Journal of Sustainability Science and Management Special Issue Number 1:
The International Seminar on the Straits of Malacca and the South China Sea 2016: 1-10
ISSN: 1823-8556

DOES HUMAN INFRINGEMENT AT THE SPAWNING GROUNDS CHALLENGE HORSESHOE CRAB EGGS AND THEIR EMBRYOGENESIS?

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Abstract: Horseshoe crabs come ashore to search for surf-protected areas with loosely packed sediments to spawn. This enables the buried horseshoe crab eggs to have sufficient oxygenation and moisture to complete their embryogenesis. Under these circumstances, Balok, on east Peninsular Malaysia (South, South China Sea) was frequently visited by *Tachypleus gigas* (Müller, 1785) for their spawning. It became doubtful whether horseshoe crab embryogenesis could complete under stressful habitat conditions onset human infringement through physical infrastructure placement on the beach. In addition, the absence of biological evaluating tools triggered the idea to evaluate the health of horseshoe crab spawning grounds using parasitic worms as sensitive indicators. In this study, field visits were made between 2009 and 2014 to trace horseshoe crab nests. Upon successful excavation, the eggs were collected carefully from the nests using plastic hand shovel. Meanwhile, nematodes (*Ascaris* spp. and *Dolicholaimus* spp.) and polychaetes (*Glycera* spp. and *Lumbrineris* spp.) as well as poor conditioned eggs including those in black (unfertilized/desiccated) or red (bacteria infected eggs), were also recorded from the horseshoe crab nests. The assumed healthy green-yellow green horseshoe crab eggs were brought back to the laboratory and acclimatized for 2 days under 28 ‰ salinity and 28 ºC prior the laboratory culture. The number of days in reaching third embryonic moult (Stage-20), hatching of first-instar trilobite larvae and moulting into second-instar was recorded for 40 days. While the field visits of 2011 documented 5-6 worms (nematode and polychaete) per horseshoe crab nest, during 2013, it increased to 6-11 worms. It was clear that physical alterations that took place at the beach during 2011 such as construction of erosion barriers, parking lot and then, the fish jetty during 2013 were responsible for the increased worm intrusion into horseshoe crab nests. The higher abundance of nematodes and polychaetes were significantly correlated ($P < 0.05$) with the increasing number of black and red eggs after the year 2011, when human infringement became active at Balok. Together, the duration for horseshoe crab embryogenesis to reaching Stage-20 and the duration to hatching into the trilobite larvae were both, delayed up to 5 days and, the egg hatching viability was reduced up to 63% after nematodes and polychaetes trespassed the horseshoe crab nests. With this, poor environment health affects the quality of buried horseshoe crab eggs. Hence, conservation and management efforts are necessary to prevent loss of *T. gigas* population and their spawning area at Balok, the most documented horseshoe crab spawning ground on east Peninsular Malaysia.

Keywords: Horseshoe crab, biological indicators, embryogenesis, aquatic worms, unregulated management, South China Sea.

Introduction

Horseshoe crabs are ‘living fossils’ that inherit appearance of trilobite arthropods from the Ordovician period (Rudkin *et al.*, 2008). Commonly found at high waters, these crabs have geographical limitations along the eastern coastlines of North America and South Asia (Sekiguchi & Shuster, 2009). Due to oviparous reproduction and their shelter-seeking behaviour from vigorous water currents,

horseshoe crabs arrive to shallow water with negligible tides during the mid and end lunar cycle for spawning (Sekiguchi & Shuster, 2009). After selecting a suitable area with loosely packed and moist sediment, horseshoe crabs release their eggs in self-made burrows at the mid-tide region of the beach (Nordstrom *et al.*, 2006). Encountering horseshoe crabs during their reproduction has made them vulnerable to poaching. In fact, adult horseshoe crabs are used as food, feed, fertilizers and biomedical products (Nelson, 2012; Nelson, 2015; Nelson *et al.*, 2015). Apart from threats to adult horseshoe crabs, their buried eggs on the beach are also vulnerable to desiccation from extreme heat exposures and, squashing and poisoning in the presence of fishing/boating activities (Burton *et al.*, 2009). Under such conditions, the surviving eggs are still able to hatch out in most of the time, but, the first instar larvae are observed to have deformed appearance and their life-span is yet unknown (Nelson, 2012).

