ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 7, Issue, 02, pp.12365-12378, February, 2015

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

REVIEW ARTICLE

EMBRYOGENESIS OF THE SEA URCHIN *STRONGYLOCENTROTUS PURPURATUS* (STIMPSON, 1857) UNDER NORMAL AND BIOACTIVE CONDITIONS

*** [1,2]Gaber Ahmed Saad and [1,3]Abdullah Hussein Bedeer**

[1]Department of Biology, College of Medicine, Dammam University, Saudi Arabia, KSA
[2]Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt
[3]Department of Zoology, Faculty of Science, Mansoura University, Mansoura, Egypt

ARTICLE INFO

Article History:
Received 07th November, 2014
Received in revised form
15th December, 2014
Accepted 17th January, 2015
Published online 26th February, 2015

Key words:
Holoblastic cleavage,
Free-swimming blastula,
Gastrula- prism stage,
Pluteus larva,
Bioactive inducers,
One-way analysis of variance (ANOVA),
Dunnett's Multiple Comparison Test.

Abbreviations

AO: Anal Opening, AS: Anal Surface,
AP: Animal pole, AR: Aimal Tier,
AT: Apical Plate, B: Blastomere
BC: Blastocoel, BD: Blastoderm,
C: Cilia, CG: Cortical Granules,
DT: Digestive Tract, E: Ectoderm,
EN: Enteron, FM: Fertilization Membrane, JC: Jelly-Coat,
LM: Larval Mouth, MA: Macromere,
ME: Mesomere, MI: Micromere,
M: Mouth, N: Nucleus, O: Ooplasm,
OS: Oral Surface, PA: Postoral Arm,
PAR: Preoral Arm, PMC: Primary Mesenchyme Cell; SN: Spine,
ST: Stomodaeum, T: Test,
VT: Vegetal Plate, VP: Vegetal Pole
VM: Vitelline Membrane, Y: Yolk

ABSTRACT

Adults of *Strongylocentrotus purpuratus* were collected during September to June (2012) and July to August (2014) along the northern estruarineharbor of the Arabian Gulf – Saudi Arabia. Procurement of gametes has been carried out by potassium chloride injection using a one ml tuberculin syringe equipped with a 26 or 30 gauge needle. Gentle shaking of the animal anticlockwise 90◦ after few minutes of injection aided in gamete shedding. Gently sperm and eggs were mixed with a clean stirring rod. The different embryonic stages till the complete metamorphic plutei were described. The time schedule for different stages has been tabulated. Embryonic stages were stained with Nile blue and were placed on glass slides with embedding mixture of PBS / glycerol / DABCO. Immediate viewing and photographing were performed under an Axiomicroscope (ZEISS-Axiophot). Sperm are very small and extremely motile while the egg (70 to 180 µm in ϕ) had yellow to orange jelly-coat. Zygote (3 min. after fertilization) has undergone radial holoblastic cleavage till the blastula stage (3.50-4.15 hrs. after fertilization).The early blastula had a blastoderm enclosing a central blastocoel (6 hr 20 min. after fertilization). Cilia were developed on the exterior of the blastoderm and gradually this embryonic stage hatched and became a free-swimming blastula (8 hr 40 min. after fertilization). At the animal pole an apical plate was formed in the form of a thickened region of epithelium with a tuft of long cilia while the epithelium at the vegetal pole flattened and thickened to form the vegetal plate (16hr 45min.. after fertilization). Ingression of primary mesenchyme cells into the blastocoel took place and a new cavity was formed referred to enteron. The larval mouth was formed (19hr 15min. after fertilization). The blastopore developed into the anal opening of the digestive tract. During the prism stage the embryo took the shape of a rounded pyramid (21 hr 20 min. after fertilization). Pluteus larva with two arms was developed (24hr 30 min. after fertilization). A pluteus larva developed into four-armedpluteus (29hr 40 min. after fertilization). The later fed and developed into eight-armed pluteus (145 hr 50 min. after fertilization). A period of extensive feeding and continued larval development was required before metamorphosis to a juvenile sea urchinr. Free swimming blastulae were subjected to certain bioactive inducers which had been claimed to induce metamorphosis, involved in signaling systems, serve in neuronal control, control morphogenetic and behavioral reactions or interact with members of several signal transduction proteins pathways under normal physiological conditions. Results of these trails were introduced to One-way analysis of variance (ANOVA) with $P < 0.05$ and Dunnett's Multiple Comparison Test with $P > 0.05$ or $P < 0.01$. It has been concluded that Acetyl choline 0.5 mM, Serotonine10 µg/ml, NOS 2.5 mM, C81 µ M, NH4ClNH4Cl and cGMP 1 µg/ml accelerated the transformation of blastula stage to an advanced pluteus larval stage with 8 arms whereas Acetyl choline 30 µ g/ml & 1 mM, NOS 1.5 mM, C8 10 µM and NH4Cl 300 µM exereted no role in metamorphosis and antibiotic sea water had negligible effect.

INTRODUCTION

Sea urchins are excellent organisms for demonstrating fertilization and early development. They have classically been used in embryology and developmental biology courses. This study would present information on echinoid development under normal and bioactive conditions. Moreover, a description of sea urchin development from fertilization to plute us larva. Thorough description of sea urchin development may be referred to the many excellent references available (**Harvey 1956; Tyler and Tyler 1966a, 1966b; Costello and Henley 1971; Guidice, 1973; Horstadius 1973; Stearns,1974; Ishikawa, 1975; Okazaki, 1975; Piatigorsky, 1975; Ruggieri, 1975; BrookBank,1978; Davenport, 1979**). Echnoid embryos have been used for more than a century to study many problems in developmental biology (**Horstadius, 1973; Piatigorsky *et al.*, 1975; Fernandez and Beiras, 2001;**

Corresponding author: Gaber Ahmed Saad
Department of Biology, College of Medicine, Dammam University, Saudi Arabia, KSA.

Brooks and Wessel, 2004; Bielmyer *et al.*, 2005; Ghorani *et al.*, 2012). Most species of echinoids are indirect developers: the embryo develops first into a swimming larva that feeds on plankton while its adult structures grow inside the larva (**Gilbert, 2003**).At metamorphosis, most larval structures are lost and the adult emerges, settles onto the substrate, and grows to sexual maturity. Superficially, the adult displays pent radial symmetry, while the larva typically has a bilaterian organization with left-right and dorsal-ventral (aboral-oral) axes (**Yajima and Kiyomoto, 2006, Yajimaand Wessel, 2011 a&b**).Echinoids use a developmental strategy where by some cells are set aside in embryogenesis as multipotent that will eventually contribute to major structures of the adult. These multipotent cells include the descendants of small micromeres, formed at the fifth cleavage, and contribute only to the coelomicsacs (**Pehrson and Cohen, 1986; Yajima, *et al.*, 2007, Yajimaand Wessel, 2012**), aprecursor of the adult rudiment in postembryonic development. The small micromeres do not contribute to any functional structures of the embryo. The micromeres are a major signaling center of the sea urchin embryo and their removal either at the 16-cell stage, or in blastulae, results in significant compensatory development and fate transitions within the embryo (**Ransick and Davidson, 1993, Yajima, *et al.*, 2013**). The micromeres are the precursor to both the large and small micromeres, and the large micromeres have a singular fate of making the larval skeletal system. Removal of the large micromeres just prior to gastrulation results in trans-fating, whereby other mesodermal cells change fate to become skeletogenic cells, a fate they would not normally display (**Ettensohn and McClay, 1988**).

The gonad is composed of five lobes located beneath the interambulacral plates of the test. Each gonad lobe contains hundreds of tubules known as acini that often give the gonad an appearance of a cluster of grapes. The parietal layer of the acinus is commonly form the germinal epithelium and is comprised of two primary cell types; germ cells (ova or sperm) and somatic cells that known as nutritive phagocytes. Nutritive phagocytes store nutrients that can be mobilized to developing germ cells or used to sustain physiological processes during periods of starvation (**Holland and Giese,1965;Unuma, 2002;Unuma*et al.*, 2003; Spirlet *et al.*, 2000; Walker *et al.*, 2001**). Nutritive phagocytes are also responsible for phagocytizing relict germ cells after spawning (**Holland and Giese, 1965**).

In the Echinoidea it is suggested that there may be a dynamic relationship between the germ cells and non germ cells , which are variously known as follicle cells, accessory cells and mutative phagocytes (**Holland and Giese, 1965; Chatlynne, 1969; Bal, 1970; Yajima *et al.*, 2013**). The annual changes that take in the accessory cells are poorly understood , and the function of the accessory cell is still under discussion (**Takashima and Tominaga, 1978**).

Thus during game to genesis, the gonad may exhibit a variety of changes in major cell populations (**Unuma, 2002; Walker *et al.*, 2007**) and biochemistry (**Chatlynne, 1969; Montero-Torreiro and Garcia--Martinez, 2003; Unuma *et al.*, 2003**). Since many echinoids exhibit an annual gonad cycle, seasonal changes in biotic and abiotic factors may play an important role in regulating the cycle. Food availability, photoperiod, and temperature can significantly influence the reproductive cycles of sea urchins (**Pearse *et al.*, 1986; Yamamoto *et al.*, 1988; Ito *et al.*, 1989; Sakairi *et al.*, 1989; Pearse and Cameron , 1991; Spirlet *et al.*, 2000; Garrido and Barber, 2001; Kelly, 2001; Shpigel *et al.*, 2004; Walker *et al.*, 2007**). **Pearse and Cameron (1991)** concluded that the effect of temperature on the game to genic cycle in echinoids is species specific. The experiments performed on sea urchin embryos using chemical agents and the classic blastomere recombination experiments performed by **Horstadius (1939; 1973**) paved the way for ideas about graded distributions of morphogenetic substances in the embryo. The sea urchin also provided useful material for studying many aspects of nucleic acid structure, complexity, and function in the early days of molecular biology (**reviewed by Davidson, 1989**). The reader interested in the historical role played by sea urchin embryos in the emergence of developmental biology, and the importance and relevance of such experiments today is referred to **Wilt (1987)**, **Davidson (19856,1989; 1990) and Livingston and Wilt (1990)**.More recently, the sea urchin embryo has been used as a convenient system for studying morphogenetic movements and cell interactions during gastrulation, the changes in gene expression associated with the establishment of tissue territories along the embryonic axes, and phylogenetic variability and associated modifications in early development (**Briggs and Wessel, 2006**). For methods of maintaining adults, obtaining gametes, and culturing embryos, (see **Hinegardner *et al.*, 1967; Hinegardner, 1975 and Leahy 1986**). General methods for culturing and experimentally manipulating embryos can be found in **Harvey (1956), Horstadius (1973) and Schroeder (1986).**

