Studies on the Ontogenic Development of a High-Valued Tropical Sea Urchin, *Tripneustes gratilla* (Linnaeus, 1758) For Seed Production and Commercial Aquaculture

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***Abstract* —** A detailed ontogenic development of the tropical sea urchin, *Tripneustes gratilla* was studied in a captive laboratory condition. Matured gametes were collected by injecting 0.5M KCl into the coelomic cavity of the adult sea urchin. Insemination was done using 10-5 'dry' sperm dilution and the mean fertilization success was estimated to be 96.6±1.4%. The resulted embryos were incubated in glass beakers containing sterilized filtered seawater (SFSW) at 26-28ºC. The 2-cell (first cleavage), 4-cell, 8-cell, 16-cell, 32-cell, and multi-cell, (morula) stages were reached at 01.22, 02.18, 2.48, 3.48 and 04.01 h post fertilization respectively. Ciliated blastulae and gastrulae were formed after 09.26 and 16.41 h of fertilization. Pluteus larvae started feeding unicellular algae (Chaetoceros calcitrans) within 2 d, grew continuously and attained metamorphic competence in 35 d after fertilization. Metamorphosis took place nearly 1.5 h from the attachment to complete resorption of larval tissues and the formation of a complete juvenile structure with adult spines and prolonged tube feet, the whole event of which usually took place within 1 d post settlement. This study represents the first successful documentation of the complete embryonic, larval and early juvenile development of *T. gratilla* in tropical Malaysia, the findings of which would greatly be helpful to understand the ontogeny and life-history strategies that will facilitate the development of breeding, seed production and culture techniques of this high-valued sea urchin for commercial aquaculture.

Keywords: Sea urchin, *Tripneustes gratilla*, embryo, larva, juvenile, developmentas.

I. Introduction

THE tropical sea urchin, *Tripneustes gratilla* (Linnaeus, 1758) (Echinodermata: Echinoidea: Toxopneustidae), belongs to the regular echinoids, is an important bioresource for research in different fields of biology, biodiversity, culture, conservation and evolution. It is one of the most common echinoids, occurring in very shallow water on a variety of hard substrates and is found at depths from 2 to 30 m [1]. *Tripneustes gratilla* grazes near the substrate, and their diet comprises of algae, periphyton and sea grass. A maximum size of 160 mm test diameter is reported [2], which corresponds to an age of four to five years. It has long tube feet and is often seen carrying all kinds of things from shells to seaweeds to cover their presence from predator such as sea otters, sea gull, trigger fish and snails. The spawning season of T. gratilla varies, i.e., the spring and the fall at the great Barrier Reef [3], the winter season in the northern Red Sea and the Gulf of Aqaba [3, 4], the summer off Japan [5], the late winter in the Indian Ocean, from the north coast of Madagascar [5], and the autumn in the Philippines and at Taiwan [6, 7].

Sea urchins are used as raw material to produce foodstuff, in particular, the product of processing gonads known as "Sea urchin roe", and is considered to be a prized delicacy in Asian, Mediterranean and Western Hemisphere countries [8, 9]. Sea urchin gonads have been using as a luxury food (i.e. prepared from fresh or a processed food) in Japan [10] and its roe could retail for as much as AUD 450/kg, therefore, it is found as one of the most expensive food stuff in the world in regards of sea foods [11]. The local community of the Asian Pacific Region has also been using it for long time as a remedy for improving general living tone, treatment for a number of diseases and strengthening of the sexual potency of the middle-aged men [12]. The high levels of AA (arachidonic acid) and EPA (eicosapentaenoic acid) recently detected in sea urchin gonads, has supported the development of aquaculture of sea urchins [13], since these polyunsaturated fatty acids (PUFAs) are important for human nutrition. In recent years, sea urchin fisheries have so advanced that the natural population of sea urchin are overfished in Japan, France, Chile, the north-eastern United States. The Canadian Maritime Provinces, and the West coast of North America from California to British Colombia meet the great demand [1, 14, 15]. Most, if not all, sea urchin fisheries have followed the rapid expansion to an unsustainable peak and also followed by an equally rapid decline. According to Carboni et al. [16], the global sea urchin production (both capture and culture) of 120,000 mt was the peak in 1995, which is in a declining state of 82 mt now. These decreasing patterns clearly reflect the overexploitation of most

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fishing grounds and highlight the need for conservation strategies, fishery management and aquaculture development.

