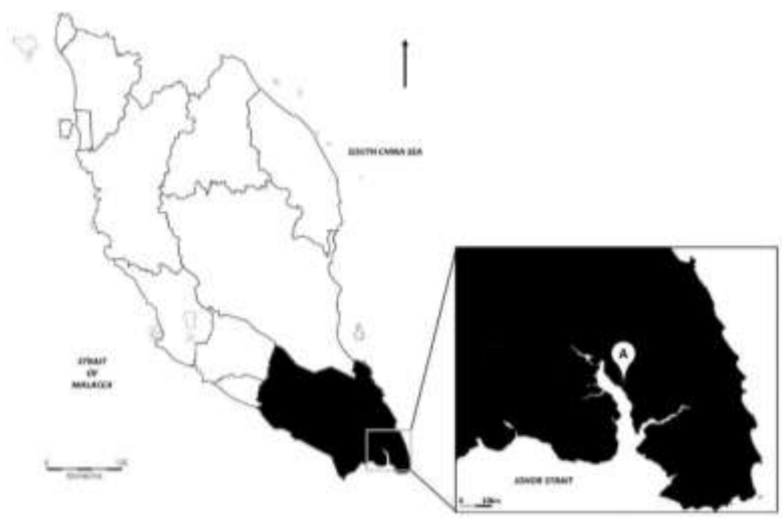

Figure 1. A: Sampling location in Johor Lama, Kota Tinggi, Johor (1°35'00''N 104°00'49''E).

Figure 2. Horseshoe crab 1st instar larvae under the sand (nest).

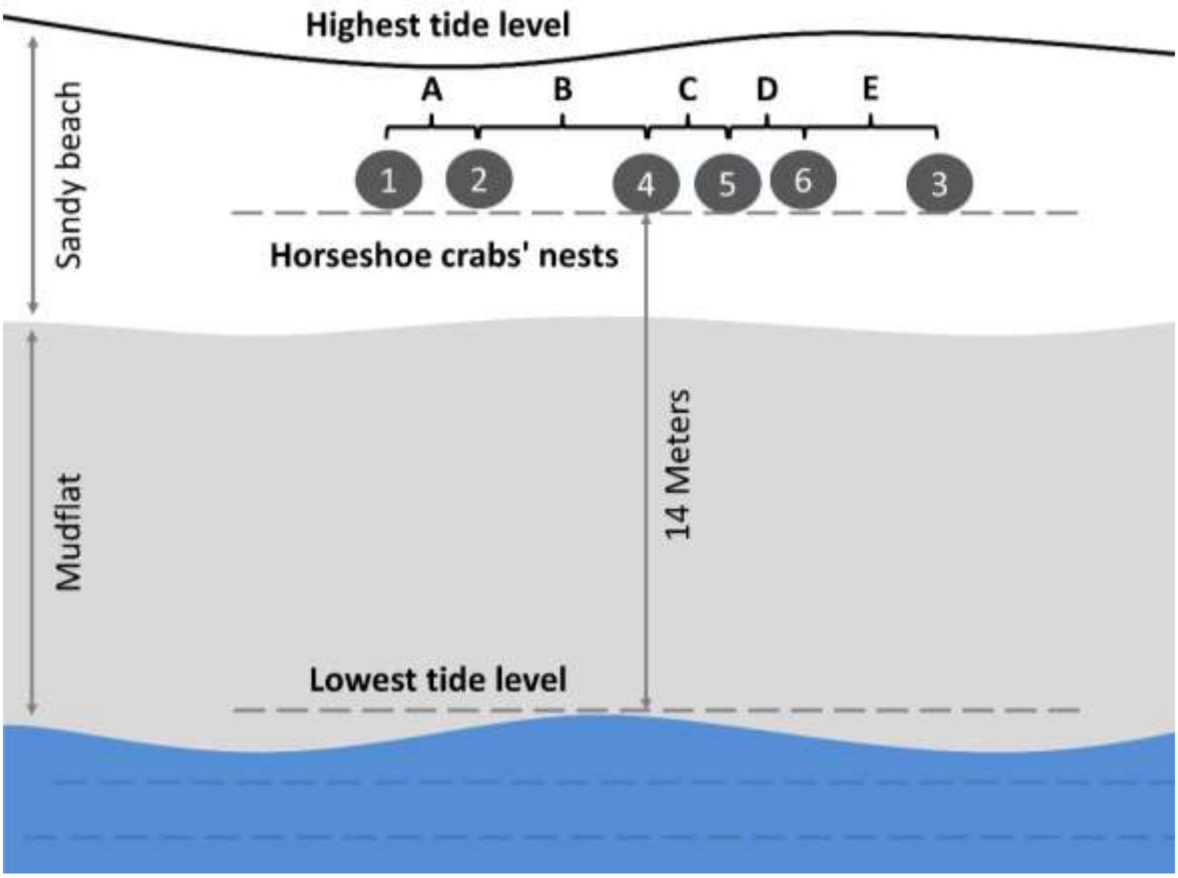

Figure 3. Distance between lowest tide level to horseshoe crabs' nests (4 August 2020) and distance between nests. (A,B,C,D,E = distance between nest; 1,2,3,4,5 = position of nest). A: 30cm. B: 100cm. C: 25cm. D: 17cm. E: 50cm.