Human-induced disturbances including beach re-nourishment, shoreline armouring with rip-rap, bulkheading and/or boat docking have resulted to degradation of horseshoe crab natal beaches in India (Chatterji & Shaharom, 2009), North America (Botton *et al.*, 2010) and Malaysia (Nelson *et al.*, 2015). After the construction of roads and bridges at Tanjung Selangor that had been destroying the horseshoe crab spawning beach in 2011, Balok is now the only existing and available horseshoe crab spawning site on the east coast of Peninsular Malaysia (Nelson, 2015; Nelson *et al.*, 2015). While horseshoe crab eggs at Balok are subjected to natural environmental stressors such as wet, dry and hot climate intervals during 2009-2010, the condition of their eggs after coastal development since 2011 including wave breakers, parking lot and jetty construction remains questionable. The present study was aimed to observe the hatching success of *T. gigas* eggs within the span of 2009 and 2014 with or without the presence of nematode and polychaete worms. These worms were then used as '*in-situ* indicators' for man-induced disturbances at horseshoe crab spawning grounds.

Methodology

Sampling Site Description

Balok, Pahang (South China Sea) (N 03°56.009' E 103°22.584') is an estuary situated at the mouth of River Balok, located on the east coast of Peninsular Malaysia (Figure 1A-B). While experiencing broad seasonal variations during the southwest (May-September) and northeast monsoons (November-March), this site experiences hot and humid climate with varying air temperature of 24-36 °C, average annual rainfall of 1567.5 mm and mixed tides that range from 0.1-3.5 m (NHC, 2014; WU, 2015). Unlike other coastal areas in Malaysia, a wet market and fish landing jetty was built in Balok to support the local fisheries. Apart from the construction of man-made structure, boating and other recreational activities were also witnessed on shore.

Collection of Eggs, Nematodes and/or Polychaetes

While the observation period covered the horseshoe crab seasonal spawning during July-December 2009, January-December 2010, January-June 2011, May-December 2012, January-May 2013 as well as April-June and September-October 2014, only two months per year with total egg count exceeded 500 eggs were selected for the present study. During that time, horseshoe crab nests were identified from their carapace imprints on the sand (Figure 1 C), nest unearthing using a hand shovel (Figure 1D) and, it followed with transferring of egg clutch from the nest into a 2 mm sieve to remove the sand. The clutches were then gently separated into egg individuals in order to search for any nematodes and/or polychaetes present surrounding the eggs or horseshoe crab nests. All worms found in each nest were placed into a container containing 10 ml, 10% v/v ethanol (each nest 1 container). The number of eggs in each horseshoe crab nest was counted and only

a total of 170 green-yellow green eggs were collected and stored in a 2-litre plastic holding tank equipped with portable aerator for future laboratory assessments if the total eggs per nest exceeded 500 eggs. The remaining eggs were returned into the nest and covered by the sand. There is no intension to include the eggs from another sympatric Asian horseshoe crab *Carcinoscorpius rotundicauda* at Balok in this study as their nests were located at upstream locations and may not be affected by the human activities.

Examination of Worms Collected from Balok, Pahang (South China Sea)

In the laboratory, nematode and polychaete samples were identified by colour, shape and size using compound microscope with scale. This was followed by 5-minute exposure to 3.3% saline for adjustments on the glass slide. Each worm was mounted on the slide using Canada balsam (1-2 drops per worm). Sketches of each worm were made under the compound microscope attached with 'Camera Lucida' (Olympus, Japan) – for taxonomic identification via available online database (http://species-identification.org; http://marinespecies.org).

Laboratory Egg Culture

Every batch of eggs was acclimatized to the culture conditions at water temperature of 28 ºC and salinity of 28 ‰ for 4 days. Only 150 eggs were used for the laboratory culture experiment, while the remaining eggs were maintained in the holding tank. These eggs were divided into 15 groups, placed on plastic sieves that were attached to the 2 litre plastic tanks and, supplied with sufficient aeration (Figure 1E). The experiment was terminated when all eggs had hatched out into trilobite larva. A total of 10 embryos/larvae were randomly selected and data of their weight, size, colour-change (based on egg moults) and development duration during embryogenesis were recorded during the days 0, 7, 14, 21, 28, 37 and 40.