Twelve species of sea urchins have been documented so far in Malaysia and among them *Tripneustes gratilla* is the most commercial species, occurs in Bum Bum Island near Semporna at Sabah, the Eastern Malaysia [17, 18]. In Sabah, it is the custom of eating "sea urchin roe". Thus, sea urchins play an important role in providing subsistence income to the local coastal communities. Due to lack of proper knowledge, and unrestrained exploitation, this important sea urchin fishery is recently under threat. However, few systematic works have been done on the abundance, distribution, population growth patterns, development and small-scale culture of some comparatively less-commercial species of echinoids in Peninsular Malaysia [19, 20] but no published information on breeding, larval development and culture techniques are available for the high-valued sea urchin, T. gratilla. The study has therefore been undertaken to investigate the detailed ontogenic development in a view to develop a viable methodology of seed production in captivity for the use in commercial aquaculture of *T. gratilla.*

II. Materials And Methods

A. Sampling and Conditioning

A total of 60 matured adults (60 to 100 mm of test diameter in size) of *T. gratilla* weighing from 80 to 200 g were collected by snorkeling (scuba dive) with the help of local fisher community at Bum Bun Island (4º27′55.08″N; 118º40′94″E) near Semporna, Sabah at low tide throughout their normal breeding season from October 2015 to January 2016. The collected live specimens were then transported with aerated plastic bucket to the laboratory of the Institute of Bioscience, Universiti Putra Malaysia (UPM) where they were maintained in an outdoor tank with flow-through seawater and fed with a diet of brown *(Sargassum sp)* macroalgae.

B. Spawning and Fertilization

Most of the urchins were used for this experiment within a week after collection. The Aristotle's lantern was removed from the healthy specimens by using scissors and forceps and rinsed thoroughly with sterilized filtered sea water (SFSW). Gametes were obtained from each sea urchin providing the injection of 0.5M KCl solution into the coelomic cavity. Eggs were then collected by overturning female sea urchin on a glass beaker filled with SFSW (Fig. 1). "Dry" sperm (Fig. 1) were also pipetted off the genetical pores and kept in concentrated form in a refrigerator at 4-5ºC for not more than 3-4 h. Diameter of eggs and length of sperm head were measured (eggs at 20 X 10x in a well-slide, sperm at 40 X 10x on a plain slide) under a compound microscope [21, 22].

Fig. 1. Collection of matured eggs and sperm of *T. gratilla* in captive condition.

In regards of fertilization, it was done by adding two drops of a diluted sperm into a petri dish containing 15 ml egg suspensions. A concentration of sperm was made at 10-5 dilution of "DRY" sperm [15, 23]. Following insemination, the sperm was kept with eggs for 5-10 minutes and then additional sperm was cleaned by 3-4 successive washes with SFSW. Six replicate fertilization experiments were performed using fresh gametes from new specimens in each time. In total, 100 eggs were counted for each trial and then recorded as fertilized if they had attained to at least 2-cell stage [14, 15].

C. Embryonic and Larval Development

The fertilized eggs were transferred into glass beakers (500 ml) and incubated in SFSW at room temperature (25-28ºC) until they reached the stage of free swimming blastula. They were transferred to DURAN standard laboratory clear (screwcap) glass bottles containing 1000 ml SFSW, which were stirred continuously by 10 rpm rotating motors. The larvae (up to the four-armed pluteus stage) were maintained at 2-3 individuals/ml in regards of stocking density [15, 23]. When the larvae attained feeding stage (four-armed pluteus), they were cultured in the same system (1000 ml DURAN standard laboratory clear screwcap glass bottles containing the larval density of 1 individual/ml). Around 90% water from the glass bottles was discarded by filtration/siphoning after 4-5 days and then filled with fresh SFSW. Larvae were fed with cultured microalgae, Chaetoceros calcitrans at concentrations of 5000, 10000, and 15000 cells/ml of cultured sea water at four-, six-, and eight- armed pluteus stage, respectively, by maintaining the food level in every three days of interval until attaining metamorphic competence [23]. All the developmental stages of embryos and larvae were observed until attaining the metamorphic competent stage [23]. At each stage, specimens were fixed in 10% formalin for more detailed studies. Observations on both living and fixed specimens, provided information on the time required for embryos to attain specific developmental stages. In each experiment, the time after insemination for 50% of the embryos to develop to 2-cell, 4-cell, 8-cell, blastula, gastrula, prism, 2-, 4-, 6-, 8-armed pluteus and competent stages was estimated [21, 24].