Sea urchin and sand dollar gametes can be obtained in large numbers by intracoelomic injection of 0.5M KCl or by electrical stimulation; this leads to the shedding of gametes into sea water (in the case of eggs) or 'dry' into a dish (in the case of sperm). Depending on the species, several millilitres of ripe eggs or sperm can be obtained from a single animal and the embryos can be conveniently reared in finger bowls or in stirring cultures. Mature sea urchin eggs, unlike eggs from many other animals, have completed meiosis and the extrusion of polar bodies in the ovary to produce a haploid gamete. Immediately opposed to the egg plasma membrane is the vitelline envelope which contains the glycoproteins essential for species-specific fusion of sperm and egg, while freshly shed eggs are surrounded by a jelly coat. Marking the jelly coat with small ink particles reveals the jelly canal, a marker for the animal pole of the egg first described by **Boveri (1901)** and more recently re-investigated, and the importance and relevance of such experiments today is referred to **Wilt (1987), Davidson (1989; 1990) and Livingston and Wilt (1990).**

MATERIALS AND METHODS

Animals

Adults of *Strongylocentrotus purpuratus* were collected during September to June (2012) and July to August (2014) along the northern estuarineharbor of the Arabian Gulf – Saudi Arabia,

transported alive in plastic aquaria containing well aerated sea water to the laboratory and placed in large glass aquaria containing well aerated sea water (changed every other day).Procurement of gametes has been carried out by potassium chloride injection (**Tyler 1949; Hinegardner *et al.*, 1967; Fuseler 1973**): A volume (0.1 to 1 .0 ml) of 0.5 M potassium chloride (isotonic to sea water) has been injected intra-coelomically using a one ml tuberculin syringe equipped with a 26 or 30 gauge needle. Gentle shaking of the animal after injection anticlockwise 90° would distribute the potassium chloride to all gonads. This injection has been given through the soft tissue on the oral surface of the animal. Gamete shedding would begin several minutes after the injection is given. An egg suspension has been prepared by placing several drops of washed eggs in a small petri dish containing app. 200 ml of sea water. A dilute sperm suspension has been prepared by placing one drop of concentrated semen in a test tube containing app. 10 ml of seawater, Mixed with a clean pipette to obtain a uniformly milky suspension. Adding two drops of the dilute sperm suspension to the egg suspension. Gently sperm and eggs were mixed with a clean large-bore pipette, wooden stick, or / and stirring rod. Eggs would be checked for fertilization app. two minutes after insemination. This can be done by placing a sample in a small dish or by making a wet mount on a slide. Successful fertilization was indicated by the presence of a fertilization envelope around each egg.The Fertilized eggs were washed and then grown at 20°C. The fertilized eggs and the different embryonic stages of development till the complete metamorphic pluteiwere observed. The time schedules for different stages were tabulated (Table 1).

Table 1. Stages of early embryonic development of *Strongylocentrotus purpuratus*. A total of 20 stages are divided into five periods. Parentheses in each period mean the start-time and end time of each period at 20°C

Stage	Time duration after fertilization	% of hatching
Fertilized egg (zygote)	0 min.	15%
Formation of fertilization membrane	3 min.	40%
Cleavage period (3.50-4.15hrs)		
2-cell	1hr10min.	25%
4-cell	1hr40 min.	20%
8-cell	2hr 50 min.	25%
16-cell	3hr 15 min.	20%
32-cell	3hr 55 min.	27%
64-cell (Morula stage)	4 hr 15 min.	29%
Blastula Period (5.50-6.20 hr)		
128-cell (Early blastula)	6 hr 20 min.	19%
Mid blastula (cilia formation)	8 hr 40 min	20%
Late blastula (hatching stage)	11 hr 10 min.	30%
Gastrula Period (26.50-29.40 hr)		
App. 150-cell, initial gastrula	13hr 50 min.	29%
early gastrula (ingression of primary mesenchyme cells)	14hr 10 min.	35%
mid gastrula (primary invagination)	16hr 45min.	30%
late gastrula (secondary invagination)	19hr 15min.	36%
Prism stage	21 hr 20 min.	50%
Pluteus larva with 2 arms	24hr 30 min.	51%
Pluteus larva with 4 arms	29hr 40 min.	49%
metamorphosed pluteus larva (145 – 146 hr)		
advanced pluteus larva with 8 arms	145 hr 50 min	55%

Macroscopic observation

Embryonic stages were then prepared for phase contrast photomacroscopy. Samples were stained with Nile blue and were placed on glass slides with embedding mixture of PBS / glycerol / DABCO. Immediate viewing and photographing were performed under an Axiomicroscope (ZEISS-Axiophot). The description of almost all stages of development till metamorphosis was carried out on live stages under Axiomicroscope since all embryonic stages are about 0.5 - 0.9µm long and transparent. Nile blue has been applied trying to clarify the transformation of the subsequent stages as well as to expose the morphological appearance of each stage. The photos did not clarify all described structures since most descriptions were carried out on live stages.

Immunocytochemical techniques

In this study a considerable number of blastulae (hatching stage) (??hr??min) were collected alive under a stereomicroscope from the culture and transferred to wells of 24-well plastic tissue culture dishes. These selected blastulae were subjected to certain bioactive inducers that enhanced metamorphosis in hydrozoans, echinoderms and ascidian species. In each well about 25blastulae were placed with sea water. The first well was left as a control, antibiotics were added in the second well and in the third well heavy metals were added to the sea water while in the other wells the sea water was sucked and replaced with NH_4Cl (2.5 mM& 300 µM), C8 (1,2-dioctanyl-rac-glycerol, (10 µ M, 1 µ M &0.1 µ M), NOS (1 mM, 1.5 mM& 2.5 mM), Serotonin (10 µg/ml) and Acetyl choline (10 mM, 1 mM and 0.5 mM) respectively. Serotonin, NOS and Acetyl choline were obtained from Sigma Chemical Corp. These blastulae were tested every 24 hrs till 114 hrs (6 days) and commented on (Table 1). The experiments were carried out in triplicate. In all wells, food suspension (Mikrozell®) has been added in equal amount. The purpose of this experiment was to investigate metamorphosis under the influence of externally applied chemicals which had been claimed to:

1. Induce metamorphosis in an ascidians (**Ammonia, Berking and Hermann, 1990**),
2. involved in signaling systems (C8, 1,2-dioctanyl-rac-glycerol, an activator of protein kinase C) (**Bishop, *et al.*, 2008**),
3. Serve in neuronal control (neurotransmitters acetylcholine and serotonine) (**Bishop *et al.*, 2008**),
4. Control morphogenetic and behavioral reactions as in the hydroid *Hydractiniaechinata* (**the neuropeptides LW-amide and RF-amide, Plickert and Schneider, 2004**). These substances were screened for putative inductive or inhibitory effects on metamorphosis of *Ascidellaaspersa* larvae **Kriegel (1996).**
5. Nitric oxide synthase (NOS) activity depends on the molecular chaperone heat shock protein 90 (HSP90) which interacts with members of several signal transduction proteins pathways under normal physiological conditions (**Bishop, *et al.*, 2008**).

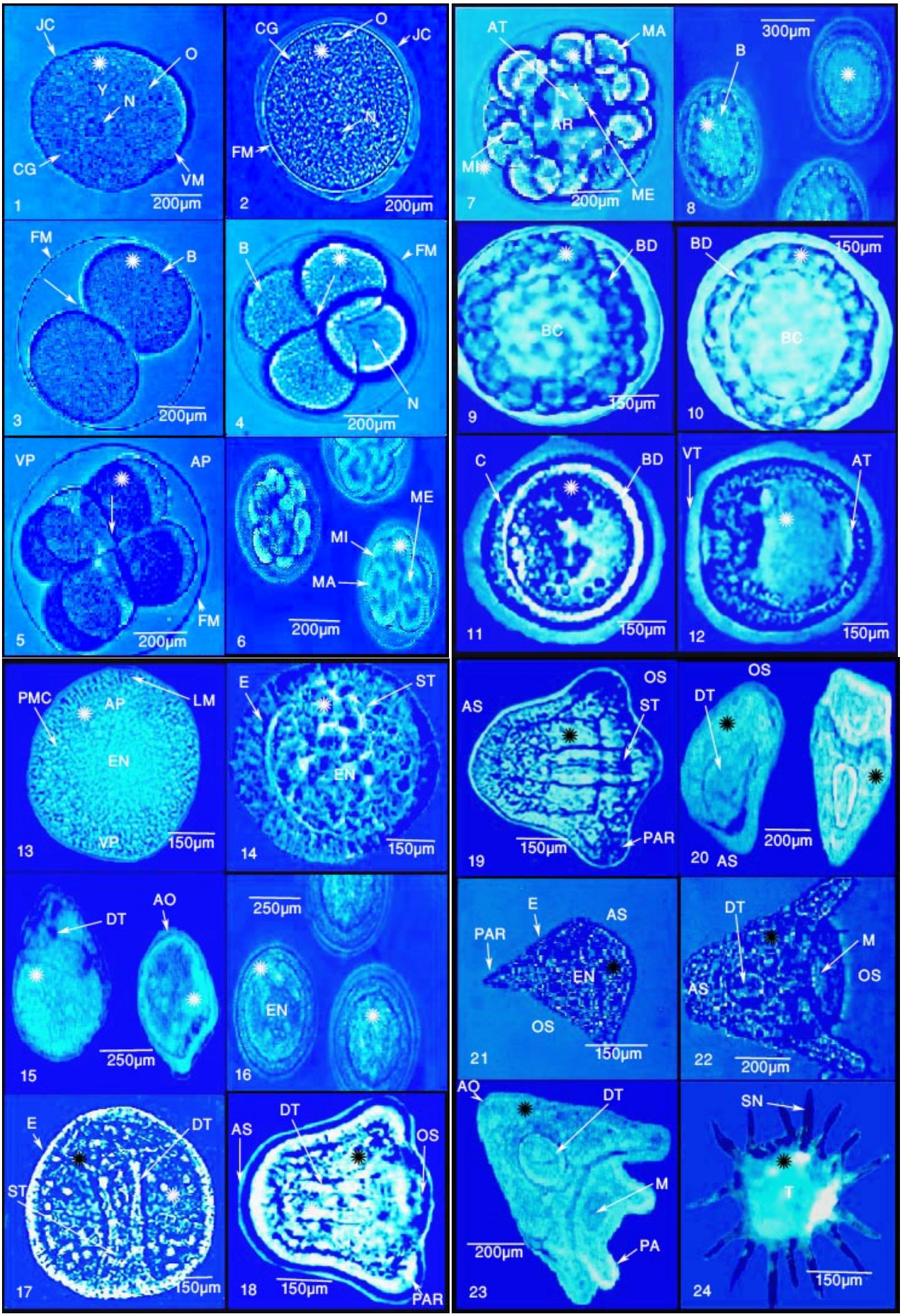
ABS = antibiotic sea water;
C8 = 1,2-dioctanyl-rac-glycerol
PPTA (stock)= PPTA is (5 ml Triton X-100, 1 g sodium azide added to 900 ml PBS
PPTA (working solution): add 10 ml horse serum to 100 ml PPTA stock solution
PBS = Phosphate buffered saline
FITC =Fluorescein-isothiocyanat (Fluorochrome)
DABCO = Diazabicyclo-(2,2,2) Octane