2.3 Sand Grain Size Analysis

Grain size analysis was conducted according to Blott and Pye (2001). 300g from each nest were transferred into three petri dishes of 100g each that were oven-dried for three days at 45 °C. The sand samples were then transferred into a series of 13 sieves ranging from 4 mm to 0.063 mm and were placed on a mechanical shaker which was set to function at 9 g acceleration for 30 minutes. The sand size was logarithmically transformed

($\emptyset = -\log_2 \mathrm{d}$; where d is the sand grain diameter in millimetres, mm). The logarithmic method of moments was implemented in estimating the mean grain size ($\bar{x}_\emptyset$) (Eq. 1) and sediment sorting properties ($\sigma_\emptyset$) (Eq. 2). Thus, f is the frequency in percentage (%), m is the mid-point of each class interval in phi ($m_\emptyset$).

$$\bar{x}_\emptyset = \frac{\sum f m_\emptyset}{100} \tag{1}$$

$$\sigma_\emptyset = \sqrt{\frac{\sum f (m_\emptyset - \bar{x}_\emptyset)^2}{100}} \tag{2}$$

2.4 Eggs Incubation

A total of 400 eggs from the first, the second, the third, and the fourth nests were incubated accordingly in four different containers hence, 100 eggs each. The water parameters of each container (15 cm x 5 cm x 10 cm) were controlled around 30ºC and 30‰ according to Cartwright-Taylor et al., (2009) and Zaleha et al., (2011). A non-circulated water system was applied in the eggs incubation tank. Observation on freshly laid eggs was conducted daily to observe the growth, the presence of infection (bacteria and fungus), the fertilizing rate, and the hatching rate. The infected eggs with bacteria and fungus were discarded promptly and recorded. The identification of the infected eggs was conducted according to Faizul et al., (2015). The unfertilised eggs were initially determined by the colour. Mostly, the fertilised eggs would change to milky greenish or yellowish. The eggs that did not change in colour were also separated into different containers for 40 days to ensure they were unfertilised. 100% of the isolated eggs did not develop. Deformities of the embryonic eggs from the fourth nest were observed until they were hatched. The normal and the deformed hatched larvae were promptly separated into different containers.

2.5 Larvae Observation

A total of 200 larvae from the fifth and the sixth nests were reared in a recirculated aquaculture system (RAS) (Chen et al., 2010). The eggs were separated into 4 different buckets with 50 larvae each according to their origin nests. The water culture parameters were controlled around 25–30ºC and 20–25‰ according to Laughlin (1983) and Zaleha et al., (2011). The system was installed according to the following figure (Figure 4). The abnormalities of the collected larvae were observed upon arrival at the laboratory. The abnormalities in larvae morphology were recorded and described. The daily observations were conducted to identify the colour changes and pigmentation of the carapace and the activeness of larvae. It is hypothesised that the pigmentation of the larvae carapace will increase as they are ready to moult. The development of the pigmentation appearance on larvae carapace was observed daily. Test on the larvae movement was conducted every five days by placing a sample on a petri dish filled with fine sand and seawater. Moving trails of a sample within one minute were measured. The tests were repeated ten times by the same samples of larvae until they had moulted to the second instar stage. The movement between days was analysed by using Analysis of Variance (ANOVA), Tukey post-hoc test. The period of the ecdysis phase was recorded once the exuviae predetermined line that was located at the edge of the anterior prosoma, split open until it had completely been discarded from the larva. The moulted larvae were separated according to their instar stages. The moulting failure of larvae were then analysed and recorded.