D. Metamorphosis

The larvae were used for settlement induction after they had reached the competent stage. The competence of larvae were indicated by the presence of large juvenile rudiment and a high rate of metamorphosis. Induction of metamorphosis was done on a mixture of coralline algal extract and Chaetoceros diatom (50:50) in the petri dishes (9.0 X 3.0 cm) containing SFSW. The density of the larvae at this stage was maintained at 1 individual/2ml of FSW [14]. In each experiment, replicate petri dishes were used and the metamorphosis rate was estimated within 24-30 h in the same environmental conditions and protocols as larval cultures.

E. Morphometric Measurement

All morphometric measurements of embryos, larvae and juveniles were made on the preserved specimens [22, 25]. Larvae were first preserved in 10% formalin with FSW in a vial tube. A few drops of 10% formalin seawater containing 10-12 larvae were put under an elevated coverslip on a microscope slide at a time. After that, they were observed and finally measured and photographed under the compound microscope (Zeiss Axioskop 2) fitted with a software (Spot Advanced Version 3.4). To identify the developmental stages, each specimen was observed four times under the microscopes [26].

III. RESULTS

A. Embryonic Development

The morphological changes, which were took place during the embryonic development of T. gratilla are summarized in Table 1, while the developmental stages are shown in Fig. 2.

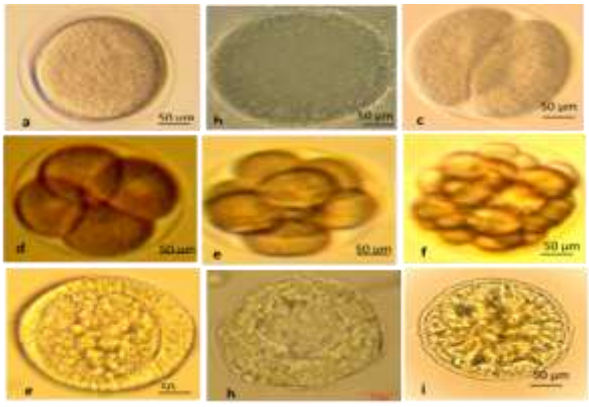

Fig. 2. Embryonic developmental stages of *T. gratilla* under compound microscopy. a. Fertilized egg showing fertilization membrane, b. Fertilized egg with complete fertilization membrane c. 2-cell stage, d. 4-cell stage, e. 8-cell stage, f. 16-cell stage, g. 32-cell stage, h. Morula stage enclosed with fertilization membrane, i. Blastula.

B. Spawning and Fertilization

The diameter of the unfertilized eggs of T. gratilla ranged between 72.60 and 95.47 µm with a mean (±SD) value of 84.74±4.05 µm. The matured eggs were transparent, spherical in shape, non-adhesive, yellowish in color and devoid of oil globules. In regards of sperm, the mean head length of matured sperm was 5.17±0.07 µm with a range value between 3.25 and 7.86 µm. The color of sperm was milky-white and at limited sperm concentration (10-5 dilution of "dry" sperm), fertilization rate ranged from 94.0 to 98.0% with a mean (±SD) value of 96.6±1.4% (n=6). The egg vitelline membrane was elevated after 30-40 sec of sperm entry and the fertilization membrane began to form (Fig. 2A). However, the complete formation of fertilization envelope took place within 5 min post-insemination (Table 1; Fig. 2B). Upon sperm penetration, the pro-nucleus of male was accelerated by microtubules near the center of egg. When the microtubules touched, the pro-nucleus of female swiftly pulled towards the pro-nucleus of male. This sperm and egg fusion held place approximately 10-12 min after the entry of sperm. During the fertilization proceedings, the cytoplasmic movement increased, and the cell surface were developed with an irregular form. Soon before the starting of the first cleavage, the membrane stopped the vibration, the cell surface became regular, and then the hyaline layer became thickened.