Data Analysis

The duration to hatch and hatching viability for each egg batch was compiled and it was compared to the presence of worms (nematode and polychaete) and, occurrence of different colour eggs (other than green-yellow green). Comparisons were carried out using Statistical Package for Social Science (SPSS) v.17 for One-way ANOVA ('*P*') and with Microsoft Excel 2013 for correlation analysis ('*X*').

Results

Field-related Observations Covering the Crab's Spawning Activity

Throughout the spanning field work, the horseshoe crab eggs were found during the southwest (May, July and August) and inter-monsoon (April and October). However, the nematodes and polychaetes *Ascaris* spp. (Figure 2A), *Dolicholaimus* spp. (Figure 2B) and *Glycera* spp. (Figure 2C) were mostly encountered in the horseshoe crab nests during inter-monsoon, followed by southwest monsoon, except for *Lumbrineris* spp. (Figure 2D) – only during the northeast monsoon, from November-March. The worms, *Ascaris* spp. and *Lumbrineris* spp. appeared in white-pink to blood red whereas, *Dolicholaimus* spp. and *Glycera* spp. were either in semi-translucent or pink-red. The prevalence of worms and poor condition eggs (black – Figure 3A and; red – Figure 3B) were evident during inter-monsoon (26 individuals, 58 eggs), but less abundant during southwest monsoon (9 individuals, 11 eggs) (Table 1). The abundance of nematodes and polychaetes as well as black and red eggs were seconded during northeast monsoon, which is the least preferred monsoon period by the spawning horseshoe crabs. Clear-cut relationships were shown by the seasonal prevalence of worms ($F = 2.86$, $P = 0.002$; $X = 0.025$) and likewise, for the prevalence of worms in the horseshoe crab nests and the occurrence of poor condition eggs ($F = 3.65$, $P = 0.03$; $X = 0.138$). While nematodes and/or polychaetes were not present during the surveys in 2009, high occurrence of *Ascaris*

spp. in the horseshoe crab nests were noted since 2010. In 2011, up to four types of nematodes and polychaetes including *Ascaris* spp. *Dolicholaimus* spp., *Glycera* spp. and *Lumbrineris* spp. were collected from the horseshoe crab egg clutches. Although *Glycera* spp. was only discovered in the crab's nests in 2011, they had the highest prevalence throughout the investigation period, gathering a total of 15 individuals for all surveys whereas, the least was *Lumbrineris* spp. at 2 individuals. Despite the existence of these worms in *T. gigas* nests since 2010, black and red eggs were only discovered in their nests during 2011. These abnormal egg appearances became intense during late 2012 and continued to worsen until the last field observation in October 2014 (Table 1).

Experiment to Assess Tachypleus gigas Egg-Hatching Viability

After 37 (during 2009 and 2010) and 40 days (from 2011 onwards), all eggs hatched into the trilobite larvae. The larvae were transferred into a holding tank and, their size and weight recorded until day 40 (Table 2). While under normal conditions, increase in size (up to 6 mm) and weight (up to 200 mg) associate to active horseshoe crab embryo development. However, when embryos do not increase in size (remain at 6 mm) and experience sudden decrease in their weight (up to 60 mg) after 37 (observed in year 2009 and 2010) and 40 days (observed onwards year 2011), the embryos have already hatched into the trilobite larvae. During the surveys in 2009 and 2010, *T. gigas* hatching viability was 100%. However from 2011 onwards, it decreased to 63-84 % because of unidentified fungal infection (Figure 3C). Correlation analysis showed that the presence of nematodes and polychaetes decreased the horseshoe crab egg hatching viability in 2011 ($F = 11.21$, $P = 0.002$; $X = 0.074$), and delayed embryogenesis ($F = 4.61$, $P = 0.001$; $X = 0.218$). The abundance of these worms found in horseshoe crab nests was also associated with the number of poor condition eggs including black, red and fungal infected eggs noted in their nests ($F = 5.73$, $P = 0.04$; $X = 0.225$). Although the duration to reach the embryo development stage of 20 varied 1-2 days in 2011, the number of days increased – up to 5 days was observed in 2014. Similarly, the egg hatching duration varied ± 1 day in 2011, but increased to 2-3 days since 2012 (Table 2).

