TIMING OF EARLY DEVELOPMENTAL STAGES IN EMBRYOS OF *Tripneustes gratilla* (LINNAEUS, 1758) (ECHINODERMATA: ECHINOIDEA)

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Abstract: Sea urchin is one of the most important subjects in developmental biology studies due to its rapid and simple development. Timing for its development is a key component for most experiments. However, available data often vary due to the effect of temperature and other external factors. With this, standardization on the timing of the early developmental stages in embryos of *Tripneustes gratilla* was conducted in this study. Induced spawning and *in-vitro* fertilization were done on the collected sea urchins. Morphology of the embryo and timing for each developmental stage including early cleavage stages, morula, and blastula were studied. Sea urchin embryos started its development at 2 minutes after fertilization and reached blastula stage after 6 hours. Developmental stages of *T. gratilla* embryos exhibited embryological distinction from one another and tend to develop rapidly after fertilization making it an appropriate model organism for biological researches.

Keywords: *sea urchin, Tripneustes gratilla, developmental stages, early embryo, timetable*

1. INTRODUCTION

Sea urchins (Echinodermata: Echinoidea) are marine invertebrates that constitute a major part of the marine benthic fauna (Levitan *et al.*, 1991). Sea urchins are dioecious that undergo sexual reproduction through external fertilization. Sperms and eggs are released into the surrounding water for fertilization where zygotes develop (Levita *et al.*, 2007). Sea urchin development is simple and very rapid. Successive cell division happens during cleavage formation until it developed into blastula, which by this time, the cells start to occupy the periphery while leaving a central space that gradually forms a central cavity or blastocoel (Briggs & Wessel, 2006; Kominami & Takata, 2003). Lastly, developing embryo will hatch and develop into pluteus larvae, wherein arms already begin to develop and started to feed on other organisms (Ghorani *et al.*, 2012).

Sea urchin development has been extensively used as a model for developmental toxicology and for detection of cytotoxic, teratogenic and antineoplastic activities of new compounds (Costa-Lotufo *et al.*, 2002). Sea urchins are more closely related to vertebrates than any other echinoderms and its genome has been completely sequenced, making it a great model organism for developmental biology (Pinto, 2009). Some of its salient features include ability of spawning induction, artificial fertilization, coordinated and rapid development, and optical clarity of embryos under the microscope made sea urchin a suitable organism for research about fertility, early embryonic development and

various biological tests (Semenova *et al.*, 2006; Shen & Bugart, 1985). With these various applications, basic knowledge on the development of sea urchin such as timing for each developmental stage under laboratory conditions would be of great help in improving and modifying its uses in different fields.

Among sea urchin species, *Tripneustes gratilla* are frequently used for various researches due to its economic importance as they are being consumed for food. This resulted to overexploitation of this species thus, led to a sharp decline in the *T. gratilla* population (Junio-Meñez *et al.*, 1998). Available studies on this sea urchin were limited to the mechanism of fertilization and their pattern of development in early embryos (Briggs & Wessel, 2006; Ernst, 1997; Mazur & Miller, 1971). In the Philippines, researches were mainly focused on growth performance and genetics, which is related to sea urchin fishery (Capinpin, 2015; Manuel Jr. *et al.*, 2013; Talaue-McManus & Kesner, 1993). Available data on early developmental stages of *T. gratilla* are still lacking and often vary due to the effect of temperature and other external factors (Moulin *et al.*, 2011). In addition, existing literature are limited to the temperate species that exhibit variation in development as compared to the tropical Philippine species. With this premise, standardization of the timing of early developmental stages in embryo of *T. gratilla* in tropical environmental conditions is necessary to maximize its use in different biological assays and other scientific applications. This study intended to monitor and document early embryonic events in *T. gratilla* from fertilization to blastula stage under laboratory conditions.

2. METHODOLOGY

2.1 Induced spawning and in-vitro fertilization

The method for the induced spawning and *in-vitro* fertilization of the collected sea urchin was a modification devised from previous works (Schatten & Hülser, 1983). Sexually matured *T. gratilla* (about 2-3 inches in diameter) were collected from Calatagan, Batanga**s,** Philippines and transferred to the Center for Life Sciences Research, Institute for Science and Technology Research (CLSR-ISTR) laboratory at Polytechnic University of the Philippines (PUP). Gametes were obtained by intracoelomic injection of 0.5 M KCl. Spawning urchins were placed over the mouth of beaker containing sterilized artificial seawater (SASW), where the oral side faced the SASW in order to collect the gametes. Collected oocytes were washed with SASW and passed through 200 µm mesh net in order to remove the jelly coatings. This diluted sperm suspension was added into the egg and the resulting solution was mixed for two (2) minutes to allow fertilization**.** This were eventually suspended at the bottom of the container. All eggs that float at the surface of the solution were discarded and considered as unfertilized. The fertilized egg suspension was then placed in a horizontal shaker (10 rpm) at ambient temperature to avoid aggregation of the eggs.