C. Cleavages

First cell division started 01.22 h after fertilization (Table 1) and was holoblastic (Fig. 2C). Second cleavage initiated 02.18 h post fertilization (Table 1) and was meridional, dividing the embryo into 4 blastomeres (Fig. 2D). The third cleavage was equatorial, separating animal and vegetal blastomeres at 02.48 h (Table 1; Fig. 2E). During the 4th division, micromeres created equally from vegetal blastomeres whereas eight mesomeres were developed by meridional cleavage of animal blastomeres (Fig. 2F) at 03.15 h after fertilization (Table 1). Equatorial division of mesomeres, meridional division of macromeres and unequal micromeres division formed the embryos with 32-cells 0.3.48 h post fertilization (Table 1; Fig. 2G). Sixty-four cell embryos were developed when blastomeres went through an equatorial division while the micromeres experienced a meridional division. The seventh cleavage happened without micromere division and formed morula with one hundred eight cells after 04.01 h following fertilization (Table 1; Fig. 2H).

TABLE I:
EVENTS OF EMBRYONIC DEVELOPMENT IN *T. GRATILLA*. THREE REPLICATE FERTILIZATION EXPERIMENTS WERE CONDUCTED AND FOR EACH DEVELOPMENTAL STAGE, 30 EMBRYOS FROM EACH EXPERIMENT WERE USED FOR THE OBSERVATION AND MEASUREMENT OF EMBRYOS (MEAN±SD WITH RANGES IN PARENTHESES).

Developmental stages	Time after insemination	Diameter (μm)
Fertilized eggs with the formation of fertilization membrane	0.01 h	84.74 ± 4.05 (72.60–95.48)
Fertilized eggs with complete fertilization membrane	0.05 h	86.42 ± 1.87 (82.91–92.28)
2-cell	1.22 h	91.29 ± 0.31 (90.60–93.07)
4-cell	2.18 h	94.12 ± 1.21 (90.57–96.09)
8-cell	2.48 h	96.59 ± 0.06 (95.32–98.01)
16-cell	3.15 h	100.09 ± 1.19 (97.69–102.27)
32-cell	3.48 h	102.37 ± 1.06 (99.94–104.30)
Multi-cell (Morula)	4.01 h	103.63 ± 1.07 (102.04–106.89)
Hatching Blastula	9.26 h	104.44 ± 0.84 (102.37–105.77)

D. Blastula

The blastula developed in a polygonal shape throughout association of the epithelium. The vegetal plate thickened, and cilia were developed on the perimeter 09.26 h post-fertilization, just before hatching (Table 1; Fig. 2I).

E. Larval Development

The morphological variations happened throughout the larval development event of T. gratilla are summarized in Table 2, while the developmental stages are depicted in Fig. 3.

F. Gastrula

The gastrula with ciliated bands formed at 16.41 h after fertilization (Table 2). In the beginning of this stage, larva experienced with primary mesenchyme cells (PMC) which were separated from the vegetal pole, became spherical in shape, and gathered in a unipolar manner on the vegetal pole. PMC then migrated through the blastocoel forming a ring connected by thin pseudopodia on the posterior end. In this stage, red-pigmented cells were first observed on the vegetal pole and then migrated through the epithelium, simultaneously with PMC, towards the apical plate. PMC formed two aggregates and began to secrete a calcareous triradiate spicule. Secondary Mesenchyme Cells (SMC) formed on the vegetal pole, spreading cytoplasmic projections in the direction of blastocoel during archenteron invagination. SMC on the archenteron then reached to the anterior pole whereas red pigmented epithelial cells reached the anterior pole, when the blastocoel was occupied by SMC (Fig. 3A).