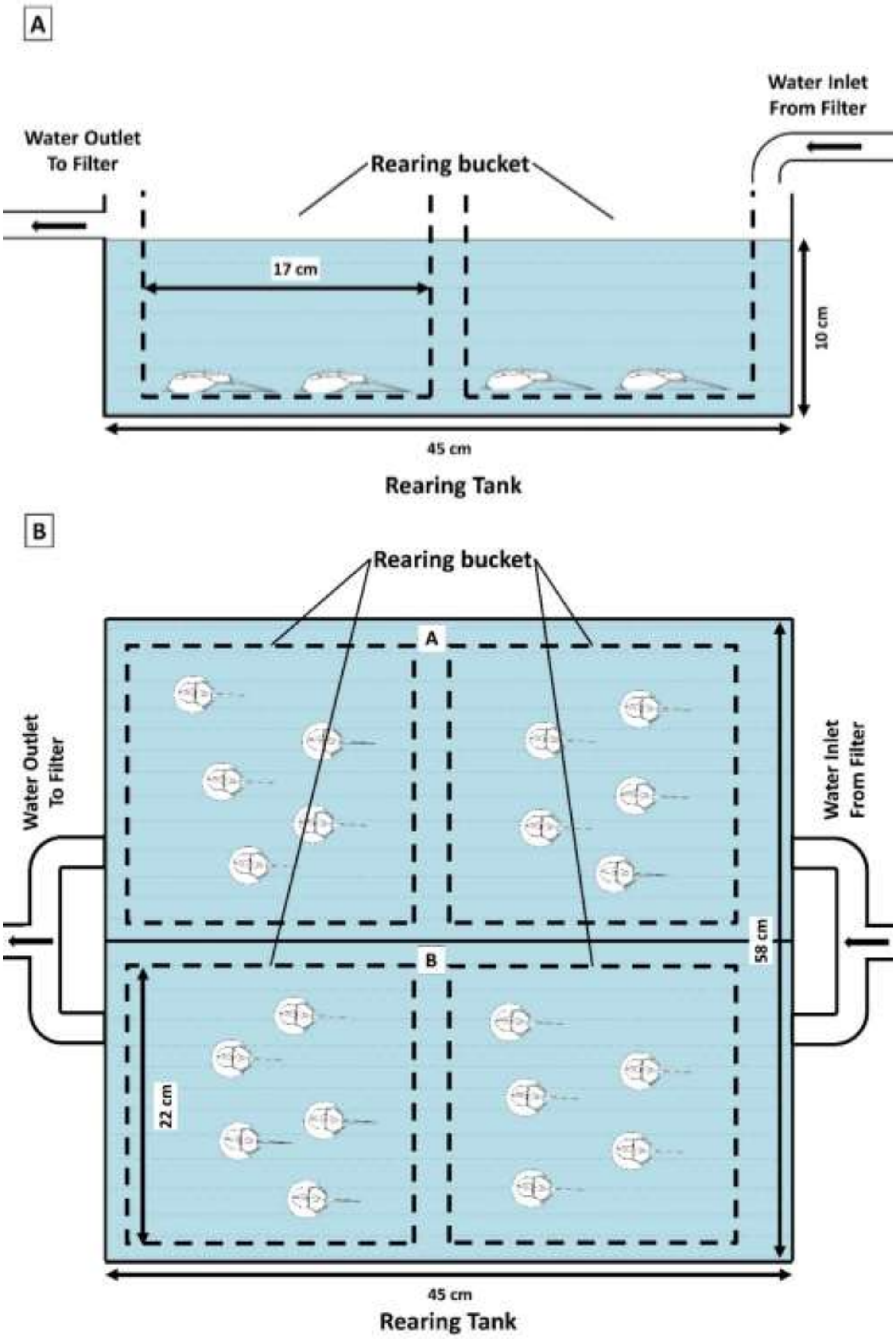

Figure 4. Recirculated aquaculture system (RAS) in rearing horseshoe crabs' larvae. A: Side view. B: Plan view. B-A: Fifth nest larvae. B-B: Sixth nest larvae.

3. RESULT

Many reclamations can be found along the Johor Lama beach. Two types of reclamation which were observed in Johor Lama were concrete walls and stacks of boulders (Figure 5). The width of the sandy beach during the lowest tide was approximately around three to five meters. The size of sand grain at all horseshoe crabs nests in Johor lama in this study was bigger (coarse sand) and poorly sorted ($\bar{x}_{\emptyset}$: 0.09± 0.01 cm; $\sigma_{\emptyset}$: 1.89 ± 0.03), (Figure 6) as compared to the well-studied horseshoe crab spawning sites in

Balok, Pahang (mid-tide mark, August 2012: $\bar{x}_{\emptyset}$: 2.38 ± 0.04 cm; $\sigma_{\emptyset}$: 0.86 ± 0.04) (Nelson et al., 2019). During the period of the study, Johor Lama had only one fisherman port and this area could be considered as low boat traffic as compared to Teluk Sengat. However, there were many rafting houses built by the locals scattered from Teluk Sengat up to Johor Lama. There was no evidence of significant mangrove deforestation in Johor Lama and most of the mangrove and mudflat areas were still in good condition. There were many horseshoe crab carcasses and exuviae found along the 600 meters of Johor Lama beach (Table 2). The highest proportion of horseshoe crab exoskeleton found on the beach were contributed by the males, followed by the females and then the exuviae. The difference in the condition for each carcass related much to the cause of death. Exuviae have a specific characteristic; a split opening that is elongated at the edge of the anterior prosoma was used to differentiate them from the carcass.

Figure 5. Wave breaker constructed along the beach to avoid beach erosion. A: Concrete wall. B: Stack of boulder.

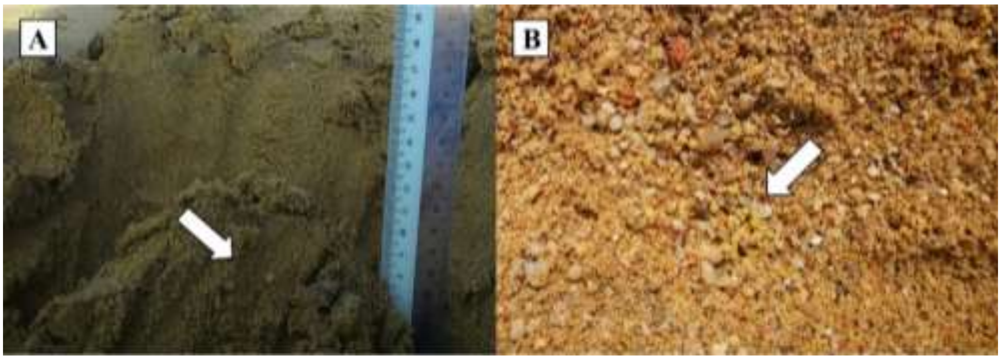

Figure 6. Horseshoe crabs spawning site at Balok, Pahang (A) and Johor Lama, Johor (B). Arrow: Horseshoe crab’s eggs.

Table 1. Quantity and condition of the stranded horseshoe crab carcasses and exuviae along the Johor Lama beach.

Species	Type	n	Condition
Carcinoscorpius rotundicauda	Exuviae	1	Juvenile, thinner exoskeleton, intact dorsal prosoma & opisthosoma, lost ventral appendages, split anterior prosoma edge (Figure 7).
	M. carcass	3	Matured male, unspecific broken (cut off) body part.
	F. carcass	2	Matured female, unspecific broken (cut off) body part.
Tachypleus gigas	Exuviae	1	Juvenile, thinner exoskeleton, severe broken dorsal prosoma, intact dorsal opisthosoma, lost ventral appendages, split anterior prosoma edge (Figure 7).
	M. carcass	8	Matured male, unspecific broken (cut off) body part.
	F. carcass	5	Matured female, intact dorsal prosoma & opisthosoma, lost ventral appendages (Figure 8).

*n: quantity (pcs), M: male, F: female.