2.2 Timing of early embryonic development of T. gratilla

Sea urchin development was observed for a period of 6 hours (point of fertilization as 0 minute). Within this time, 1.0 mL of embryo suspension was collected from the culture container at 10 minutes interval. Collected aliquot was preserved in 5% buffered

formalin and the developmental stages were observed under the microscope. A total of 100 embryos from the samples were randomly picked and the number of each developmental stages, 2-cell stage, 4-cell stage, 8-cell stage, morula, and blastula were counted. In order to standardize the timing of early embryo development of *T. gratilla*, the range of time distribution for each developmental stage and its average time of appearance were determined. Data from three (3) trials with three (3) replicates were used to describe the timing of early embryo development.

3. RESULTS AND DISCUSSION

3.1 Early development of T. gratilla embryo

Figure 1 illustrates the stages of early embryonic development of *T. gratilla*. The measured average size for the *T. gratilla* embryo is 129.08±0.68 µm (n=50). Formation of the fertilization envelope differentiates fertilized egg from the unfertilized ones (Figure 1A and 1B, respectively). The thick fertilization envelope elevated from the egg surface two minutes post fertilization time (pft). Lifting of the vitelline layer few seconds after fertilization was due to the formation of two additional coating, which are the fertilization envelope and the hyaline layer (Kominami & Takata, 2003; Shen & Bugart, 1985). These layers serve for protection and supporting structures that anchor dividing blastomeres of the embryo (Adelson & Humphreys, 1988). During cleavage, the single-celled zygote is converted into a multi-cellular embryo through rapid and repeated mitotic cell divisions (Figure 1C-1H). Cleavage formation of *T. gratilla* embryos is in radial holoblastic pattern wherein radially symmetrical embryo has cleavage furrow that cuts through the entire dividing cell. Primary reason for the complete cell division of the blastomeres would be the absence of yolk in oocyte while unknown mechanism of cell-cell communication is the important factor for the radial symmetry of the embryo (Briggs &Wessel, 2006).

The first cleavage producing the 2-cell stage (Figure 1C) cuts the fertilized egg in meridional axis, passing along the animal to vegetal pole axis. The 2-cell stage embryo consists of two equal-sized blastomeres with distinct nuclei. The second cleavage producing the 4-cell stage (Figure 1D) is also meridional but occurs at a right angle to the first cleavage. The 4-cell stage embryos can be characterized by two perpendicular meridional cuts resulting to four equal sized blastomeres. For the formation of 8-cell stage embryo, an equatorial cleavage cuts perpendicularly across the polar axis creating two tiers of cells (Figure 1E). The equatorial cleavage makes two tiers of cells namely upper (animal pole) and lower (vegetal pole) tiers. At the 8-cell stage, a small cavity appears between the blastomeres as preparation for the formation of the blastocoel. All the cells of the embryo in each of the first three cleavages are equal in size, cleavage up to this point was observed to be equal. The fourth cleavage yields the 16-cell stage embryo. The four cells in the upper tier divide equally and meridionally to produce eight mesomeres. The four cells of the lower tier divide unequally and horizontally to produce four larger macromeres and four smaller micromeres located at the vegetal pole of the embryo. From this point, individual blastomeres become increasingly difficult to distinguish. The 32-cell stage embryo results from fifth cleavage. The mesomeres from the upper tier divide horizontally and equally, producing another two tiers of cells. The macromeres and micromeres on the lower tier divide equally and meridionally. The sixth

cleavage produces the 64-cell stage embryo containing eight tiers or layers of cells. The micromeres on the animal pole divide horizontally while the macromeres on the vegetal pole divide horizontally, producing additional tiers of cells. The 16-cell to 64-cell stage embryos fall under the morula stage (Figure 1F) (Masuda & Sato, 1984). The early blastula stage (Figure 1G) is characterized by the presence of a developing fluid-filled blastocoel. A declining rate of cell division was observed due to the difference between the sizes of cells found in the animal and vegetal poles. The cells of the early blastula are arranged in few layers around the blastocoel.

3.2 Time table of the early embryonic development

Timing for the appearance of the early embryonic stages of *T. gratilla* is presented in Figure 2. The figure includes the first and last time appearances of each stage and the mean±standard error time, which represents the probable time when cleavage is expected to occur. The formation of fertilization envelope was first observed right after fertilization until 107 minutes post fertilization time (pft) at 70.00±10.00 min. This is followed by the appearance of 2-cell stage embryos that appeared at 30 minutes until 150 minutes pft (80.00±10.00) and 4-cell stage at 80 minutes to 180 minutes pft (130.00±10.00 min). From 90 minutes to 270 minutes pft, 8-cell stage was noted (160±10.00 minutes). Lastly, the morula and blastula stages first appeared at 100 minutes to 300 minutes pft (190±10.00 minutes) and 190 minutes to 300 minutes (247.27±11.13 minutes), respectively. Observed overlapping of time between appearances of each embryonic stage indicates asynchronous development of individual fertilized egg of *T. gratilla*.