Statistical analysis

Analysis of variance (ANOVA) is a broad group of techniques for identifying and measuring different sources of variation within the data set. It consists of a set of procedures by which a variance of the random variable is broken down by certain sources of variation of its value. With the components of variance, depending on the sources, one can conclude if there is a significant difference between the values of dependent variable for different levels of the observed factor variables. In the present study, a one-way analysis of variance is used to compare the different developmental stages of the sea urchin from blastula stage to newly metamorphosed echinoid. If the above-mentioned assumptions for ANOVA are not met, the Newman-Keuls Multiple Comparison Test is used for determining whether four or more independent samples originate give a clear cut differences. When this test leads to significant results, at least one sample differs from the others. A principal component analysis is a standard tool in modern data analysis. It is a simple, nonparametric method for extracting relevant information out of confusing data sets. Principal component analysis is concerned with the interpretation of the variance and covariance structure of the original set of variables through a small number of their linear combinations. The general objectives of principal component analysis are data reduction and interpretation. In order to reduce the number of variables.

RESULTS

The sea urchin *Strongylocentrotus purpuratus* (2.5-10 cm in ϕ) hasa purple color (juveniles may be light green). It may be found intertidally or sub tidally. Ambulacral pore pairs arranged in curving arcs of eight on upper side of test. Identification of the sea urchin under investigation has been carried out according (**Ziegler *et al.*, 2014**). External dimorphism is absent and sexes can be determined only after gamete shedding has begun or following biopsy of the gonads. Macroscopically, the gonad is lobulated and is located beneath the interambulacral plates. Each gonadal lobule consists of manyacini. Germ cells and somatic cells constitute the exterior lining of the acinus. In other expressions the germinal epithelium can be differentiated into two primary cell types; germ cells and somatic cells. The histological pictures of the ovary showed that from December to March, the main bulk of the ovary is filled with accessory cells containing numerous globules (15 -20 µm in ϕ). Small oocytes about 10µm in ϕ are observed along the ovarian wall. From March to May the growing oocytes enlarged rapidly and many yolk granules of various size were observed in the ooplasm. During May and June, the full-sized oocytes undergo reduction divisions and move toward the centre of the ovary to be stored as mature ova until they are spawned. As the reproductive season begins, the ovary is almost completely filled with ova and very few accessory cells. After the end of the spawning season, the space occupied by accessory cells increased and the ovary contained unshed ova which are in the process of autolysis. During metamorphosis dramatic developmental changes occur, transforming the larval form into an adult sea urchin. Sea urchin sperm are very small and extremely active, consisting of a head and a tail. The larger ova were yellow to orange and measured 70 to 180 µm in ϕ with eccentrically located nucleus (Fig. 1).Polar bodies were not observed. Many cortical granules of different sizes were observed underneath the plasma membrane. The ovum was enclosed in a vitelline membraneand a thick jelly coat outside it. Few drops of sperm suspension were added to the ova culture in the laboratory and both gamete fusion and nuclei fusion with penetration and copulation paths were observed. After fertilization the metabolically quiescent egg was stimulated to begin its embryogenesis. Following the fusion of the gametes, the vitelline membrane was transformed into a thick fertilization membrane (3 min. after fertilization), and the cortical granules distributed randomly in the ooplasm (Fig. 2). This caused a folding of the ovum surface and the fertilization membrane to be lifted away from the plasma membrane. It was observed that the sperm nucleus united with the ovum nucleus by the time the fertilization membrane has been completely elevated from the plasma membrane. Zygote has undergone radial holoblastic cleavage (3.50-4.15 hrs after fertilization) (see Table 1). The first two cleavages are meridional and perpendicular to one another, passing through the animal and vegetal poles to produce first two and then four blastomeres of equal size (1 hr 40 min. after fertilization) (Figs. 3-4). The third cleavage is equatorial, separating the cleaved zygote into an animal and vegetal quartet (2 hr 50 min. after fertilization) (Fig. 5).The fourth cleavage (3hr 15 min. after fertilization) was very different from the first three ones. The four blastomeres of the animal tier divide meridionally into eight blastomeres, each with the same size. These blastomeres were called mesomeres. The vegetal tier underwent unequal equatorial cleavage to produce four large macromeres, and four smaller micromeres (Fig. 6). At the fifth cleavage (3hr 55 min. After fertilization) (Fig. 7). The mesomeres divide equatorially and the producing micromeres lie at the vegetal tier produced two animal tiers. At the sixth cleavage (4 hr 15 min after fertilization), all blastomeresdivided equatorially to produce a 64-cell stage with five tiers (Fig. 8). At the seventh cleavage (6 hr 20 min. after fertilization), all cells divide meridionally toproduce a 128-cell blastula. During the blastula stage, cells no longer cleave synchronously: as development proceeds(11 hr 10 min. after fertilization) (Fig. 9). Divisions of local groups of blastomeres remain synchronous. The early blastula has a blasoderm of epithelial cells enclosed a central blastocoel. The blasttoderm eventually became impermeable by the mid-blastula stage (8 hr 40 min. after fertilization). At the mid-blastula stage, cilia were developed on the exterior of the blastoderm and this stage began to rotate within the fertilization membrane, and later membrane autolyzed gradually allowed this embryonic stage to hatch and become a free-swimming blastula (8 hr 40 min. after fertilization) (Figs. 10-11). After blastulation and hatching, there was an observable decrease in the rate of blastomere division (13hr 50 min.hrsafter fertilization). At the animal pole an apical platewas formed in the form of a thickened region of epithelium with a tuft of long cilia while the epithelium at the vegetal pole flattened and thickened to form the vegetal plate (16hr 45min.after fertilization) (Fig. 12). Ingression of primary mesenchyme cells into the blastocoel took place and a new cavity was formed referred to enteron. Blasomeres at the tip of the enter on protruded, extending elongations in to the blastocoel. Eventually the enter on elongated across the blastocoel, and its tip attached to the ventral ectoderm near the animal pole


JC
O
Y
N
CG
VM
200µm
1
CG
O
JC
N
FM
200µm
2
AT
MA
AR
MI
ME
200µm
7
300µm
B
8
FM
B
200µm
3
B
FM
N
200µm
4
BD
BC
150µm
9
BD
150µm
BC
10
VP
AP
FM
200µm
5
ME
MI
MA
200µm
6
C
BD
150µm
11
VT
AT
150µm
12
PMC
AP
LM
EN
VP
150µm
13
E
ST
EN
150µm
14
OS
AS
ST
PAR
150µm
19
OS
DT
200µm
20 AS
DT
AO
250µm
15
250µm
EN
16
E
AS
PAR
EN
OS
150µm
21
DT
M
AS
OS
200µm
22
E
DT
ST
150µm
17
DT
AS
OS
150µm
18
PAR
AQ
DT
M
200µm
PA
23
SN
T
150µm
24