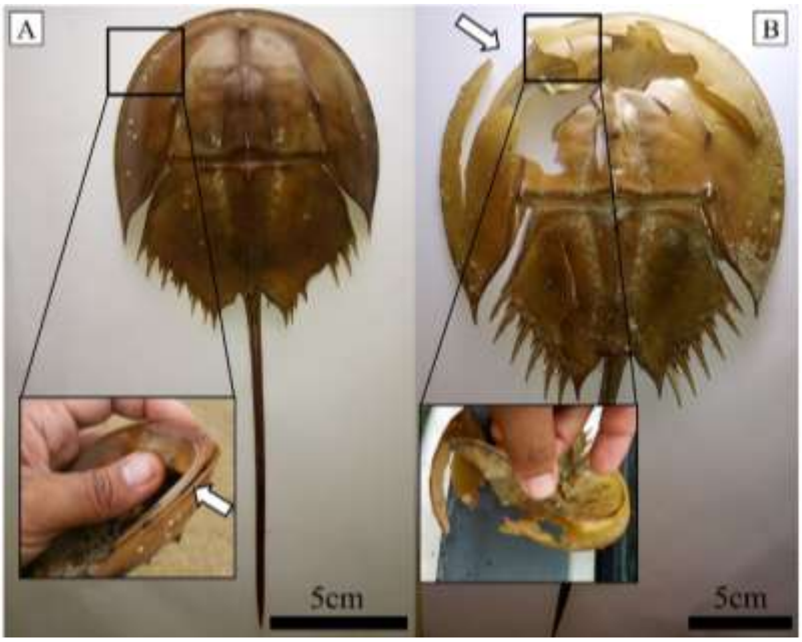

Figure 7. Horseshoe crab exuviae. A: *C. rotundicauda*, B: *T. gigas*, Arrow: Split opening at the anterior prosoma edge.

Figure 8. Female's (*T. gigas*) carcass with lost ventral appendages, intact dorsal carapace, and no split opening at the anterior prosoma.

Table 3 shows the number of eggs and larvae, the stage of development, the hatching rate of the eggs, the moulting rate of the larvae, and the infection rate for each nest. There were six horseshoe crab nests found in Johor Lama beach. Three out of six nests contained freshly laid eggs; one with embryonic eggs, and the final two nests contained first instar stage larvae. The eggs hatched after 40 ± 5 days in incubation. The hatching rate for the second nest was higher (66.1%) as compared to the first (33.1%) and third (31.8%) nest. The hatching rates for Johor Lama horseshoe crab eggs in this study were lower than the ones reported in Zaleha et al., (2011) and Biswal et al., (2016) studies. The moulting rate for both the fifth and the sixth larvae stage nests could be considered as high with 8% in slight difference (fifth nest: 90.9%, sixth nest: 82.9%). Larvae started to moult to second instar on day 25^{th} until 100^{th} under the laboratory environment after they hatched. No fungus and bacteria infestation observed on the fourth, the fifth, and the sixth nest samples (first nest: 24%, second nest: 9.6%, third nest: 28%, fourth nest: 0%, fifth nest: 0%, sixth nest: 0%). There were two types of infection detected infesting the eggs during the incubation period: (i) bacteria and (ii) fungus (Figure 9).

Bacterial infection can be determined by the presence of reddish colour followed by increase in size during the incubation period, while fungus infection can be determined by the presence of whitish mould that will later turn to green or black (Faizul et al., 2015). In this study, most of the infected eggs were caused by the once unfertilized and would further be infected as soon as day-two in the incubator. The unfertilized eggs were identified from the colour as unfertilized eggs will remain greenish after three days, while the fertilised ones turned milky greenish or yellowish. Many types of abnormalities were identified during the embryonic development stage such as a conjoined twin larva caused by genetic impairment (Figure 10).

Figure 10-A,B shows a diprosopus twin larva that shared a single abdomen and head, but bore a two-face region. Both twins had one separated eye but shared the other eye. They also had completely separated gnathobases located at the ventral of each prosoma. Figure 10-C,D shows a dipygus twin that had a single prosoma with two abdomens. The divarication of the abdomens started from the hinge until the end of the telson. All genetically impaired larvae failed to moult to the second instar stage. Besides genetic

abnormalities that occurred during embryonic development, some of the abnormalities happened due to external physical factors as well.

Figure 11 shows the same abnormal larva during the first and the second instar stages which were collected from the sixth nest. However, the morphology was not completely different as compared to the normal larva. Although the shape of the exoskeleton was slightly distorted, the basic Xiphosura's body plan was still in a good form. Besides that, the morphology had improvised as the larvae moulted to the second instar. Normal larvae take 1 hr 5mins ± 6 mins to complete their ecdysis phase. However, the minor deformed larvae from this study took approximately 30 ± 5 minutes longer to discard the exuviae during ecdysis, due to some of the larvae body parts getting stuck in their exuviae which eventually caused the impaired exoskeleton shape. Moulting failure is one of the problems that can lead to horseshoe crab mortality. This can be divided into three types of failure: prosomatic, opisthosomatic, and appendages (Figure 12).

Prosomatic moulting failure can be determined by the condition of larvae's exuviae being stuck at the prosomatic region and the opisthosomatic failure happens when the exuviae are completely discarded from the prosomatic region but somehow failed to pull the opisthosomatic region out of the exuviae. Meanwhile, appendage failure happens when the exuviae are only stuck at the larvae's appendages. Based on the observation, the development of pigmentation on larvae carapace had significantly increased as they nearly moult (Figure 13). Pigmentation had significantly developed along the posterior ridge of the dorsal prosoma and the midline of opisthosoma. Table 3 shows the abnormalities of larvae in the fourth nest was highest (GF: 1.48%, EPF: 2.22%) as compared to the fifth (EPF: 2.1%) and sixth (GF: 0.9%, EPF: 1.8%).

Table 2. Record on daily observation on horseshoe eggs and larvae

Nt	n	Stage	NtD (cm)	HcR (%)	MtR (%)	IfR (%)	Abl (%)
1	272	FL	11	33.1	–	24	–
2	233	FL	12	66.1	–	9.6	–
3	157	FL	11.5	31.8	–	28	–
4	135	EE	12.5	47.4	–	37	GF: 1.48%, EPF: 2.22%
5	143	1^{st} InS	11.5	–	90.9	0	EPF: 2.1%
6	111	1^{st} InS	12	–	82.9	0	GF: 0.9%, EPF: 1.8%

* –: no data. Nt: nest, n: quantity, NtD: nest depth, HcR: hatching rate, MtR: moulting rate, IfR: infection rate, Abl: abnormalities, FL: freshly lay eggs, EE: embryonic egg, InS: instar stage, GF: genetical factor, EPF: external physical factor. Units, cm: centimetre(s).