Discussion

Changing Environmental Conditions at Balok

When the field work initiated in 2009, Balok beach was already occupied by a wet market. Several small-scale fish landing jetty were placed along River Balok to support the local fisheries. However, erosion of the river banks and estuarine beach in 2011 called for the construction of erosion barriers and wave breakers. This was followed by parking lot construction in 2011 and concrete fishing jetty in 2013. During the surveys in 2014, Balok beach was elevated by 10 % (previously ±10 % mean sea level (MSL); Nelson *et al.*, 2010), making the beach very steep and the distance from the highest water mark during low tide became <10 m (previously >20 m, Nelson *et al.* unpublished). Due to reduced oxygenation, moisture entrapment and heat exposure during daytime low tides, the buried eggs were succumbed to desiccation and infection and thus, raising the prevalence of black eggs in the horseshoe crab nests (Nordstrom *et al.*, 2006; Burton *et al.*, 2009).

Comparison Between Field and Laboratory Findings

The previous studies on Asian horseshoe crabs and their relationship to other associated organisms on shore are limited to feeding, which suggested that nematodes and polychaetes are important diets for horseshoe crabs (Hsieh & Chen, 2009; Kwan *et al.*, 2015). Meanwhile, the prevalence of these worms in fin-fish (Marcogliese & Pietrock, 2011) and vegetables (Uhuo, 2015) were also used to assess the health condition of habitat environments. In the case of Balok, the deteriorating beach conditions were beneficial to nematode and polychaete worms,

in which the decaying horseshoe crab eggs can provide food sources for them. While *Ascaris* spp. and *Dolicholaimus* spp. are parasitic nematodes and *Glycera* spp. and *Lumbrineris* spp. are carnivorous polychaetes (Gaston *et al.*, 2015), their occurrence in horseshoe crab nests at Balok was most likely to scavenge on poor condition and black eggs. Perhaps, after the scavenging, leftover egg pieces were infected by bacteria during the decaying process. Ultimately, infection spread throughout the horseshoe crab nests and contributed towards the prevalence of red eggs. Thus, infection and changing beach conditions from natural and/or human infringement at Balok stressed other horseshoe crab eggs, deteriorated their quality and negatively affected their hatching viability in the laboratory.

The shift of Balok beach sediments from medium to fine sand was thought to improve the female crab's spawning output (Nelson, 2015). However, this gave rise to seasonal nematode and polychaete accumulation in the crab's nest. For instance, *Lumbrineris* spp. only occurred during the wet northeast monsoon because of the nutrient-rich waters (Gaston *et al.*, 2015).

Horseshoe crab eggs cultured at the laboratory were vulnerable to secondary infections, such as fungal infection, which may be due to captivity stress (Nelson, 2012; Faizul *et al.*, 2015). However, it is noted that all the embryos can be successfully hatched out given that the horseshoe crab eggs had reached the development stage of 20 (Nelson, 2012). While early horseshoe crab embryogenesis stages are sensitive to their environment, proper management at Balok will not only make this horseshoe crab spawning beach favourable for the crab's embryogenesis and hatching but also, reduce the opportunistic trespassing of nematodes and polychaetes in horseshoe crab nests.

Management Strategies at Balok

Because all three Asian horseshoe crabs are currently listed as 'data deficient' in the IUCN Red List (IUCN, 2015), horseshoe crabs and their spawning sites in Malaysia are therefore not protected by Malaysian Wildlife Conservation Act 2010 and Malaysian Fisheries Act 1985. Wild releasing laboratory cultured horseshoe crab larvae into their habitats to increase *in-situ* populations are not that effective and feasible because of the long embryogenesis (37-40 days) and larval-juvenile development periods (9-11 years to attain maturity; Shuster & Sekiguchi, 2003; Nelson, 2012; Nelson, 2015; Hu *et al.*, 2015).

In this study, nematodes and polychaetes are demonstrated to be sensitive *in-situ* biological indicators to assess the health condition of horseshoe crab spawning grounds. Collaboration between researchers and fisherman at Balok may be feasible to identify 'boat-docking areas' on the beach away from horseshoe crab spawning areas, to place sign-boards of prohibiting catch and selling of horseshoe crabs, and to use the infrastructure of Balok fish market as a gathering venue to deliver conservation messages of horseshoe crabs to the locals. In addition, through community-based conservation programmes, such as touch and feel, larval release, catch-tag-release of mating amplexus, identification of spawning sites and translocation to other beaches, conservation awareness towards horseshoe crabs can be raised. Restoration of spawning grounds, similar to that described in Hsieh and Chen (2009), may also help to provide more favourable condition of habitat for the spawning of horseshoe crabs at Balok.