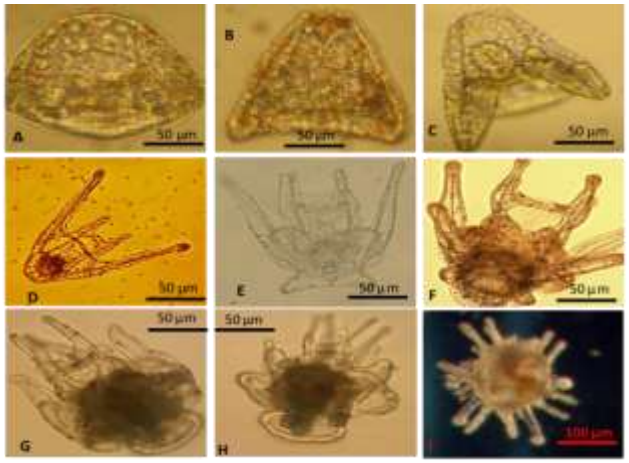

Fig. 3. Larval developmental stages of *T. gratilla* under compound microscopy. A. Gastrula, B. Prism, C. 2-arm pluteus, D. 4-arm pluteus, E. 6-arm pluteus, F. 8-arm pluteus, G. Pre-competent larva with ciliated ring and growing rudiment, H. Competent larva with complete rudiment growth, I. Juvenile (1 d after metamorphosis).

G.Prism

Prism stage initiated 22.25 h post fertilization (Table 2). Epithelial red-pigmented cell was not present on the oral (ventral) region at prism stage. During the formation of complete prism larval stage, the surface of them were covered with cilia apical tuff on the anterior pole and a ciliated ring around the anus (Fig. 3B).

H.2-arm pluteus

The 2-arm pluteus stage was formed after 34.00 h post-fertilization (Table 2; Fig. 3C). The mouth was opened at this stage, but the larvae cannot able to feed; the larvae captured microalgae and gradually forwarded in the direction of the mouth with the help of larval arm, but were deflected away possibly by an opposing current. There were three portions identified in the gut under a compound microscope, such as esophagus, stomach, and intestine but were not functioning. Esophagus muscles began to contract; the stomach developed in diameter whereas it's epithelium became thinner.

I. 4-arm pluteus

Within 48.00 h of fertilization, 4-arm pluteus larva was formed with two well-developed post oral arms (Table 2; Fig. 3D). At this stage, pluteus larva attained with complete digestive tract and also capable of feeding unicellular algae. The well-defined opening in the lower half of the larva represented the anus. The tips of arms and the arched oral lobe behind them represents the leading front of the swimming larva under the oral lobe which directed algae into the mouth.

J. 6-arm pluteus

During the onset of the 6-arm pluteus larval development, post oral arm further elongated and the post oral and anterolateral arms supported by well-formed skeletal rods (Fig. 3E). The digestive tract moves centrally through the larva. The mouth, the clear opening at the base of the shorter (anterolateral) arms, was followed by a constricted muscular esophagus, which exhibited peristalsis during feeding. Posterodorsal arms, the third pair of arms were appeared to form this stage (Fig. 3E). The darkened appearance of the bulbous stomach section is due to the concentration of engulfed algae (Fig. 3E).

K.8-arm pluteus

In 8-arm pluteus larva, postetodorsal arms further elongated and the preoral arms, the fourth pairs of arms appeared to form this stage after 16.00 d of fertilization (Table 2; Fig. 3F). Arched pigmented ciliated bands between the post oral arms began to develop. Immediately above the bands was the mouth cavity enclosed on the lower surface by a small concave edged lobe and on the upper surface by a largest overhanging fold, from which two small preoral arms was protruded. These preoral arms were the fourth pair of larval arms (Fig. 3F). In this event, characteristic thorns of the skeletal rods were also visible in the post oral arms.

L. Pre-competent and Competent

The pre-competent (premature) larval stage began to form 28.00 d after fertilization (Table 2). In this stage, the basal portion of the larva was enlarged, and the pigmented arches appeared to form and the pedicellaria was encircled with a ciliated ring (Fig. 3G). Increased differentiation of adult tissue accounted for the dense appearance of the interior portion of the larva.

The rudiment developed spines and tube feet, which still grow inside the larval body (Fig. 3H). Pedicellariae were not formed on the surface of the larva, as commonly showed in competent larvae of regular echinoids. A continued degeneration of larval tissue and arms accompanied by the emergence of the adult spines and tube feet could be seen slightly below the left corner of the larva. Under the temperature of 25ºC, this stage reaches at approximately 35 days (Table 2).