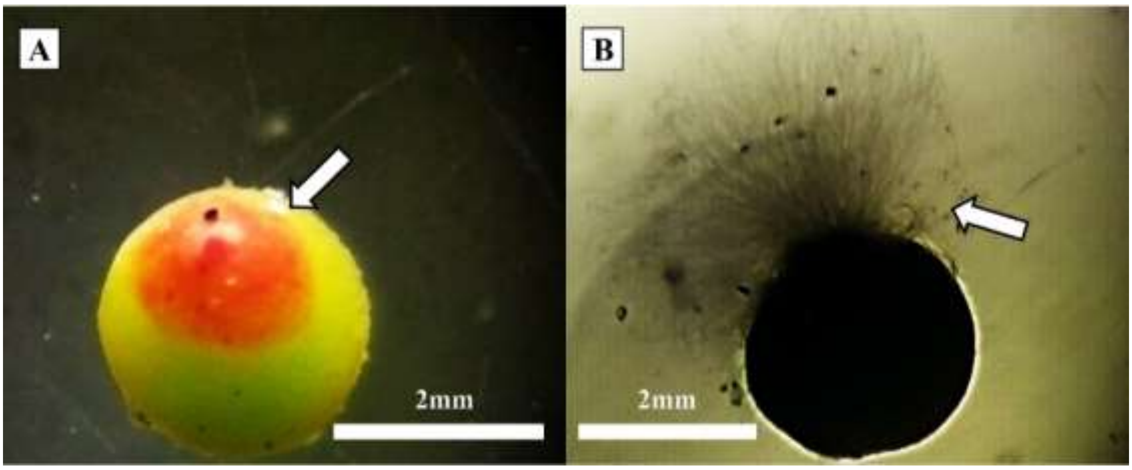

Figure 9. Infection of bacteria (A) and fungus (B) on horseshoe crab eggs during incubation.

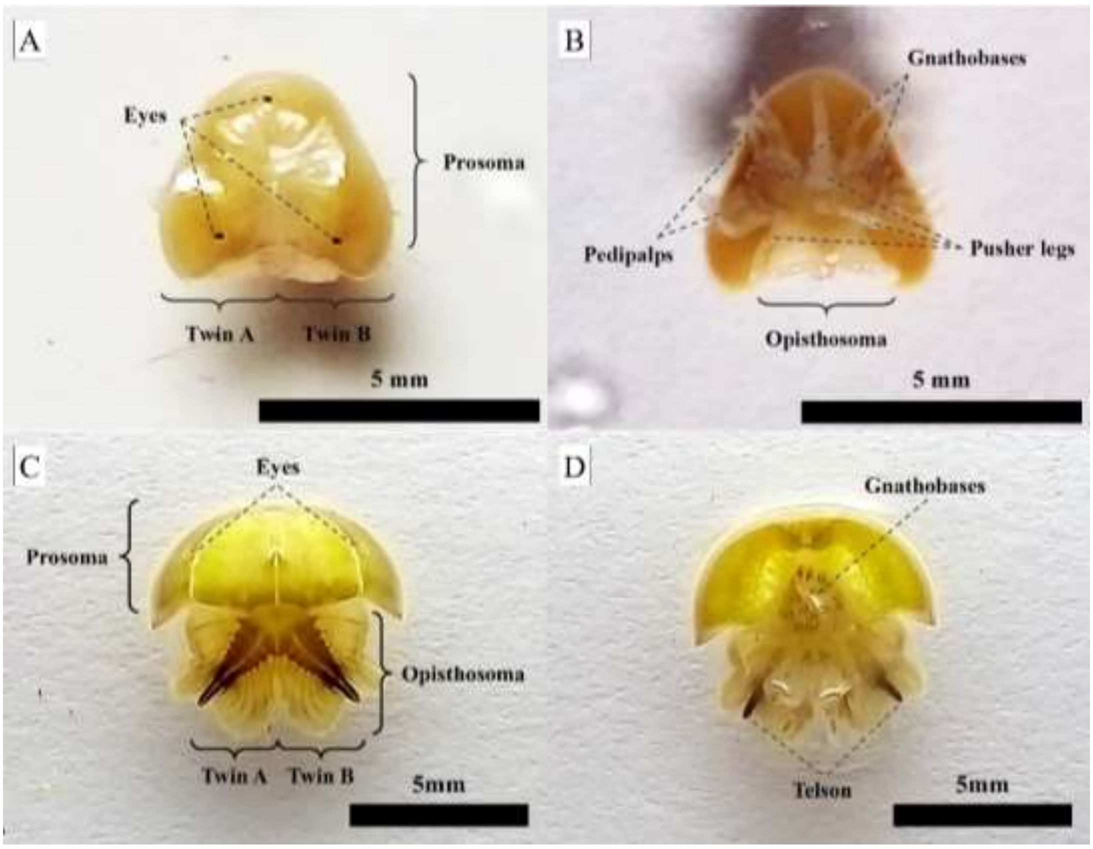

Figure 10. Conjoined twin larvae. A: Dorsal diprosopus twin. B: Ventral diprosopus twin. C: Dorsal dipygus twin. D: Ventral dipygus twin.

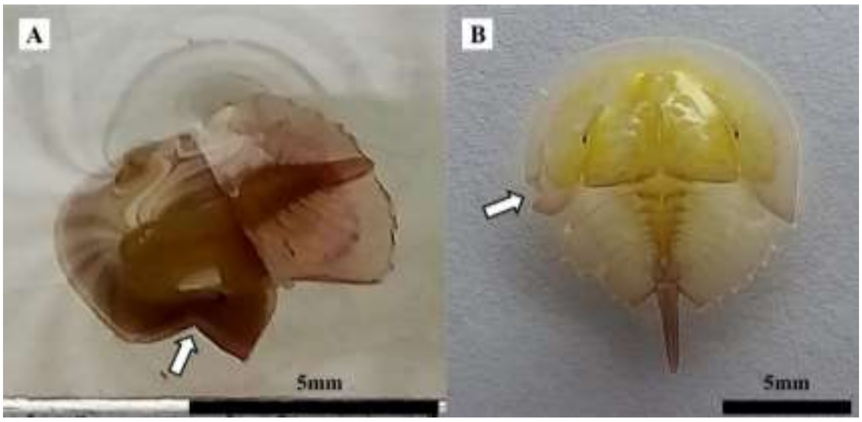

Figure 11. Distortion of larvae exoskeleton that caused by the external factor. A: First instar stage. B: Second instar stage.