Conclusion and Recommendations

The importance of horseshoe crabs as living fossils must be preserved as natural heritage for future generations. While *in-situ* biological indicators such as nematodes and polychaete were developed in this study to indicate environmental stress towards buried horseshoe crab eggs, the evaluation of health condition of horseshoe crab spawning grounds especially those near to the human infringement becomes possible. It is also important that future studies can be done to identify: these worms in the

horseshoe crab nests, the bacteria that caused red eggs and, the type of fungus that fouled the eggs. Collaborative helps from fishermen, local communities and government officers may enable Balok to become the first protected horseshoe crab spawning area in Malaysia, under guidance by the local laws.

Acknowledgements

The present study was administratively supported by Institute of Tropical Aquaculture (AKUATROP), Institute of Oceanography and Environment (INOS) and the School of Marine and Environmental Sciences (PPSMS) at the Universiti Malaysia Terengganu. Authors wish to thank the Ministry of Higher Education, Malaysia for providing a scholarship (MyBrain-15) to B.R.N.

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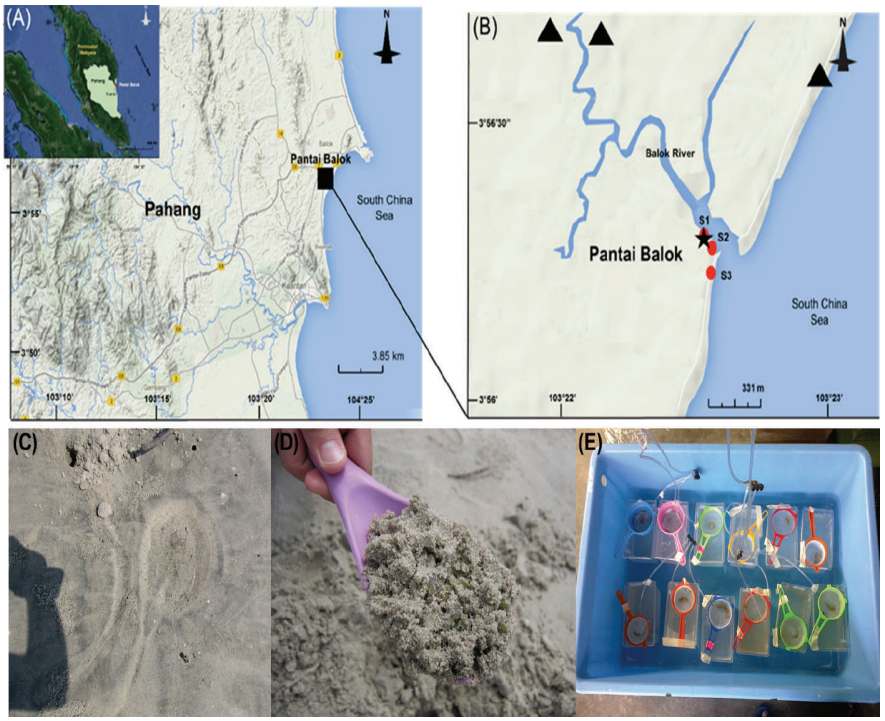

Figure 1: Location of *Tachypleus gigas* spawning site, the crab's nest appearance and laboratory setup for the crab's embryogenesis. Symbols: '▲' = Sand mining, '★' = man-made structure construction and S1-3 = Sites 1-3. (A) = The location Pahang State and (B) = the horseshoe crab spawning site Balok along the east coast of Peninsular Malaysia. (C) = Horseshoe crab carapace imprint on the sand. (D) = Horseshoe crab eggs after exhumation of nests. (E) = The arrangement of 2-litre tanks and sieve setup for *Tachypleus gigas* egg incubation experiment