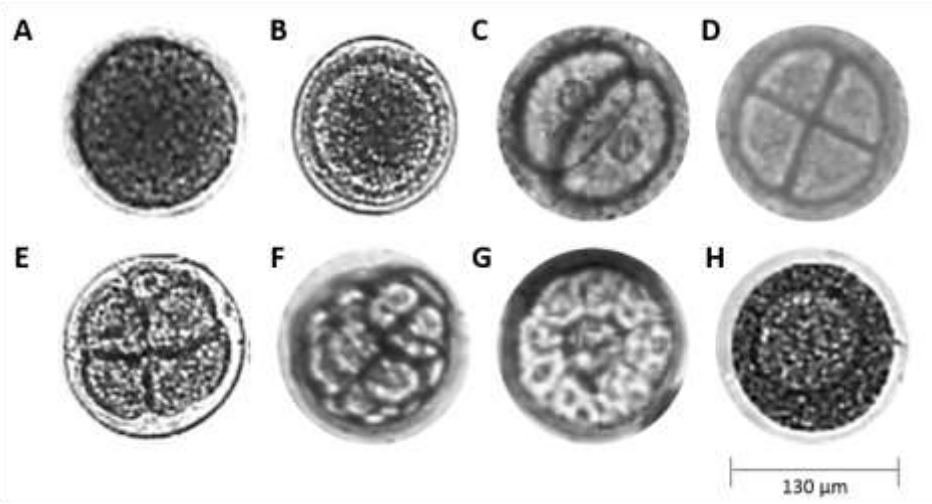

Figure 1. Different developmental stages resulted from *in-vitro* fertilized sea urchin egg. (A-B) embryo with distinct fertilization envelope; (C) 2-cell stage embryo formed from first cleavage; (D) 4-cell stage embryo (second cleavage formation); (E) 8-cell stage embryo from third cleavage; (F) morula stage with 16-32 cells; (G) early blastula characterized by the formation of single-layered arrangement of cell and the formation of central cavity; and (H) mid-blastula stage with distinct blastocoel.

According to the presented data, 70 minutes after fertilization, the first cleavage occurred which is comparable to the *Arbacia punctulata* (Shimek, 2018). Furthermore, occurrence of the blastula stage embryo in *T. gratilla* is earlier compared to temperate species such as *Sterechinus neumayeri* and *Paracentrotus lividus* with a timing of 2 to 3 days pft and 12 hours pft, respectively (Ruso *et al.*, 2003; King & Riddle, 2001). Variations on the timing on different species can be associated to the environmental conditions that lead to the different adaptation of the species on its environment (Ghorani *et al*., 2012). Also, the ambient temperature in laboratory condition in the Philippines is warmer (27^{o}C to 31^{o}C), thus, one important environmental factor to consider would be the temperature. Reports have indicated that sea urchin embryos in cool regions tend to develop sooner than similar species from warmer regions (King & Riddle, 2001).

4. CONCLUSIONS

In conclusion, the current study demonstrated that *T. gratilla*, as other species of warm regions, has relatively shorter developmental timing compared to other studied species. Thus, the time of early developmental stage obtained from this study should be used in tropical and warmer regions when used as experimental model for biological studies. Also, the results of this study support the suitability of sea urchins as models for biological studies due to its ability of spawning induction and artificial fertilization, rapid development and also optical clarity of embryos. *T. gratilla* may be used as a bioindicator for biological tests, including toxicity bioassays and other ecotoxicology tests.

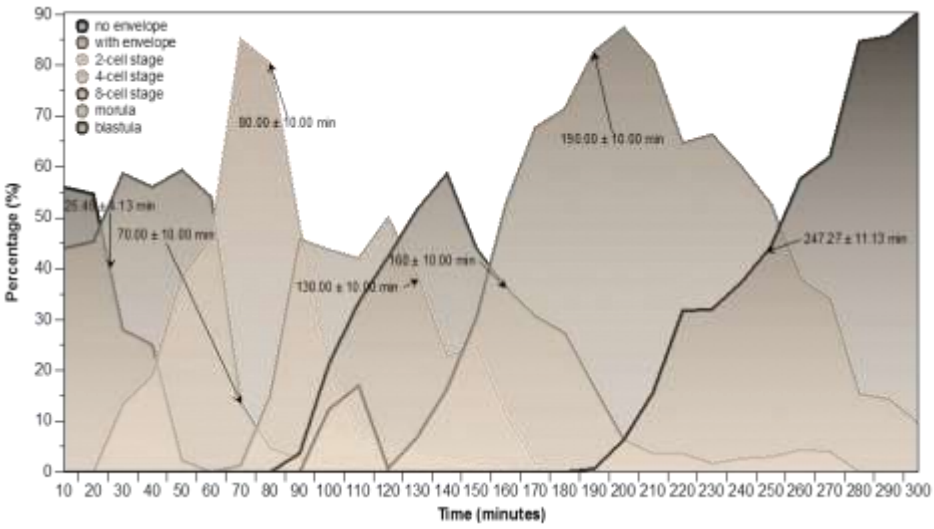

Figure 2. Time of the different stages of early developmental stages in *Tripneustes gratilla.* Arrow indicates the mean time for the appearance of a specific developmental stage.

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