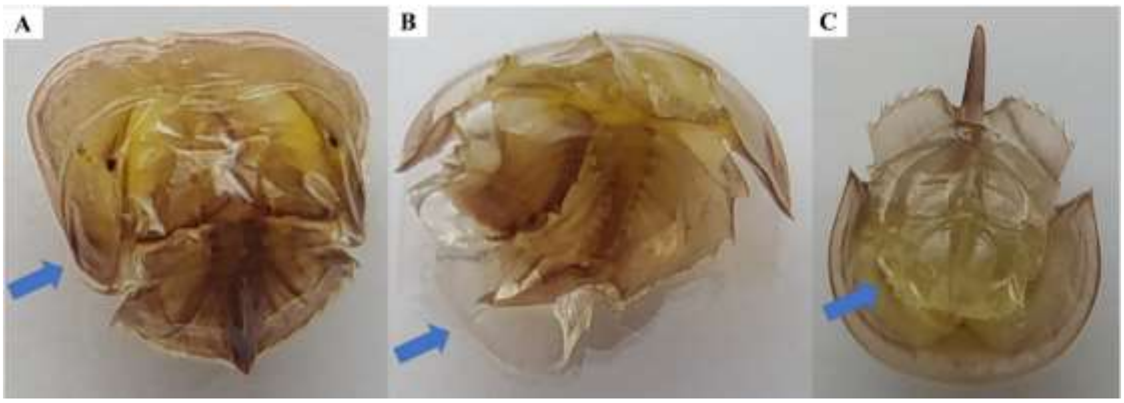

Figure 12. Larvae moulting fails. A: Prosomatic failure. B: Opisthosomatic failure. C: Appendage's failure.

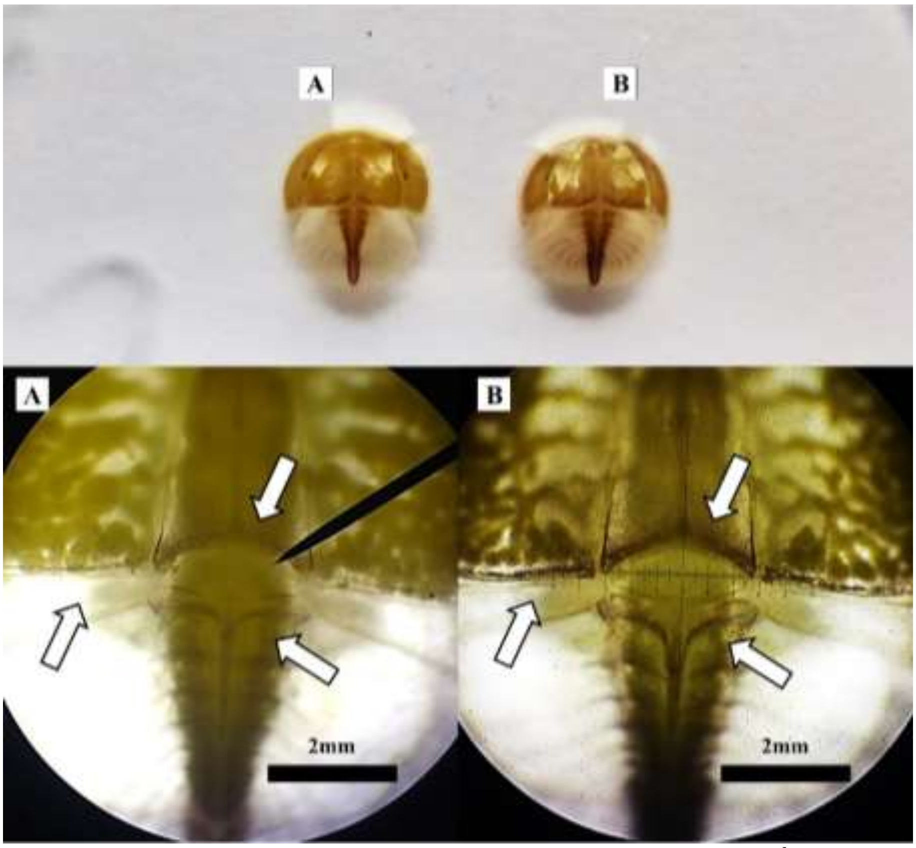

Figure 13. Comparison of larvae exoskeleton pigmentation between day 5th (A) and day 15th (B) in rearing system.

The movement of the larvae also decreased as their life cycles had almost entered the ecdysis phase (Figure 14). The result shows the movement of the lightly pigmented larvae was higher than the darker ones. The larvae movement was significantly decreased every five days in rearing captivity ($p<0.05$). On day 15th, the larvae were not significantly moving and preferred to stay under the sand until day 25th, where the ecdysis process started to occur rapidly ($p>0.05$).