TABLE II:
EVENTS OF EMBRYONIC DEVELOPMENT IN *T. GRATILLA*. THREE REPLICATE FERTILIZATION EXPERIMENTS WERE CONDUCTED AND FOR EACH DEVELOPMENTAL STAGE, 30 LARVAE FROM EACH EXPERIMENT WERE USED FOR THE OBSERVATION AND MEASUREMENT OF LARVAE (MEAN±SD WITH RANGES IN PARENTHESES).

Developmental stages	Time after insemination	Length (µm)
Gastrula	16.41 h	120.12 ± 12.79 (95.79 – 142.36)
Prism	22.25 h	131.18 ± 1.75 (109.51 – 149.14)
2-arm pluteus	34.00 h	173.16 ± 21.93 (121.53 – 167.62)
4-arm pluteus	48.00 h	514.05 ± 103.64 (339.46 – 690.65)
6-arm pluteus	10.00 d	608.54 ± 54.86 (506.35 – 683.86)
8-arm pluteus	16.00 d	668.44 ± 124.55 (394.63 – 835.31)
Pre-competent larva with ciliated ring and growing rudiment	28.00 d	742.42 ± 11.90 (722.50 – 762.67)
Competent larva with complete rudiment	35.00 d	380.31 ± 19.43 (332.65 – 486.54)

M. Metamorphosis

Competent larvae showed a characteristic substrate-test behavior that involved in swimming near the bottom. In this stage, well-formed spines and extended tube feet were evident. Larval structures were discarded or absorbed at this point (Fig. 3I). Metamorphosis happened when the larvae attached firmly to the bottom with the protruding tube feet and the larval tissues started to degenerate and gather on the aboral surface of the rudiment. During this process, larval spicules became exposed and broke off, and the larval tissues gathered on the aboral surface of the rudiment forming a globoid structure. At least 1.5 h is usually required to complete metamorphosis from attachment to full regression of the larval tissues.

N. Juvenile

Metamorphosis was followed by the resorption of larval tissues and the development of complete juvenile structure with adult spines, extended tube feet and well developed pedicellaria (Fig. 3I) and the whole event usually took place within 1d post-settlement (Table 3; Fig. 4A). There was no skeleton on the aboral surface of early post larval juveniles except fragments of larval rods. The gut was not yet formed and neither mouth nor anus were present. However, during the resorption of larval tissues, the Aristotle's lantern rudiments and teeth were visible in the oral region detected under polarized lights in the laboratory. The newly formed juvenile appeared with a complete adult structure (mouth, gut anus, spine, tube feet, etc.) then grew on coralline algae to one month (Fig. 4B), 2- month (Fig. 4C) and 3-month ages juvenile (Fig. 4D) by increasing the overall juvenile body, spine and tube foot lengths (Table 3). The 3-month old juvenile produced through the above developmental and growth stages represents the sea urchin seed (Fig. 4D) for stocking in grow-out culture.

O. Complete Lifecycle of T. gratilla

Various developmental stages documented in the present investigation (as above) have been utilized to determine the complete life cycle of *T. gratilla.* These are: i) pelagic stage (fertilized eggs with the formation of fertilization membrane, fertilized eggs with complete fertilization membrane, 2-cell, 4-cell, 8-cell, 16-cell, 32-cell, multicell-morula, hatching blastula, gastrula, prism, 2-arm pluteus, 4-arm pluteus, 6-arm pluteus, 8-arm pluteus, pre-competent larvae, and ii) benthic stage (competent larvae, metamorphosis, juvenile and adults), the details of which are illustrated in Figure 4.