LEGENDS

Fig. 1. Phase contrast photomicrograph of a whole mount of a secondary oocyte of the sea urchin *Strongylocentrotus purpuratus*. Note, it had yellow to orange jelly-coat and measured 70 to 180 μm in ϕ with eccentrically located nucleus. Polar bodies were not observed. Many cortical granules of different sizes were observed underneath the vitelline membrane. **Fig. 2.** Phase contrast photomicrograph of a whole mount of a fertilized ovum of the sea urchin *Strongylocentrotus purpuratus*. Note, a fertilization membrane was formed and the cortical granules were randomly distributed in the ooplasm. **Fig. 3.** Phase contrast photomicrograph of the onset of cleavage division in the sea urchin *Strongylocentrotus purpuratus*. Zygote has undergone radial holoblastic cleavage. The first cleavage furrow (indicated by an arrow) was meridional passing through the animal and vegetal poles to produce first two blastomeres of equal size. **Fig. 4.** Phase contrast photomicrograph of the second cleavage division in the sea urchin *Strongylocentrotus purpuratus*. The second cleavage furrow (indicated by an arrow)was meridional and perpendicular to the first one, passing through the animal and vegetal poles to produce four blastomeres of equal size. **Fig. 5.** Phase contrast photomicrograph of the third cleavage division in the sea urchin *Strongylocentrotus purpuratus*. The third cleavage (indicated by an arrow)is equatorial, separating the cleaved zygote into an animal and vegetal quartet **Fig. 6.** Phase contrast photomicrograph of the fourth cleavage division in the sea urchin *Strongylocentrotus purpuratus*. Note, the four blastomeres of the animal tier divide meridionally into eight blastomeres, each with the same size. These blastomeres were called mesomeres. The vegetal tier underwent unequal equatorial cleavage to produce four large macromeres, and four smaller micromeres. **Fig. 7.** Phase contrast photomicrograph of the fifth cleavage division in the sea urchin *Strongylocentrotus purpuratus*. Note, at the fifth cleavage the mesomeres divide equatorially and the producing micromeres lie at the vegetal tier produced two animal tiers. **Fig. 8.** Phase contrast photomicrograph of the sixth cleavage division in the sea urchin *Strongylocentrotus purpuratus*. Note, at the sixth cleavage, all blastomeres divided equatorially to produce a 64-cell stage with five tiers. **Fig. 9.** Phase contrast photomicrograph of the seventh cleavage division in the sea urchin *Strongylocentrotus purpuratus*. Note, at the seventh cleavage all cells divide meridionally to produce a 128-cell blastula. During the blastula stage, cells no longer cleave synchronously. **Figs. 10-11.** Phase contrast photomicrographs of the blastula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, Divisions of local groups of blastomeres remain synchronous. The blastoderm became thick. Cilia were developed on the exterior of the blastoderm. **Fig. 12.** Phase contrast photomicrograph of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, there was an observable decrease in the rate of blastomere division. At the animal pole an apical plate was formed in the form of a thickened region of epithelium with a tuft of long cilia while the epithelium at the vegetal pole flattened and thickened to form the vegetal plate. Cilia were overlooked because of ligh. **Fig. 13.** Phase contrast photomicrograph of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, ingression of primary mesenchyme cells into the blastocoel took place and a new cavity was formed referred to enteron. Blasomeres at the tip of the enteron protruded, extending elongations into the blastocoel. Eventually the enteron elongated across the blastocoel, and its tip attached to the ventral ectoderm near the animal pole. Ultimately the tip of the enteron fused with the ectoderm to form the larval mouth. **Fig. 14.** Phase contrast photomicrograph of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, at the end of gastrulation the enteron tip bended and began to shift position toward one side of the embryo. At the point of contact between the enteron tip and the overlying ectoderm, the ectoderm invaginated to form the future stomodaeum. At the stomodaeum the larval mouth and the anterior opening of the digestive tract developed. **Fig. 15.** Phase contrast photomicrograph of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, the blastopore developed into the anal opening of the digestive tract. This tract later differentiated into an eosophagus, stomach, and intestine. During the prism stage the embryo took the shape of a rounded pyramid. **Figs. 16-18.** Phase contrast photomicrographs of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, The side of the embryo containing the stomodaeum becomes flattened forming the oral surface of the developing larva. The blastoporal side of the embryo also becomes flattened and forms the anal surface of the developing larva. A band of cilia developed around the stomodaeum. As this occured the apical tuft disappeared and the embryo elongates slightly along the dorso-ventral axis. **Figs. 19-22.** Phase contrast photomicrographs of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, As development proceed two arms developed, the preoral arms. **Fig. 23.** Phase contrast photomicrographs of the gastrula stage of the sea urchin *Strongylocentrotus purpuratus*. Note, two additional arms, the postoral arms, appeared and extend out from the junction of the oral and anal surfaces. **Fig. 24.** Phase contrast photomicrographs of a newly juvenile stage of the sea urchin *Strongylocentrotus purpuratus*. Note, a period of extensive feeding and continued larval development is required before metamorphosis to a juvenile sea urchin occur.

Ultimately the tip of the enteron fused with the ectoderm to form the larval mouth(19hr 15min. after fertilization)(Fig. 13). The eversion of the late gastrula is a complex event. At the end of gastrulation the enteron tip bended and began to shift position toward one side of the embryo. At the point of contact between the enteron tip and the overlying ectoderm, the ectoderm invaginated to form the future stomodaeum. At the stomodaeum the larval mouth and the anterior opening of the digestive tract developed (19hr 15min.after fertilization) (Fig. 14). The blastopore developed into the anal opening of the digestive tract. This tract later differentiated into an eosophagus, stomach, and intestine. During the prism stage the embryo took the shape of a rounded pyramid (21 hr 20 min. after fertilization) (Fig. 15). The side of the embryo containing the stomodaeum becomes flattened forming the oral surface of the developing larva. The blastoporal side of the embryo also becomes flattened and forms the anal surface of the developing larva. A band of cilia developed around the stomodaeum.

As this occurred the apical tuft disappeared and the embryo elongates slightly along the dorso-ventral axis (Figs. 16-18). As development proceed two arms developed, the preoral arms (24hr 30 min. after fertilization) (Figs. 19-22).Two additional arms, the postoral arms, appeared and extend out from the junction of the oral and anal surfaces(29hr 40 min. after fertilization) (Fig. 23). When completely formed the postoral arms are longer than the preoral arms. Because of the changes in the shape of the larva, the developing digestive tract is bent into a J-shape. The stomach enlarged and filled a large part of the body of the pluteus. A pluteus larva at this stage of development is referred to as the fourarmed pluteus larva. As the four-armed pluteus fed it developedinto the eight-armed pluteus larva (145 hr 50 min. after fertilization). A period of extensive feeding and continued larval development is required before metamorphosis to a juvenile sea urchin occur (Fig. 24).

Table 2. Effect of bioactive inducers on metamorphosis of free swimming blastula stage till advanced pluteus larva with 8 arms

Bioactive chemical control	Its conc.	No. tested blastulae	Obtained stage	after 24 hr			after 48 hr			after 146 hr		
				1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail
Sea water	--	26* 25** 23***	B	5	5	4	2	3	2	0	0	0
			G	7	3	4	4	2	3	0	0	0
			P	3	2	1	3	3	2	1	0	1
			PS1	0	0	0	3	1	1	1	3	1
			PS2	0	0	0	1	1	1	1	2	4
			A	0	0	0	0	0	0	4	2	2
			D	11	15	14	2	2	7	8	3	1
Antibiotic sea water	--	21* 27** 26***	B	10	10	11	5	3	6	0	0	0
			G	2	6	3	4	5	7	0	0	0
			P	3	3	4	2	7	7	1	2	1
			PS1	0	0	0	2	1	2	3	5	4
			PS2	0	0	0	1	2	1	4	7	6
			A	0	0	0	0	0	0	4	5	5
			D	6	8	8	1	1	2	3	0	3
cGMP	1 µ M	23* 28** 25***	B	4	4	6	1	1	1	0	0	0
			G	3	3	3	1	1	1	0	0	0
			P	13	15	11	4	2	1	0	0	0
			PS1	0	0	0	6	4	9	1	0	0
			PS2	0	0	0	12	21	13	0	1	0
			A	0	0	0	0	0	0	19	21	19
			D	3	6	5	1	0	1	0	0	1
NH4Cl	**2.5 mM**	26* 25** 24***	B	6	4	2	1	1	1	0	0	0
			G	2	1	2	1	1	1	0	0	0
			P	11	15	14	2	2	1	0	0	0
			PS1	0	0	0	5	6	2	0	0	0
			PS2	0	0	0	11	10	12	0	0	1
			Y	0	0	0	0	0	0	19	19	17
			D	7	5	6	1	0	0	0	0	0
	300 µM	29* 22** 25***	B	6	6	6	3	3	2	0	0	0
			G	3	2	1	3	3	3	0	0	0
			P	1	1	1	3	1	1	1	0	0
			PS1	0	0	0	1	0	1	2	3	2
			PS2	0	0	0	0	0	0	2	3	3
			A	0	0	0	0	0	0	5	3	3
			D	19	13	17	0	0	1	0	0	0
C8	10 µ M	27* 26** 30***	B	6	5	6	3	2	3	0	0	0
			G	3	2	1	2	2	2	0	0	0
			P	1	2	2	1	1	1	2	1	1
			PS1	0	0	0	2	1	1	2	2	2
			PS2	0	0	0	0	0	1	3	2	4
			A	0	0	0	0	0	0	2	3	1
			D	17	17	21	2	3	1	1	1	
	1 µ M	24* 27** 22***	B	4	3	2	1	1	2	0	0	0
			G	3	3	4	1	1	1	0	0	0
			P	13	15	11	4	1	1	0	0	0
			PS1	0	0	0	3	2	3	0	0	0
			PS2	0	0	0	11	17	10	0	0	0
			A	0	0	0	0	0	0	20	20	17
			D	4	6	5	0	0	0	0	1	0
	0.1 µ M	26* 25** 27***	B	5	6	14	2	2	9	0	0	0
			G	3	2	4	3	2	3	0	0	0
			P	1	2	1	3	5	6	2	3	4
			PS1	0	0	0	1	1	1	1	4	5
			PS2	0	0	0	0	0	0	2	5	6
			A	0	0	0	0	0	0	3	4	3
			D	17	15	8	0	0	0	1	0	1
NOS	1 mM	28* 23** 21***	B	5	4	3	1	1	1	0	0	0
			G	2	3	4	2	2	1	0	0	0
			P	15	13	12	4	3	1	0	0	0
			PS1	0	0	0	4	2	3	0	0	0
			PS2	0	0	0	11	11	13	22	19	18
			Y	0	0	0	0	0	0	22	19	18
			D	6	3	2	0	0	0	0	1	1
	1.5 mM	25* 24** 28***	B	7	7	6	3	2	2	0	0	0
			G	3	2	3	2	3	2	0	0	0
			P	1	1	2	2	2	3	0	0	0
			PS1	0	0	0	1	1	2	3	2	3
			PS2	0	0	0	1	1	1	3	4	5
			A	0	0	0	0	0	0	4	3	3
			D	14	14	17	2	1	1	1	0	0
	2.5 mM	26* 28** 24***	B	3	6	5	0	0	1	0	0	0
			G	2	3	2	1	1	1	0	0	0
			P	15	12	11	1	1	1	0	0	0
			PS1	0	0	0	6	1	2	0	0	0
			PS2	0	0	0	12	17	13	0	0	0
			A	0	0	0	0	0	0	20	21	17
			D	6	7	6	0	1	0	0	0	1