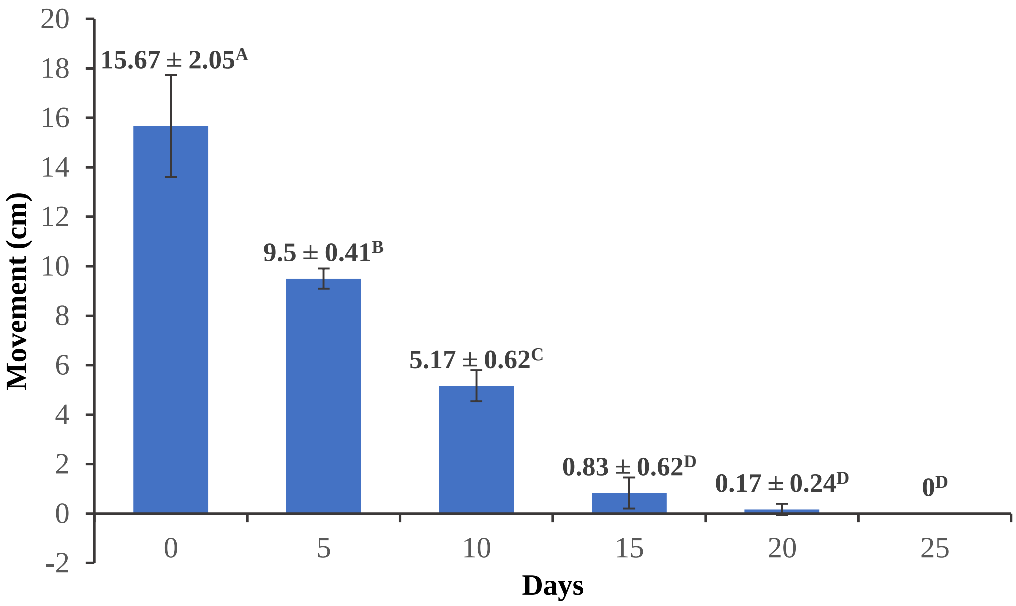

Figure 14. Larvae activeness at every five days during first instar stage. Different capital letters between days indicate the significant differences ($p<0.05$).

4. DISCUSSION

Johor Lama is well-known as a horseshoe crab habitat, nursery, and spawning site by the locals. Unfortunately, it is not a preferred site for horseshoe crab study in Malaysia since the location is isolated and among the remote area with 35 km distance from the town of Kota Tinggi. Two common species of horseshoe crabs, *T. gigas* and *C. rotundicauda*, can be found in Johor Lama and lived sympatrically along the beach area. Based on the regular observation at the beach area, the horseshoe crab population in Johor Lama can be categorized as endangered. Although the number of fisherman port in Johor Lama is lower as compared to the neighbouring settlement, Teluk Sengat, there are dozens of raft houses built at the front of Johor Lama beach that some were used by the locals as a facility to store the harvested horseshoe crabs from the wild before being transported to the land (personal communication). This would eventually lead to overexploitation of horseshoe crab natural resources in Johor Lama. According to John et al., (2018), the harvested horseshoe crabs from Kota Tinggi are gathered at Tanjung Sedili, Johor, before being exported to Thailand for consumption.

Besides that, the horseshoe crab spawning site in Johor Lama is also facing another anthropic activity such as reclamation. Although the location is strategic and protected from direct southwest and northeast monsoon sea current, the existence of local facilities that are located nearby the beach area has led to the construction of the wave breaker along the place. Reclamations in Johor Lama have significantly decreased the width of the beach that subsequently decreases the availability of the exposed sandy beach area for the horseshoe crab to conduct their mating ritual (Mohd Razali & Zaleha, 2017). Reclamation of wave breakers along the beach has altered the condition of the beach as well as the size of the sand grain at the horseshoe crab spawning site. Sand grain size in Johor Lama was bigger and poorly sorted as compared to the ones reported by Nelson et al., (2019) in Balok. The construction of the wave breaker has increased the steepness of the shoreline and further changed the natural wave refraction that would subsequently increase the current turbulence at the beach area.

According to Pilkey et al., (2004), higher wave energy moves the smaller grains offshore to quieter water, leading to the accumulation of coarser sand throughout the reclamation. Interestingly, horseshoe crabs in Johor Lama have adapted to the changes to the size of the sand grain at their spawning site. This result would impact the previously assumed condition of the horseshoe crab spawning site. Despite anthropic activities, the ecology of Johor Lama can still qualify this place to be considered as a good nursery ground for horseshoe crab larvae and juveniles. Mangrove and mudflat in Johor Lama are still preserved and there is no rapid development reported in this area that led to a reduction of shoreline deforestation. According to Kota Tinggi Local Plans 2020 (2017) and State Structure Plan 2030 of Johor (2016), the location of horseshoe crab spawning sites in Johor Lama is within the agricultural zone and possesses a low development suitability index (0.44 to 0.466). Additionally, the district also has been drafted in 2006 as a heritage tourism area. However, the future of this spawning site is still doubted since there are many ports and industrial zones which are currently located at the opposite side of the river in Johor Bahru district, nearby the opening of Johor River, (17 km from Johor Lama) that could lead to severe water pollution.

Through this study, it was recorded that male horseshoe crabs were the higher contributor to the quantity of stranded horseshoe crab carcasses in Johor Lama beach than that of the females. Based on the observation, most of the male *T. gigas* and both sexes of *C. rotundicauda* have unspecifically broken on their body parts that might be caused by external forces. According to Kassim et al., (2008), John et al., (2010) and Manca et al., (2016), only female horseshoe crabs have the commercial value and are being marketed in Malaysia. Male *T. gigas* and both sexes of *C. rotundicauda* are bycatch horseshoe crabs and are often released back to the sea. Most of the released horseshoe crabs are injured due to the action of fishermen cutting off some parts of their bodies to disentangle them without destroying the net. Female *T. gigas* carcasses on Johor Lama beach have no ventral prosomatic and opisthosomatic appendages. This condition is caused by the action of predators in the wild. The predators would be ripping off the thinner exoskeleton layer which is located at the ventral region since it is the most convenient way to assess the eggs and muscle that is inside.