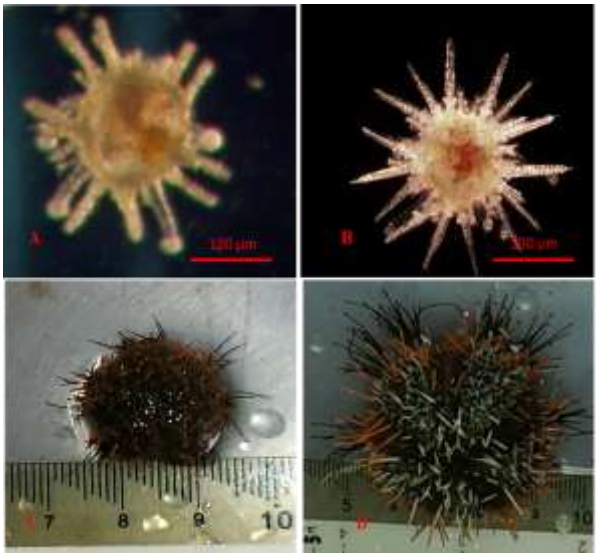

Fig. 4. Development of juvenile stages of *T. gratilla* under a compound microscope. A. Juvenile (1 day after metamorphosis), B Juvenile (1 month after metamorphosis), C. Juvenile (2 months after metamorphosis), D. Juvenile (3 months after metamorphosis)

TABLE III:
DEVELOPMENT OF JUVENILE STAGES OF *TRIPNEUSTES GRATILLA*. THREE REPLICATE FERTILIZATION EXPERIMENTS WERE CONDUCTED FOR EACH DEVELOPMENTAL STAGE. TWELVE JUVENILES FROM EACH REPLICATE WERE USED FOR STUDY AND MEASUREMENT (MEAN±SD WITH RANGES IN PARENTHESES).

Development of juvenile stages	Body Length (mm)	Spine Length (mm)	Tube feet Length (mm)
Juvenile (1 day after metamorphosis)	0.428±0.03 (0.387-0.474)	0.464±0.12 (0.334-0.707)	0.577±0.10 (0.426-0.758)
Juvenile (1 month after metamorphosis)	3.95±0.20 (3.65-4.44)	2.19±0.47 (2.12-2.98)	4.96±0.91 (3.62-5.55)
Juvenile (2 month after metamorphosis)	6.50±0.25 (6.15-6.88)	3.13±0.57 (2.28-3.54)	5.93±1.06 (5.41-8.84)
Juvenile (3 month after metamorphosis)	9.66±0.41 (9.05-10.32)	5.18±0.95 (4.38-6.34)	7.03±1.09 (6.32-7.45)

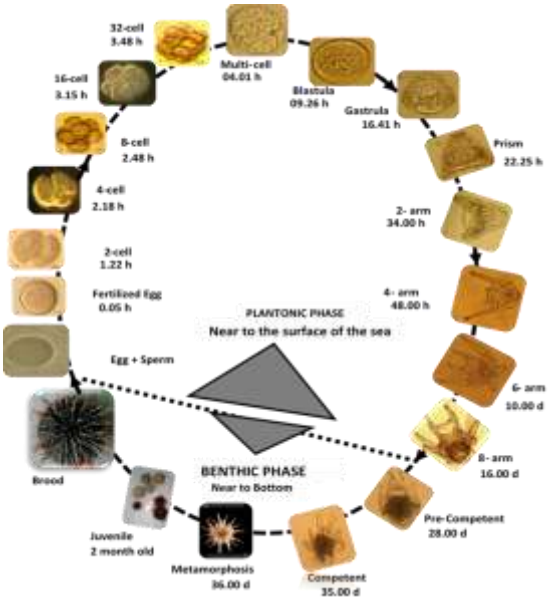

Fig. 5. A complete life cycle of *Tripnuestes gratilla*

IV. DISCUSSION

In the planktonic phase, the cleavages and development of embryo and larva of *T. gratilla* were similar to other echinoids having pelagic mode of development [27-32]. The developmental time of hatching blastulae was 09.26 h at 24ºC, which is longer than those of *Lytechinus* variegatus (6 h at 23ºC) [28] and in *Clypeaster subdepressus* (7 h at 26ºC) [32]. The time for development of later stages were also followed the same trends but slightly differed from those of Caribbean species, L. variegatus at 23oC [28] and the Pacific Species of *Colobocentrotus mertensii* at 27oC [31], respectively.