Bioactive chemical control	Its conc.	No. tested blastulae	Obtained stage	after 24 hr			after 48 hr			after 146 hr		
				1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail
Seratonin	10 µg/ml	27* 22** 26***	B	5	6	5	0	0	0	0	0	0
			G	2	1	2	1	1	1	0	0	0
			P	15	11	14	1	2	1	0	0	0
			PS1	0	0	0	4	3	2	0	0	0
			PS2	0	0	0	16	12	17	0	0	0
			A	0	0	0	0	0	0	22	16	20
			D	5	4	5	0	0	0	0	2	1
Acetyl choline	1 mM	23* 27** 28***	B	9	10	7	4	4	3	0	0	0
			G	3	3	3	3	4	3	0	0	0
			P	1	2	1	3	5	2	2	3	1
			PS1	0	0	0	2	1	2	3	4	3
			PS2	0	0	0	0	0	2	4	6	2
			A	0	0	0	0	0	0	5	3	4
			D	10	12	17	1	1	0	0	0	1
	0.5 mM	30* 27** 26***	B	7	5	3	1	1	1	0	0	0
			G	4	2	1	1	2	1	0	0	0
			P	16	15	17	1	2	1	0	0	0
			PS1	0	0	0	6	4	1	0	0	0
			PS2	0	0	0	18	13	17	0	0	0
			A	0	0	0	0	0	0	27	21	19
			D	3	5	5	0	0	0	0	0	2
	30 µ g/ml	25* 24** 28***	B	7	7	7	3	3	2	0	0	0
			G	2	3	1	3	4	3	0	0	0
			P	1	1	1	1	1	1	2	1	0
			PS1	0	0	0	1	1	1	2	2	2
			PS2	0	0	0	0	0	0	3	4	3
			A	0	0	0	0	0	0	4	4	3
			D	15	13	19	2	1	2	1	0	1

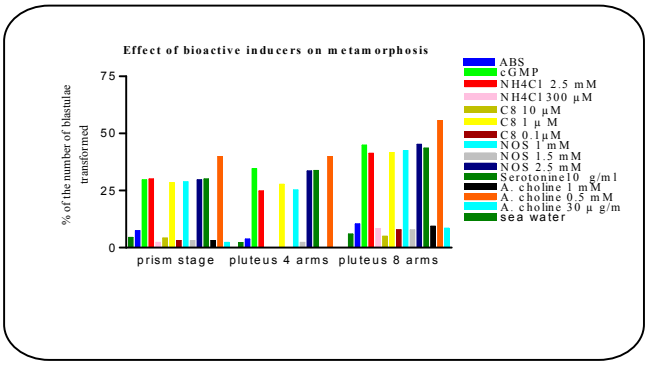
* = No. of tested blastulae in the first trail B = blastula P = Prism stage PS1 = Pluteus larva with 2 arms ** = No. of tested blastulae in the second trial G = gastrulaA = Advanced pluteus PS2 = Pluteus larva with 4 arms *** = No. of tested blastulae in the third trial d = died stage

Table 3. Percentage of prism stage, pluteus larva with 4 arms and advanced stage of pluteus larva with 8 arms of the total number of blastulae transformed (sum of the three replicates) subjected tocontrol or treatment

Bioactive chemical control (or)	Its conc	Number of blastulae	After 24 hr	After 48 hr	After 146 hr
			% of prism stage	% of pluteus larva with 4 arms	% of advanced stage of pluteus larva with 8 arms
sea water	--	74	4.44 %	2.22 %	5.92 %
ABS	--	74	7.4 %	3.7 %	10.36 %
cGMP	1 µ M	76	29.64 %	34.5 %	44.84 %
NH4Cl	2.5 mM	75	30 %	24.75 %	41.25 %
NH4Cl	300 µM	76	2.28 %	0 %	8.36 %
C8	10 µM	83	4.15 %	0 %	4.98 %
C8	1 µ M	73	28.47 %	27.74 %	41.61 %
C8	0.1µM	78	3.12 %	0 %	7.8 %
NOS	1 mM	72	28.8	25.2 %	42.48 %
NOS	1.5 mM	77	3.08 %	2.31 %	7.7 %
NOS	2.5 mM	78	29.64 %	33.54 %	45.24 %
Serotonine	10 µg/ml	75	30 %	33.75 %	43.5 %
Acetyl choline	1 mM	78	3.12 %	0 %	9.36 %
Acetyl choline	0.5 mM	83	39.84 %	39.84 %	55.61%
Acetyl choline	30 µ g/ml	77	2.31 %	0 %	8.47 %

Table 4. One-way analysis of variance and Dunnett's Multiple Comparison Test to analyze the total number of blastulae succeeded to undergo metamorphosis under control and bioactive conditions.

Table Analyzed				
Data Table-total number of blastulae transformed				
One-way analysis of variance				
P value	P<0.0001			
P value summary	***			
Are means signif. different? (P < 0.05)	Yes			
Number of groups	15			
F	21,34			
R squared	0,9087			
ANOVA Table	SS	df	MS	
Treatment (between columns)	11530	14	823,6	
Residual (within columns)	1158	30	38,6	
Total	12690	44		
Dunnett's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
sea water vs ABS	-2,96	0,5835	P > 0.05	-18.16 to 12.24
sea water vs cGMP	-32,13	6,335	P < 0.01	-47.33 to -16.93
sea water vs NH4Cl 2.5 mM	-27,81	5,482	P < 0.01	-43.01 to -12.61
sea water vs NH4Cl 300 µM	0,6467	0,1275	P > 0.05	-14.55 to 15.85
sea water vs C8 10 µM	1,15	0,2267	P > 0.05	-14.05 to 16.35
sea water vs C8 1 µ M	-28,41	5,601	P < 0.01	-43.61 to -13.21
sea water vs C8 0.1µM	0,5533	0,1091	P > 0.05	-14.65 to 15.75
sea water vs NOS 1 mM	-27,97	5,513	P < 0.01	-43.17 to -12.77
sea water vs NOS 1.5 mM	-0,17	0,03351	P > 0.05	-15.37 to 15.03
sea water vs NOS 2.5 mM	-31,95	6,298	P < 0.01	-47.15 to -16.75
sea water vs Serotonine10 g/ml	-31,56	6,221	P < 0.01	-46.76 to -16.36
sea water vs A. choline 1 mM	0,03333	0,006571	P > 0.05	-15.17 to 15.23
sea water vs A. choline 0.5 mM	-40,9	8,064	P < 0.01	-56.10 to -25.70
sea water vs A. choline 30 µ g/m	0,6	0,1183	P > 0.05	-14.60 to 15.80



Histogram 1. Showing the % of prism stage, pluteus larva with 4 arms and advanced pluteus larva with 8 arms after 24, 48 and 146 hours treatment respectively

Immuno-cytochemical results

After 24, 48 and 146 hours treatment with all bioactive inducers, it was shown that Acetyl choline 0.5 mM, Serotonine10 µg/ml, NOS 2.5 mM, C81 µ M, NH4ClNH4Cl and cGMP accelerated the transformation of blastula stage to a prism stage, pluteus larval stage with 4 arms and an advanced pluteus larval stage with 8 armsrespectively. Acetyl choline 30 µ g/ml & 1 mM, NOS 1.5 mM, C8 10 µM and NH4Cl 300 µM exereted no role on metamorphosis and antibiotic sea water had negligible effect (see Tables 2- 4 and Histogram 1 for review). The advanced pluteus larval stage with 8 arms took about 5 weeks in the culture to transform to a newly metamorphosed echinoid. It was very difficult to test the effect of the bioactive inducers on this transformation as many pluteus larvae died in the tissue culture plate after 7-10 days.

DISCUSSION

Echinoids have external fertilization anddevelopment allowing for continuous monitoring in sea water. Adults are very fertile, anda single female can produce over 50 million eggs in a single spawning (**Young, 2002; Revilla-i-Domingo and Davidson, 2003; Oliveri *et al.*, 2003; Oliveri and Davidson, 2004; Levine and Davidson, 2005**). More modestnumbers of gametes are available year-round from several readily available speciesthroughout the world. Being able to *in vitro* fertilize the oocytes allows for thesynchronous culture of many embryossimultaneously, making the collection ofembryological material simple (**Leahy, 1986; Wessel, 1985, 2013**). The eggs of echinoderms have yolk evenly distributed throughout the egg. Echinoids, such as *P. miliaris*, have an annual reproductive cycle (**Hyman, 1995**) and the spawning season is in the early summer months. Natural spawning occurs over a period of one to several months and gametes may be shed several times during a season (**Srathmann, 1987**). However, most of the eggs are released at the first spawning (own observation). The reproductive systemconsists of five gonads, suspended from the aboral side of the test via mesenterial strand (**Hyman, 1995**). Echinoid embryos are considered a living laboratory for studying development and morphogenesis. Fertilization is the first of a series of events in the several changes within development of echinoderm larvae. During fertilization an increase in the intracellular pH by 0.3 units, from 7.3to7.6 (**Johnson *et al.*, 1976; Payan *et al.*, 1983**) and free calcium ions gives rise to the elevation of the fertilization membrane. This study showed that there was a series of cells divisions, in which the three first divisions produces eight equal blastomeres, while the fourth cell division produces sixteen cells of different sizes. Eight cells called mesomeresat the animal pole, four macromeres and four micromeres at the vegetal pole. It should be highlighted that most of the lineage founder cells and many tissues are established at the 64- cells stages, well before the appearance of any morphological difference of the later embryo (**same result in Matranga, 2005**). However, Cleavages and development of embryo and larva of the investigated sea urchin *Strongylocentrotus purpuratus* were similar to those reported in other echinoidswith planktotrophic larvae (**Emlet, 1986; Vellutini and Migotto, 2010**). The developmental timing of hatching blastulae took longer period (08.45 h at 24°C)than those in *Lytechinusvariegatus* (6 h at 23°C) (**Strathmann, 1987**) andin *Clypeastersubdepressus* (7 h at 26°C) (Vellutini and Migotto, 2010). Developmental timing of later stages followed the same trends but slightly differed from those of Caribbean species of *L. variegates* at23°C (**Strathmann, 1987**) and the Pacific species of *Colobocentrotusmertensii* at 27°C (**Thet *et al.*, 2004**).