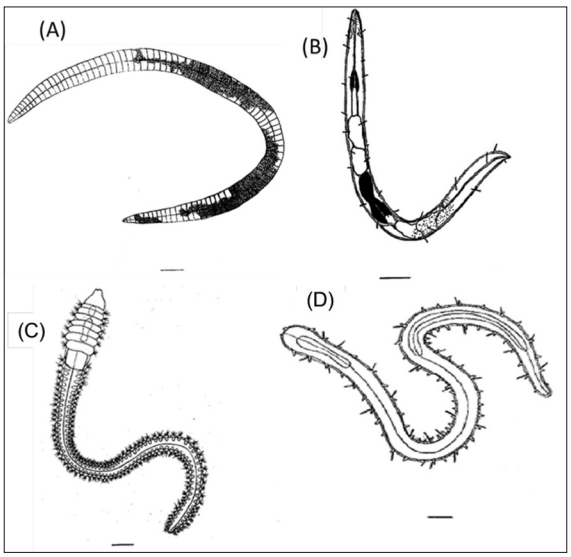

Figure 2: Type of worms present in *Tachypleus gigas* nests: nematodes (A) = *Ascaris* spp. and (B) = *Dolicholaimus* spp. as well as polychaete (C) = *Glycera* spp. and (D) = *Lumbrineris* spp.

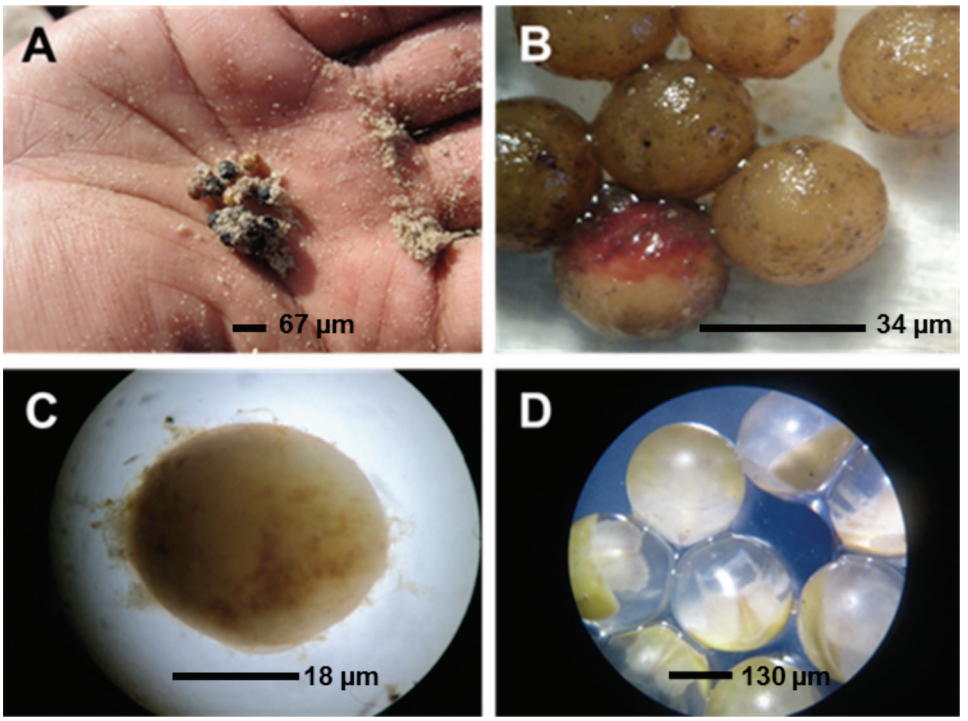

Figure 3: The poor-conditioned eggs discovered at Balok and at the laboratory as well as appearance of bloated eggs after removal of chorion layer (embryonic shell). The appearance of (A) = black eggs, (B) red eggs, (C) = fungal infection on horseshoe crab eggs during the egg incubation assay and (D) = *Tachypleus gigas* embryogenesis at development stage of 20

Table 1: Nematode, polychaete and poor conditioned egg abundances per nest at Balok in 2009-2014