The gastrulation become full-bodied with the relationship between different types of gastrulation and the migration strategy of red colored cells in *T. gratilla*. Red-colored cells formed on the area of vegetal pole and migrate through the ectoderm to the apical plate, whereas elongation of the archenteron is constant. Similar phenomena were observed during the commencement of gastrulation in another tropical sea urchin, *Salmacis sphaeroides* [19], *Diadema setosum* [20] and *Echinometra mathaei* [33], and in sea biscuit *Clypeaster subdepressus* [32]. Some regulatory roles show in red pigmented cells and are recognized to trigger gastrulation in E. *mathaei* and *S. sphaeroides* [19, 33]. The participation of these cells on morphological changes might occur during the formation of prism and early axis specification of *pluteus* larvae [20, 31, 32]. Furthermore, the first sign of larval skeleton in the form of triradiate spicules, were formed during gastrulation in *T. gratilla*, the events of which were more or less similar to those observed in other regular echinoids [19, 31, 32].

In the competent larval stage, *T. gratilla* has been observed to demonstrate substrate test behavior, which are similar to those documented in other echinoids [19, 32, 34-37]. The early post larval juveniles look like a regular urchin, having a spherical body shape, bilateral symmetry could be identified soon after the resorption of larval tissues and was possibly determined during the formation of rudiment as those observed recently in sea urchins [19, 20] and sea biscuits [32]. Larval arms were found to be completely absorbed together with the skeletons and epidermis in the newly metamorphosed juvenile of *T. gratilla* (Fig. 3I). In contrast, tissue resorption in *Eucidaris thouarsi* and *Paracentrotus lividus* was achieved by the retraction of only epidermis resulting in the naked skeleton [27, 36]. However, the naked skeletal rods will eventually be broken down and such kinds of discrepancy may be related to the species differences [31].

After the induction of complete metamorphosis, *T. gratilla* juveniles had 4 primary spines per interambulacrum (20 totals), similar to those documented in P. lividus [36], *Strongylocentrotus purpuratus* (Miller and Emlet, 1999), *S. sphaeroides* [19] and *E. mathae*i [20]. However, a greater number of key spines per interqanbulacrum was documented after metamorphosis in the asymmetrical echinoid *E. cordatum*. They are also differed from *T. gratilla* by the presence of subsidiary spines and a sub-anal facsciole and 4 primary spines [37]. The newly metamorphosed juveniles of *T. gratilla* had one tube-feet per ambulacrum as similar to those occurring in *S. fanciscanus* and *S. purpiratus* [38], *P. lividus* [36] *E. cordatum* [37] and *S. sphaeroides* [19]. On the *contrary*, *C. subdepressus* inimitably displayed 3 podia just after metamorphosis [32]. The competent larvae of *T. gratilla* have pedicellariae during the late larval period and after metamorphosis as those documented in other regular urchins, such as *P. lividus* [36], *S. fanciscanus* [38] and *S. sphaeroides* [19], while sometimes appear in *S. purpuratus* after metamorphosis. Quite the opposite, spines or pedicellariae are not exhibited in competent larvae of *E. cordatum* [37], while *C. subdepressus* do have spines but lacking any pedicellariae in this stage [32].

In the young juvenile stage, *T. gratilla* has neither a mouth nor anus and no guts at all. Similar event was also observed in other sea urchins [31, 39, 40] and sea biscuits [19, 20, 27, 32]. At this stage, the dorsal half is necessarily a rounded lump of larval tissue punctured by the three pedicellaria. The dorsal organs appear to develop out of these tissues. For the first 2 days, the larval tissue can easily be picked off the urchin. The digestive system and probably other internal organs appear at about 4-5 days after settlement induction and then the urchin begins to feed, as those documented in *L. pictus* [39], *P. lividus* [36], *Colobocentrotus mertensii* [31] and *S. sphaeroides* [19] and *D. setosum* [20]. On the contrary, the juveniles of *C. subdepressus* and *C. rosaceus* start feeding 7 and 10 days, respectively, when the Aristotle's lantern and mouth become functional [27, 32]. Nevertheless, the consequences of these developmental events in progressing juvenile stages of *T. gratilla* deserve further study.

V.CONCLUSION

The present study reveals the first successful documentation of the embryonic, larval and post-metamorphic juvenile

development of *T. gratilla* in Malaysia. The findings of the designated study would greatly be helpful towards the understanding of the ontogeny and life-history strategies, which will ultimately assist in the development of mass seed production techniques for culture and conservation of this commercially important sea urchin to a greater extent.

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