As cell division continue, the embryo develops and becomes a free-swimming blastula. Form this stage, a new rearrangement of the embryo begins. The primary mesenchyme cells migrate towards two ventro- lateral sites and the archenteron is formed, which is the future intestine. Gastrulaionrobusts with the correlation between thetypes of gastrulation and the pattern of migration of red-pigmented cells in *S. sphaeroides*. Red-pigmented cells originate on the vegetal pole and migrate through the ectodermto the apical plate while the archenteron elongationis continuous. Similar phenomena were observed during the onset of gastrulation in tropical sea urchin *Echinometramathaei* (**Takata and Kominami, 2004**) and the sea biscuit *Clypeastersubdepressus* (**Vellutini and Migotto, 2010**). Red-pigmented cells can have regulatory role and are knownto trigger gastrulation in *E. mathaei* (**Takata and Kominami, 2004**). These cells might participate on the morphological changes occurring during the formation of prism and early axis specification of pluteuslarvae (**Takata and Kominami, 2004, Thet *et al.*, 2004**). This study showed that the late gastrula transformed into the prim stage of pluteus after 24hrs 30 min, post-fertilization. Subsequently the larval mouth opens and a full intestine develops along a complex skeleton . Larval arms began to form on both sides and grow longer. The post- oral pair of the arms is the first to project anteriorly and is the best indicator of divergence of larval form (**Strathmann *et al.*, 1992**). The second pair of arms to develop is the anterolateral arms. The posterodorsal arms are the third pair arising, and lastly the fourth pair of arms the preoral arms presented in all echinoplutei larvae (**Emlet, 2002**). Once the larvae has opened the mouth and the digestive system has been completed the larvae must consume food to acquire energy and continue development to produce a juvenile sea urchin through metamorphosis. Additionally, the triradiate spicules, the firstsign of larval skeleton, were formed during gastrulation in *S. sphaeroides*, which were more or less similar to those observed in other regular echinoids (**Thet *et al.*, 2004, Vellutini and Migotto,**

2010).Competent larvae of *S. sphaeroides* demonstrated substrate-test behavior similar to those documented in otherechinoid species (**Caldwell, 1972, Nunes and Jangoux, 2007,Vellutini and Migotto, 2010**). Although early postlarvaljuveniles resemble regular urchins with a spherical body, bilateral symmetry could be identified soon after theirsorption of larval tissues and was probably determined during rudiment formation as those observed recently in sea biscuits (**Vellutini and Migotto, 2010**). In newly metamorphosed juvenile of *S. sphaeroides* (Figure 2(i)), larval arms were completely absorbed together with the skeletons and epidermis. On the contrary, in Eucidaristhouarsi (**Emlet, 1988) and *Paracentrotuslividus* (Gosselin and Jangoux, 1998**), tissue resorption is achieved by the retraction of only epidermis resulting in the naked skeleton. The naked skeletal rods will eventually be broken down. Such type of discrepancy may be related to the species differences (**Thet *et al.*, 2004**). Following the induction of complete metamorphosis, *S.sphaeroides* juveniles had 4 primary spines per interambulacrum (20 totals), similar to those documented in *P. lividus* (**Gosselin and Jangoux, 1998)** and *Strongylocentrotus purpuratus* (**Miller and Emlet, 1999**). The irregular echinoid *Echinocardiumcordatum* has a greater number of primary spines per interqambulacrum after metamorphosis and also differs from *S. sphaeroides* by the presence of secondary spines and a subanalfacsciole and 4 primaryspines (**Nunes and Jangoux, 2007**). Similar to the events in *S. fanciscanus* and *S. Purpuratus* (**Miller and Emlet, 1999)**, *P. lividus* (**Gosselin and Jangoux, 1998**), and *E. cordatum* (**Nunes and Jangoux, 2007**), the newly metamorphosed juveniles of S. spheroidshad one tube foot per ambulacra. In contrast, *C. subdepressus*uniquely displayed three podia after metamorphosis (**Vellutini and Migotto, 2010**). Competent larvae of *S. sphaeroides* have pedicellariaeduring the late larval period and after metamorphosis as those documented in other regular urchins, *P. lividus* (**Gosselin and Jangoux,1998**) and *S. fanciscanus* (**Miller and Emlet, 1999**), while pedicellariae of *S. purpuratus*appeared some time after metamorphosis. On the contrary, competent larvae of *E. cordatum* do not exhibit spines orpedicellariae (**Nunes and Jangoux, 2007**), while those of C. subdepressus do have spines but devoid of any pedicellariae (**Vellutini and Migotto, 2010**).The young juvenile of *S. sphaeroides* has neither a mouthnor anus and no guts either. Similar event was also observed in other sea urchins (**Hinegardner, 1969, Mazur and Miller, 1971; Thet *et al.*, 2004)** and sea biscuits (**Emlet, 1986, Vellutini and Migotto, 2010**). At this stage, the dorsal half is essentially a rounded lump oflarval tissue punctured by the three pedicellaria. The dorsal organs appear to develop out of this tissue. For the first 2days, the larval tissue can easily be picked off the urchin.The digestive system and probably other internal organsappeared at about 4-5 days after settlement and then theurchin began to feed, as similar to those documented in *Colobocentrotusmertensii* (**Thet *et al.*, 2004**), *P. lividus* (**Gosselin and Jangoux, 1998**), and L. pictus(Hinegardner, 1969). In contrast, the juveniles of *C. subdepressus* and *C.rosaceus* start feeding 7 and 10 days, respectively, when theAristotle's lantern and mouth become functional (**Emlet, 1986,Vellutini and Migotto, 2010**). However, the consequences of these developmental basis in progressing juvenile stages of *S. sphaeroides* deserve furtherinvestigations.In conclusion, this study demonstrates the first successful investigation on the embryonic, larval, and postmetamorphic juvenile development of *S. sphaeroides* from Peninsular Malaysia. The findings obtained from the designated study would immensely be helpful towards the understanding of ontogeny and life-history strategies, which will eventually assist us in the development of breeding, larval rearing, seed production, and culture techniques of sea urchins for aquaculture industry.

To compare the effect of the bioactive inducers on metamorphosis of blastulae to plutei. Free swimming blastulae were subjected to certain bioactive inducers which had been claimed to induce metamorphosis, involved in signaling systems, serve in neuronal control, control morphogenetic and behavioral reactions or interact with members of several signal transduction proteins pathways under normal physiological conditions. It has been concluded that Acetyl choline 0.5 mM, Serotonine 10 µg/ml, NOS 2.5 mM, C81 µ M, NH_4Cl NH_4Cl and cGMP 1 µg/ml accelerated the transformation of blastula stage to an advanced pluteus larval stage with 8 arms whereas Acetyl choline 30 µ g/ml & 1 mM, NOS 1.5 mM, C8 10 µM and NH4Cl 300 µM exereted no role in metamorphosis and antibiotic sea water had negligible effect. Similar immune cytochemical protocols yielded different results were applied to larvae of *Ascidellaaspersa, Phallusiamammilata* and *Molgulamanhattensis* (**Niermann-Kerkenberg and Hofmann, 1989, Wahl und Lafargue, 1992,Kriegel, 1996, Saad, 2002 , Hofmann*et al.*, 2008,Saad, 2014**).

Authors of the present manuscript would like to introduce the different methods of procurement of gametes in marine inveretebrates: Several methods have been successfully used to induce shedding of gametes: (1) Potassium chloride injection (**Tyler 1949; Hinegardner, et al. 1967; Fuseler 1973**): A volume (0.1 to 1 .0 ml) of 0.5 M potassium chloride (isotonic to sea water) is injected intracoelomically using a one ml tuberculin syringe equipped with a 26 or 30 gauge needle. Gentle shaking of the animal after injection will distribute the potassium chloride to all gonads. This injection should be given through the soft tissue on the oral surface of the animal. Gamete shedding will begin several minutes after the injection isgiven. (2) Acetylcholine injection (**Hinegardner 1961, 1975**): Approximately 0.1 ml of 0.01 M acetylcholine (see Appendix C for source) in sea water is injected intracoelomically using a one ml tuberculin syringe equipped with a 26 or 30 gauge needle. This injection should also be given through the soft tissue on the oral surface of the animal. Gentle shaking of the animal after injection will distribute the acetylcholine to all gonads. The acetylcholine should be freshly prepared; the actual concentration of acetylcholine is not as critical as the freshness of the solution. **Hinegardner (1967)** recommends that once you have an idea of how much dry acetylcholine is needed to make up several milliliters of solution you need not bother weighing out the solid but obtain a suitable amount with a spatula and dissolve in sea water. (3) Electrical stimulation (**Harvey 1953; Osanai 1975**): This involves placing a pair of electrodes on the test (body) of the animal and passing an electric current through the electrodes. An adjustable physiological stimulator (e.g., Grass square-wave stimulator) can be employed at a frequency of 60 hertz.

For those urchins which have small tests (e.g. *A. punctulata, L. pictus*) the electrodes should be placed near the gonopore region. Adding an appropriate volume of freshly prepared sperm suspension to an egg suspension in a large container (e.g., beaker or finger bowl). This procedure is recommended when large numbers of eggs are to be fertilized for observational or experimental purposes. (A) Prepare an egg suspension by placing several drops of washed eggs in (B) container containing approximately 200 ml of sea water. (C) Prepare a dilute sperm suspension by placing one drop of concentrated semen in a test tube containing approximately 10 ml of seawater. Mix with a clean pipette to obtain a uniformly milky suspension. (D) Add two drops of the dilute sperm suspension to the egg suspension. Gently mix the sperm and eggs with a clean large-bore pipette, wooden stick, or stirring rod. (E) The eggs should be checked for fertilization approximately five minutes after insemination. This can be done by placing a sample in a small dish or by making a wet mount on a slide. Successful fertilization is indicated by the presence of a fertilization envelope around each egg.

REFERENCES

Bal, A. K. 1970. Ultrastructural changes in the accessory-cells and the oocyte surface of the sea urchin *Strongylocentrotusdrobachiensis* during vitellogenesis. Z. Zellforsch Mikrosk. Anat., 111:1-14.

Berking, *S.* and Herrmann, K. 1990. Dicapryloylglycerol and ammonium ions induce metamorphosis of ascidian larvae. Roux's *Arch. Dev. Biol.,* 198:430–432.

Bielmyer, G.K.; Brix, K.V., Capo, T. R. andGrosell, M. 2005. The effect ofmetals on embryo-larval and adult life stages of the sea urchin, *Diademaantillarum*. *Aquat. Toxicol.,* 74:254–263.