	Period	Nematode		Polychaete		Egg condition	
		Ascaris spp.	*Dolicholaimus* spp.	*Lumbrineris* spp.	*Glycera* spp.	Black	Red-stained
SW	August 2009	0	0	0	0	0	0
IM	October 2009	0	0	0	0	0	0
NE	March 2010	1	0	0	0	0	0
SW	July 2010	0	0	0	0	0	0
NE	March 2011	0	2	1	0	0	0
IM	April 2011	1	3	0	2	3	12
SW	May 2012	2	1	0	0	1	2
SW	August 2012	2	2	0	2	2	6
NE	March 2013	3	2	1	0	4	9
IM	April 2014	0	4	0	5	8	15
IM	October 2014	5	0	0	6	7	13

Sample collection period: SW = southwest monsoon, IM = inter-monsoon, NE = northeast monsoon

Table 2: Size and weight changes during *Tachypleus gigas* embryogenesis, the duration for embryos to reach the development stage of 20 and hatching viability of eggs during the egg incubation experiment in 2009-2014

Batch / Period	Weight (g)							Size (mm)							S-20 (Days)	Hatching viability (%)	Fungus infected eggs (%)	Hatching duration (Days)
	Day 0	Day 7	Day 14	Day 21	Day 28	Day 37	Day 40	Day 0	Day 7	Day 14	Day 21	Day 28	Day 37	Day 40				
August 2009	30±2	40±2	160±18	270±12	310±11	60±1^{A}	80±3^{B}	3.4± 0.2	3.8±0.2	4.5±0.5	5.3±0.3	7.4±0.3	6.4±0.1^{A}	7.5±0.3^{B}	15	100	0	37
October 2009	30±2	40±4	170±6	230±10	270±10	60±5^{A}	80±2^{B}	3.6±0.2	3.7±0.4	4.8±0.2	4.6±0.2	6.4±0.2	6.5±0.5^{A}	7.6±0.2^{B}	16	100	0	37
March 2010	30±2	40±5	130±42	230±28	260±28	60±5^{A}	80±2^{B}	3.6±0.2	3.6±0.4	3.7±1.2	4.5±0.6	6.3±0.7	6.4±0.5^{A}	7.6±0.2^{B}	17	100	0	36
July 2010	30±1	40±5	70±57	230±17	270±17	60±1^{A}	80±2^{B}	3.5±0.1	3.5±0.4	2.1±1.7	4.6±0.3	6.4±0.4	6.3±0.1^{A}	7.6±0.2^{B}	16	100	0	37
March 2011	30±1	30±2	130±43	220±19	250±19	70±4	70±3	3.6±0.1	3.2±0.2	3.8±1.2	4.4±0.4	6.1±0.5	8.6±2.4	7.1±0.3^{B}	17	84	16	36
April 2011	30±2	40±4	130±52	230±39	270±39	130±94	70±8	3.6±0.2	3.5±0.4	3.6±1.5	4.6±0.8	6.4±0.9	10.4±5.4	6.9±0.5	17	79	21	37
May 2012	30±1	40±5	140±42	230±22	270±22	90±31	80±8	3.6±0.1	3.7±0.4	4.0±1.2	4.7±0.4	6.5±0.5	8.7±3.1	7.2±0.8^{B}	18	91	9	38
August 2012	30±1	40±4	130±37	220±28	260±28	100±48	70±9	3.5±0.2	3.5±0.3	3.7±1.1	4.4±0.6	6.1±0.7	8.7±3.8	6.5±0.2	19	87	13	39
March 2013	30±1	40±4	100±39	230±15	260±15	70±29	70±10	3.6±0.2	3.3±0.4	3.0±1.1	4.5±0.3	6.2±0.4	9.1±4.9	6.3±0.2	18	78	22	38
April 2014	30±1	40±4	80±46	230±28	260±28	80±53	70±9	3.7±0.1	3.7±0.4	2.3±1.1	4.5±0.6	6.3±0.7	11.2±5.3	6.5±0.2	20	63	37	40
October 2014	30±1	40±3	150±22	230±12	270±12	130±82	70±9	3.6±0.1	3.4±0.3	4.3±1.6	4.7±0.3	6.5±0.3	10.7±5.3	6.4±0.2	20	66	34	39

A = Weight (mg) and size (mm) of *Tachypleus gigas* first instar (trilobite larvae); B = Weight (mg) and size (mm) of *Tachypleus gigas* second instar (larvae with telson). S-20 = Stage 20 of embryogenesis (opaque chorion splits, egg expands – filled with perivitelline fluid and, embryo is visible).