This study also shows an alarming condition regarding the horseshoe crab population in Johor Lama. The hatching rate for the horseshoe crab eggs from Johor Lama with optimum water parameters was lower than the previously reported. In addition to this, the infection on the eggs during the incubation period were higher as compared to the ones studied previously. This condition might happen due to the difficulties during fertilisation caused by the lack of sperm during mating due to low male ratio (Pennington, 1985; Levitan, 1993). Fungus and bacterial infections started to occur on the fourth day and would initially infect the unfertilised eggs under laboratory conditions. Fertilisation rate is closely related to the ratio of the male to female during the mating ritual. The presence of more than one male (attached and satellite males) might increase the fertilisation rate of the eggs. According to Smith et al., (2013), the spawning ritual for the male is highly competitive. Nevertheless, the paternity of attached and satellite males are not significantly different (Brockmann et al., 2000). This finding shows that the presence of both attached and satellite males during the spawning ritual is crucial and at the same time complementary.

Nonetheless, the infections from the bacteria and fungus remain the common issue and concern that happened to horseshoe crab eggs during the incubation period that could cease the eggs from developing. According to Faizul et al., (2015), four species of bacteria (*Shewanella putrefaciens*, *Enteroccus faecalis*, *Bacillus cereus*, and *Corynebacterium* sp.), and fungi (*Aspergillus* sp., *Aspergillus niger*, *Peniclillium* sp. and *Gliocladium* sp.) could be found on the infected eggs and larvae. Interestingly, the implementation of Chen et al., (2010) method (constant water flow) in the larvae rearing system has succeeded in inhibiting bacteria and fungus infection on larvae.

Larvae abnormality is another concern that can always occur during embryonic development and post-hatch. There are two factors that could lead to abnormality of larvae: (i) genetic impairment and (ii) external physical factors. The genetic abnormality in aquatic organisms could be caused by biological inheritance and triggered by an external factor such as chemical pollution that could lead to DNA mutation (Belfiore & Anderson, 2001; Amoatey & Baawain, 2019; Sultana et al., 2019; Hong et al., 2020). According to Itow et al., (1998) who studied the effect of heavy metals on the horseshoe crab embryonic development, Zn exposure could lead to double embryo (conjoined twin) abnormality on horseshoe crab larvae. The genetic disorder would disadvantage the larvae to live and survive during the post-hatch phase. Apparently, larvae would die before reaching the second instar stage, that is when the limited vitelline content inside the carapace depleted. Impaired morphology will also hinder the larvae from performing the normal ecdysis process that leads to the unsuccessful moulting condition. According to Phlippen et al., (2000), death is inevitable if ecdysis could not be completed successfully. Abnormalities during embryonic development could be one of the indicators of heavy metal pollution in Johor Lama. Higher abnormality frequency (>0.6%) indicates that water might be polluted. Additionally, Liang et al., (2020) reported that the heavy metal concentrations in Johor River were found to be exceeding Class II, which is more than the allowable limit.

Nevertheless, another factor of morphological abnormality that could be caused by the external physical force include the impact of the substrate on the larvae in the nest or failure to remove the chorion and exuviae completely from the body after hatching and during ecdysis, respectively before they enter the intermoult phase. Horseshoe crab larvae exoskeleton are soft and malleable during the initial period of post-hatch and post-ecdysis. Haemolymph content will be increased to unfold the exoskeleton and maintain the shape before the endocuticle form to harden the exoskeleton during the intermoult phase (Chung et al., 1999). The external physical force would prevent the exoskeleton from expanding well and subsequently distort the shape of the carapace during the intermoult phase. However, some abnormalities which could be caused by the external physical force do not show prominent differences than the normal larvae as compared to the genetic abnormalities. The non-severe distortion does not interfere with the activity that is commonly conducted by the larvae during the post-hatch phase. Besides that, some of the abnormalities will be gone as the larvae are moulted. Nonetheless, severe distortion will lead to mortality if it hinders the moulting process or occurs at the vital organs such as gills, gnathobases, etc.

Horseshoe crabs are known as successful species throughout history. All four species have reached the peak of evolution that led to the common ecdysis behaviour between all populations. Increased pigmentation as the larvae is nearing ecdysis is known as the same common behaviours of horseshoe crab (Yamamichi et al., 1983; Skinner, 1985; Ewer & Truman, 1996) that takes place during pre-moult phases due to the increase of folded cuticle composition within the old cuticle. Arthropods would forage intensively during the intermoult phase before the next moulting phase; pre-moult, to store their energy. They commonly will stop feeding and decrease their movement at some point in time during pre-moult. This stage begins with an increase in the concentration of moulting hormone in the hemolymph (Chang, 1992). The old cuticle would be withdrawn from the epidermis in an apolysis process. Hypertrophy of cells would take place to form a new epicuticle underlying the endocuticle layer. The material for cuticle synthesis is derived from two sources; accumulated reserves due to feeding and resorption from the old cuticle (Dall et al., 1990).

5. CONCLUSION

Johor Lama is among the isolated and the least studied horseshoe crab spawning sites. Reclamation in this area is severe, but there are still a few areas that could be considered as suitable for the horseshoe crab to conduct their spawning ritual. As the mangrove in this area is preserved well, it is concluded that the nursery for the horseshoe crab larvae and juveniles remains undisturbed. Besides, the location is also far from the urbanisation that subsequently means the least deforestation area. However, the water quality in this area is still in doubt and further study needs to be carried out. This study also revealed the adaptation of horseshoe crabs to the different sedimentation properties as their spawning site, since the quantity of the eggs in each nest is almost the same as the other populations. Genetic impairment and external force factors that lead to larva morphological abnormality and deformation are normal in all populations. Nevertheless, the reason that could cause all these complications should be first studied and understood. Normal larvae might have common growth behaviour (movement becomes slower and pigmentation turns darker) the same as other populations in the west and east coast of Peninsular Malaysia. A strict regulation should be enforced in Johor Lama to preserve the spawning site of this precious species.

6. ACKNOWLEDGEMENT

This study was funded by The Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme (FRGS19–162–0771)

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