Bishop, C. D., Pires, A., Shong-Wan, b,. Norby, d., Boudko, D., Leonid, L., Moroz, C. and Michael G. 2008. Analysis of nitric oxide-cyclic guanosine monophosphate signaling during metamorphosis of the nudibranch *Phestillasibogae* Bergh (Gastropoda: Opisthobranchia). *Evolution and Development,* 10(3): 288–299.

Boveri, T. 1901. Merogoneoie (Y. Delange) und Ephebogenesis (B. Rawitz),neue Namen für eine alte *Sache. Anat. Anz.,* 19: 156–172.

Briggs, E. 1. and Wessel, G. M. 2006. In the beginning animal fertilization and sea urchin development. *Dev. Biol.,* 1; 300(1):15-26.

Brookbank, J. W. 1978. Histone synthesis by cleavage arrested sea urchin eggs. *Cell Differ.,* 7(3):153-8. PMID: 299702. [PubMed - indexed for MEDLINE]. {8}

Brooks, J. M. and Wessel, G. M. 2004. The major yolk protein of sea urchins is endocytosed by a dynamin-dependent mechanism. *Biol. Reprod.,* 71: 705–713.

Caldwell, J. W. 1972. Development, metamorphosis, and substrate selection of the larvae of the sanddollar, *Mellitaquinquesperforata* Leske, 1778. M.Sc., Thesis, University of Florida, Gainsville, 63 pp.

Chatlynne, L. 1969. A histochemical study of oogenesis in the sea urchin*Strongylocentrotus purpuratus. Biological Bulletin.* 136: 167-184.

Costello, D. P. and Henley, C. 1971. Methods of obtaining and handling marine eggs and embryos. MBL Press. Woods Hole, MA. 185-190.

Davenport, R. 1979. An Outline of Animal Development. Ch. 15. The sea urchin embryo. Reading, MA. Addison-Wesley Publishing Company, 1979. 259-293.

Davidson, E. H. 1989. Lineage-specific gene expression and the regulative capacities of the sea urchin embryo: A proposed mechanism. *Development,* 105:421-445.

Davidson, E. 1986. "Gene Activity in Early Development." 3rded.Academic Press, Orlando, FL.

Davidson, E.H. 1990. How embryos work: a comparative view of diverse modes of cell fate specification. Development, 108: 365-389.

Emlet, R. B. 1986. "Facultative planktotrophy in the tropicalechinoid *Clypeasterrosaceus* (Linnaeus) and a comparisonwith obligate planktotrophy in *Clypeastersubdepressus* (Gray) (Clypeasteroida: Echinoidea)," *Journal of Experimental Marine Biology and Ecology,* vol. 95, no. 2, pp. 183–202.

Emlet, R. B. 1988. "Larval form and metamorphosis of a "primitive"sea urchin, Eucidaristhouarsi (Echinodermata: EchinoideaCidaroida), with implications for developmental and phylogeneticstudies," *Biological Bulletin,* 174(1). 4–19.

Emlet, R.B. 2002. Ecology of adult sea urchins. In: Yokota, Y., Matranga, V., Smolenicka, Z. (Eds.), Sea Urchin: From Basic Biology to Aquaculture. *Balkema Publ.,* Rotterdam, pp. 111–114.

Ettensohn, C.A. and McClay, D.R. 1988. Cell lineage conversion in the sea urchin embryo. *Dev. Biol.,* 125:396–409.

Fernandez, N. and Beiras, R. 2001. Combined Toxicity of Dissolved Mercury with Copper, Lead and Cadmiumon Embryogenesis and Early Larval Growthof the *Paracentrotuslividus*Sea-Urchin. Ecotoxicology, 10: 263-271.

Fuseler, J. W. 1973. Repetitive procurement of mature gametes from individual sea stars and sea urchins. *J. Cell Biol.,* 57:879–881.

Garrido, C.L. and Barber, B.J. 2001. Effects of temperature and foodration on gonad growth and oogenesis of the green sea urchin,*Strongylocentrotusdroebachiensis*. *Mar. Biol.,* 138: 447–456.

Ghorani, V. Mahdavi, S. Nasser, I. Ghassemzadeh, F. Mortazavi, M. S. Mohammadi, E. andSadripour, E. 2012. The effect of lead toxicity on embryonic development and early larval growth of the *Echinometramathaei*sea urchin (Persian Gulf), morphologic andmorphometricstudiesAnnals of Biological Research, 3(7):3321-3327. *(http://scholarsresearchlibrary.com/archive.html).*

Gilbert, S. F. 2003. The morphogenesis of evolutionary developmental biology. *The International Journal of developmental Biology,* 47: 467 – 477.

Gosselin, P. and Jangoux, M. 1998. "From competent larvatoexotrophic juvenile: a morph functional study of theperimetamorphic period of *Paracentrotuslividus* (Echinodermata, Echinoida)," Zoomorphology, vol. 118, no. 1, pp. 31–43.

Giudice, G. 1973. Developmental biology of the seaurchin embryo. Academic Press. New York.

Harvey, E. B. 1953. A simplified electrical method of determining the sex of sea urchins and other marine animals. *Biol. Bull.,* 105: 365.

Harvey, E. B. 1956. The American Arbacia and Other Sea Urchins. Princeton University Press. Princeton, New Jersey. xiv + 298 pp. 16 pls.

Hinegardner, R. T. 1961. The DNA content of isolated sea urchin egg nuclei. Exp. Cell Res., 25:341-347.

Hinegardner, R. T. Echinoderms. Wilt, F. H.; Wessels, N. K. 1967. Methods in Developmental Biology. New York Thomas Y. Crowell; 139-155.

Hinegardner, R. T. 1969.Growth and development of the laboratory cultured sea urchin. *Biol. Bull.,* 137:465-475.

Hinegardner, R. 1975. Care and handling of sea urchin eggs, embryos, and adults (principally North American species). Czihak, G. ed. The Sea Urchin Embryo. Biochemistry and Morphogenesis. New York: Springer-Verlag,10-25.

Hofmann, D.K. Michael, M. I. Khalil, S.H. El-Bawab, F.M. and Saad, G.A. 2008. Larval metamorphosis in *Ascidiella aspersa* Müller, 1776 and *Phallusia mammilata* (Cuvier, 1815) Urochordata, Ascidiacea - An experimental study including an immunocy to chemical approach. Proc. 5th *Int.Conf. Biol.,* (Zool,.) 5:235-248.

Holland, N. D. and Giese, A. C. 1965. An autoradiographic investigation of the gonads of the purple sea urchin (*Strongylocentrotus purpuratus*)**.** Biol Bull 128: 241-258.

Hörstadius, S. 1939. The mechanics of sea urchin development, studied by operative methods. *Biol. Rev. Cambridge Philos. Soc.,* 14: 132-179.

Hörstadius, S. 1973. In Experimental Embryology of Echinoderms. Oxford University Press (Clarendon): London and New York.

Hyman, L. 1995. Echinoderm. Pp. "505-506" in Collier's Encyclopedia Vol.8. New York: P.F. Collier.

Ishikawa, M. 1975. Fertilization. Czihak, G. ed. The Sea Urchin Embryo. Biochemistry and Morphogenesis. New York Springer-Verlag, 1975. 99-147.

Ito, K. Hirota, Y. and Akiyama, Y. 1989, Temperature-sensitive sec mutants of Escherichia coli: inhibition of protein export at the permissive temperature. *J. Bacteriol.,* 171:1742-1743.

Johnson, J. D. Epel, D. and Paul, M. 1976. Intracellular pH and activation of sea urchin Eggs after fertilization. *Nature, Lond.,* 262: 661-664.

Kelly, M.S. 2001. Environmental parameters controlling gametogenesis in the echinoid *Psammechinusmiliaris*. *Journal of Experimental Marine Biology and Ecology*, 266 (1): 67 – 80 - See more at:http://www.sams.ac.uk/maeve-kelly/publications#sthash.doXhptmA.dpuf

Kriegel, N. 1996. Immunocytochemische Versuche zur Darstellung von Nervenelementen in Entwicklungsstadien von *Cassiopea spp*. (Cnidaria: Scyphozoa. Schriftliche Hausarbeit Im Rahmen der ersten Staatsprüfung für das Lehramt für die Sekundarstufe II. Staatliches Prüfungsamt Dortmund.Themensteller: Prof. Dr. D. K. Hofmann. Fachbereich Entwicklungsbiologie (unpublished thesis).

Leahy, P. S. 1986. Laboratory culture of *Strongylocentrotus purpuratus* adults, embryos, and larvae. *Methods Cell Biol.,* 27:1–13.

Levine, M. and Davidson, E.H. 2005. Gene regulatory networks for development. Proc. Natl. Acad. Sci., U. S. A., 102:4936–4942.

Livingston, B.T. and Wilt, F.H. 1990. Determination of cell fate in sea urchin embryos. Bio essays, 12: 115-119.

Matranga, V. (Ed.) 2005. Echinodermata. Progress in Molecular and Sub cellular Biology. *Marine Molecular Biotechnology, 39*: 85-110.

Mazur, Jane E.; Miller, John W. A 1971. Description of the Complete Metamorphosisof the Sea Urchin *Lytechinus Variegatus* Cultured in Synthetic Sea Water. *Ohio Journal of Science*, Volume 71, Issue 1: 30-36. http://hdl.handle.net/1811/5588

Miller, B.A. and Emlet, R. B. 1999. Development of newly-metamorphosed juvenile sea urchins(Strongylocentrotusfranciscanus and S. purpuratus): Morphology, the effects of temperature and larval food ration, and a method for determining age. *Journal of Experimental Marine Biology and Ecology,* 235: 67-90.

Montero-Torreriro, M.F. and Garcia-Martinez, P. 2003. Seasonal changes in the biochemical composition of body components of the sea urchin, *Paracentrotuslividus*, in Lorbé (Galicia, norh-western Spain). *J. Mar. Biol. Assoc.,* U. K. 83: 575-581.

Niermann-Kerkenberg, E. and Hofmann, D.K. 1989. Fertilization andnormal development in *Ascidiellaaspersa* (Tunicata) studied with Nomarski-optics. Helgoländer Meeresunters. 43: 245-258

Nunes, C. D. A. P. and Jangoux, M. 2007. "Larval growth andperimetamorphosis in the echinoid *Echinocardiumcordatum* (Echinodermata): The spatangoid way to become a sea urchin,"Zoomorphology, vol. 126, no. 2, pp. 103–119.

Okazaki, K. 1975. Normal development to metamorphosis. In The Sea Urchin Embryo: Biochemistry and Morphogenesis (ed. G. Czihak), pp. 177-232. NewYork: Springer-Verlag.

Oliveri, P. Davidson, E. H. and McClay, D. R. 2003. Activation of pmar1 controls specification of micromeres in the sea urchin embryo. Dev Biol., 258, 32-43.

Oliveri, P. and Davidson, E.H. 2004. Gene regulatory network controlling embryonic specification in the sea urchin. *Curr. Opin. Genet. Dev.,* 14: 351-360.

Osanai, K. 1975. Handling Japanese sea urchins and their embryos. Czihak, G. ed. The SeaUrchin Embryo. Biochemistry and Morphogenesis. New York: Springer-Verlag, 1975: 26-40.

Payan,P. ; Girard, J. P. and Ciapa, B. 1983. Mechanisms regulating intracellular pH in sea urchin eggs. *Dev. Biol.,* 100: 29-38.

Pearse, J. S. Pearse, V. B. and Davis, K. K. 1986. Photoperiod regulation ofgametogenesis and growth in the sea urchin *Strongylocentruspurpuratus. J. Exp. Zool.,* 237:107–118.

Pearse, J. S. and Cameron, R. A. (1991): Echinodermata: Echinoidea. In: GieseAC, Pearse JS, Pearse VB (eds) Reproduction of marine invertebratesVolume VI

Echinoderms and Lophophorates. The Box wood Press, California, pp 513–662.

Pehrson, J. R. and Cohen, L. H. 1986. The fate of the small micromeres in seaurchindevelopment. *Dev. Biol.,* 113: 522-526.

Piatigorsky, J. 1975. Gametogenesis. Czihak, G. ed. The Sea Urchin Embryo. Biochemistry and Morphogenesis. *New York Springer-Verlag,* 1975. 42-98.

Ransick, A. and Davidson, E.H. 1993. A complete second gut induced bytransplantedmicromeres in the sea urchin *embryo. Science,* 259: 1134-1138.

Revilla-i-Domingo, R. and Davidson EH. 2003. Developmental Gene Network Analysis. *Int. J. Dev. Biol.,* 47(7-8):695-703.

Ruggieri, G. D. 1975. Echinodermata Smith, W. L. Chanley, M. H. eds. Culture of Marine Invertebrate Animals. New York: Plenum Press. 1975. 229-243.

Saad, G. A. 2002. Comparative studies of the nervous and reproductive systems of some species of urochordates with emphasis on the role of the nervous system on reproduction and larval metamorphosis. Ph.D. *Thesis, Fac. Sci.,* Alexandria University

Saad, G. A. 2014. Embryonic development of *Molgula manhattensis* (de kay, 1843) with emphasis on the effect of some bioactive inducers and HSP 90 on larval metamorphosis, 6(1):945-967

Sakairi, K., Yamamoto, M., Ohtsu, K. and Yoshida, M. 1989. Environmental control of gonadal maturati on inlaboratory-reared sea urchins, *Anthocidariscrassispina* and *Hemicentrotuspulcherrimus*. *Zool. Sci.,* 6:721– 730.

Shpigel, M., S.C., McBride, S., Marciano, A. and Lupatsch, I. 2004.The effect of photoperiod and temperature on the reproduction of European sea urchin *Paracentrotuslividus. Aquaculture,* 232: 343-355.

Schroeder, T.E. 1986. *Echinoderm Gametes and Embryos.* Orlando, Academic Press, Inc.

Spirlet, C., Grosjean, P. and Jangoux, M. 2000. Optimization of gonad growth by manipulationof temperature and photoperiod incultivated sea urchins, *Paracentrotuslividus* (Lamarck Echinodermata). Aquaculture,185: 85–99.

Stearns, L.W. 1974. Sea Urchin Development: Cellular and Molecular Aspects.Dowden, Hutchinson and Ross, Inc., Stroudsburg, PA.

Strathmann, M. F. 1987. Echinoidea. In M. F. Strathmann, (ed.). Reproduction and Development of Marine Invertebrates of the Northern Pacific Coast. University of Washington Press, Seattle.

Strathmann, R. R., Fenaux, L. and Strathmann, M.F. 1992. Heterochronic developmental plasticity in larval sea urchinsand its implications for evolution of non-fecdinglarvae. Evolution, 46: 972-986.

Takashima, Y. and Tominaga, K. T. 1978. Formation, and behaVor of the nurse cell giant granules in the sea urchin ovary. In: 9th Int. Congr. Electron. Microsckop. Toronto, V. 2, p. 554-555. http://www.dissercat.com/content /bioindikatsiya-zagryazneniya-morskikh-vod-tyazhelymi-metallami#ixzz3QaDTWIVQ

Takata, H. and Kominami, T. 2004. "Behavior of pigment cells closely orrelates the manner of gastrulation in sea urchin embryos, "*Zoological Science,* Vol. 21(10): 1025–1035.

Thet, M. M., Noguchi, M. and Yazaki, I. 2004. "Larval and juvenile development of the echinometrid sea urchin *Colobocentrotusmertensii*: emergence of the peculiar form of spines," *ZoologicalScience,* Vol. 21(3): 265–274, 2004.

Tyler, A. A. 1949. simple, non-injurious method for inducing repeated spawning of sea urchins and sand dollars. *Coll. Net.,* 19:19-20.

Tyler, A. and Tyler, B**.** 1966a.The gametes; some procedures and properties. Boolootian, R. A. ed. Physiology of Echinodermata. New York: Interscience Publishers; 683-741.

Tyler, A. and Tyler, B. 1966b. Physiology of fertilization and early development. Boolootian, R. A.ed. Physiology of Echinodermata. New York: InterscicencePublishers; 639-682.

Unuma, T. 2002. Gonadal growth and its relationship to aquaculture in sea urchins. In Yokota Y, Matranga V, Smolenicka Z (eds.) The sea urchin: from basic biology to aquaculture,Lisse: Balkema.

Unuma, T., Yamamoto, T., Akiyama, T. and Ohta, H. 2003. Quantitativechanges in yolk protein and other components in the ovary andtestis of the sea urchin *Pseudocentrotusdepressus*. *J. Exp. Biol.,* 206: 365–372.

Vellutini, B. C. and Migotto,A. E. 2010. "Embryonic, larval, and juvenile development of the sea biscuit Clypeastersubdepressus (Echinodermata: lypeasteroida)," *PloS One,* Vol. 5, no. 3, article e9654.

Wahl, M. and Lafargue, F. 1992. Ascidien: Entwicklung. In: Emschermann, P., Hoffrichter, O., Körner, H. und D. Zissler (eds.): Meeresbiologische Exkursion: Beobachtung und Experiment, Abschnitt 1.3.28. Gustav Fischer, Stuttgart, pp.163-168

Walker, C.W. and Lesser, M.P. 1989. Manipulation of food and photoperiod promotes out-of-season gametogenesisin the green sea urchin *Strongylocentrotusdroebachiensis*: implications for aquaculture. *Mar. Biol.,* 132: 663–676.

Walker, C.W., Unuma, T., McGinn, N. A., Harrington,L.M. and Lesser, M.P. 2001. Reproduction of sea urchins. In: J.M. Lawrence (ed.), Edible Sea Urchins: Biology and Ecology, pp.5-26. Elsevier, Amsterdam, Netherlands.

Walker, C. W., Unuma, T. and Lesser, M. P. 2007. Game to genesis and reproduction of sea urchins in JM588 Lawrence (ed.) Edible Sea Urchins: Biology and Ecology, 2nd Edition. Developments in 589 *Aquaculture and Fisheries Science,* 37. Elsevier, 11-33.

Wessel, G. M. and McClay, D. R. 1985. Sequential expression of germ-layer specific molecules in the sea urchin embryo. *Dev. Biol.,* 111:451-463.

Wessel, G. M., Brayboy, L., Fresques, T., Gustafson, E. A. ;Oulhen, N., Ramos, I., Reich, A., Swartz, S. Z., Yajima, M. and Zazueta, V. 2013. The biology of the germ line in echinoderms. *Mol Reprod Dev., [*Epubahead of press].

Wilt, F.H. 1987. Determination and morphogenesis in the sea urchin *embryo. Develop.,*100: 559-575.

Yajima, M. and Kiyomoto, M. 2006. Study of larval and adult skele to genic cells in the developing sea urchin larvae. *Biol. Bull.,* 211:183–192.

Yajima, M., Kiyomoto, M. and Akasaka, K. 2007. Ars insulator protectstransgenesFromlong-term silencing in sea urchin larvae. *Dev. Genes Evol.,* 217:331–336.

Yajima, M. and Wessel, G. M. 2011a. Small micromeres contribute to the germline in Thesea urchin. *Development,* 138:237–243.

Yajima, M. and Wessel, G. M. 2011b. The DEAD box RNA helicase Vasa functions in embryonic mitotic progression in the seaurchin. *Development,* 138:2217–2222.

Yajima, M. and Wessel, G. M. 2012. Autonomy inspecification of primordial germ cells and their passive translocation in thesea urchin. *Development,* 139:3786–3794.

Yajima, M., Suglia, E., Gustafson, E. A. and Wessel, G. M. 2013. Meiotic gene expression initiates during larval development in the sea urchin. *Dev. Dyn.,* 242:155–163.

Yamamoto, M., Ishine, M. and Yoshida, M. 1988. Gonadal maturation independent of photic conditions in laboratory-reared sea urchins, Pseudocentrotusdepressus and Hemicentrotuspulcherrimus. *Zool. Sci.,* 5: 979– 988.

Young, O. R. 2002. The institutional dimensions ofenvironmental change: fit, interplay, and scale. MIT Press,Cambridge, Massachusetts, USA.

Ziegler, A., Cornelius, F., Susanne, M., Nina, N. and Leif, S. 2014. A dataset comprising 141 magnetic resonance imaging scans of 98 extant sea urchin *species. Giga Science,* 3:21 doi: 10.1186/2047-217X-3-21 (Openaccess).

****