ISSN 2347-6893

EMBRYONIC DEVELOPMENT OF *MOLGULA MANHATTENSIS* (DE KAY, 1843) WITH EMPHASIS ON THE EFFECT OF SOME BIOACTIVE INDUCERS AND HSP 90 ON LARVAL METAMORPHOSIS

Gaber Ahmed Saad Ibrahim
Department of Biology, College of Medicine, Dammam University, Saudi Arabia, KSA
Department of Zoology, Faculty of Science, Alexandria University, Alexandria, Egypt
Email: gaibrahim@uod.edu.sa

ABSTRACT

Specimens of *Molgula manhattensis* (De Kay, 1843) were collected during March to June (2013) along the northern estruarine harbour of the Arabian Gulf - Saudi Arabia. Sperm and eggs were sucked from gonoducts and placed separately in suitable Petri dishes containing sea water and antibiotic. Artificial heterologous insemination has been carried out. The different embryonic stages were studied alive and tabulated. Retrogressive metamorphosis of the larval stage till the newly metamorphosed squirt were studied normally and tabulated. Larvae with long tail were placed in certain bioactive chemicals with different concentrations to test whether metamorphosis would be affected. These bioactive chemicals were claimed to induce metamorphosis in an ascidians (Ammonia) or involved in signaling systems (1,2-dioctanyl-rac-glycerol) or serve in neuronal control (neurotransmitters acetylcholine and serotonine) or control morphogenesis (NO). Tukey's Multiple Comparison Test with $P > 0.05$ and One-Way analysis of variance (ANOVA) revealed that NH_4Cl (2.5 mM), C8 (1 µ M), NOS (2.5 mM), seratonin (10 µg/ml) and Acetyl choline (0.5 mM) accelerated the transition of larval stage from stage II with long tail to a newly metamorphosed sessile squirts stage IV. The other bioactive compounds did not show a remarkable observation. This study tried to test whether NO and HSP 90 play a role in the development of the larval and/or metamorphosing stages. It was concluded that the heat shock protein 90 is present in the brain vesicle, the nerve cord and all neurons supplying the different parts of the larval body of the larvae with long and short tails. Gradually this protein diminished from the brain vesicle as the larva lost almost its tail. Newly metamorphosed young showed fluorescence without illumination in its external surface.

Keywords

Artificial heterologous insemination; embryonic stages; retrogressive metamorphosis; bioactive chemicals; NO and HSP 90

ISSN 2347-6893

INTRODUCTION

Ascidian embryos and larvae have a small number of cells. At the beginning of gastrulation, embryos contain only about 110 cells whereas amphibian gastrulae contain about 10 000 cells (Gilbert and Raunio, 1997). Ascidian tadpole larva consists of a few thousand cells and only six different tissues. Ascidian larvae develop rapidly. Swimming larvae hatch after 12-18 hrs after fertilization. Distribution of coloured cytoplasmic regions of the zygote to specific blastomeres allows cell fates to be followed by routine microscopy. Ascidians have small genomes that facilitate cloning genes involved in developmental process. Researches concerning the embryonic development of ascidians in the available literature are very limited. Conklin (1931) studied the development of centrifuged eggs of ascidians, Cloney (1964) studied the development of the ascidian notochord, Jia (1987) investigated the larval nervous system and its development in *Ciona intestinalis*, Nakayama et al. (2005) gave a limited monograph to describe the second larval stage of *Ciona intestinalis*. The metamorphosis of the ascidian tadpole larva has long attracted interest of many investigators. Using tadpoles of *Ciona* , *Phallusia* , *Ascidia* ,*Styela*, *Styelopsis*, *Distomus*, *Clavelina* and *Distaplia* Berrill (1947a) concluded that ascidians spread only during larval stage and acid metabolites produced by the activity of the tail were the inducing agent of metamophosis. Moreover, he identified two factors during the metamorphosis of the ascidian tadpole, namely 'aging factor' which is the progressive exhaution of the yolk reserves of the epidermis. The epidermis, muscles and notochord are affected. 'Nutritive exhaustion' culminates in the centripetal contraction of the epidermal envelope, a contraction that appears to have a disruptive effect upon the tissues within. In the work of Cloney (1961), it was mentioned that the resorption of the tail of *Boltenia vilosa*, *Pyura haustorlo* and *Styela gibbsii* begins proximally and progresses distally. The principal structures of solitary ascidian larvae are catagorized into three groups Cloney (1990b): transistory larval organs, prospective juvenile organs and larval juvenile organs. The former one is phagocytized or destroyed at metamorphosis while the later two become the functional parts of the juvenile or oozooid. The larvae of ascidians have a dorsal tubular nervous system and a tadpole like body, with a tail supported by an axial notochord. The notochord is flanked by rows of muscle cells that are responsible for tail movements such as swimming (Kowalewsky, 1866; Mast, 1921; Kats, 1983; Bone, 1992). Ascidian tadpoles actively swim by bending their tail with alternating symmetrical contractions. They also produce asymmetrical contractions, or 'tail flicks', which help the larvae escape from the chorion membrane and, later, to change direction (Mast, 1921). The size of newly hatched larva varies from one species to another. They range in length from 0.6mm (*Molgula manhattensis*), 0.7 mm (*Ciona intestinalis*), 1.5 mm (*Halocynthia roseria*), to 11 mm (*Eudistoma digitatum*). The length of the swimming period in oviparous species can range from only a few minutes to several hours. while in most ovivivipiparous species, it ranges from a few minutes to several days. The larva swims for 6hrs to several days and during this swimming period, it prepares for the onset of metamorphosis. It alters its response to light and gravity. The larva is first negative geotactic and positive phototactic. Soon Immediately before settlement it avoids light and prefers to settle on dark or shaded surfaces. Metamorphosis begins with settlement and is followed by series of coordinated morphogenetic movements that rearrange cells, tissues and organs. The axial complex of the tail, the visceral ganglion and the sensory organs of the cerebral vesicle are destroyed and engulfed by phagocytes (Cloney, 1990b).

The most attracting field in the available literature concerning the reproduction of ascidians was that concerning their breeding seasons. The comprehensive study was that concerning the growth, maturation and seasonal variation of gonads, spawning, ecologiocal factors affecting breeding and geological distribution of the genus or/and species. It was found that *Ciona intestinalis* and *Styela plicata* were the most favourable ascidians chosen to study one or more of the above aspects (Berrill,1947a; Sabbadin,1957; Dybern,1965; Lambert,1968 and Yamaguchi,1970 &1975). Sabbadin (1957) studied in addition, that of another solitary ascidian *Molgula manhattensis*. In another publication, Berrill (1947b) studied the metamorphosis of *Ciona intestinalis, Ascidiela aspersa, Diplosoma listerianum* and *Botryllus schlosseri*. Diehl (1957) studied the effect of the ecological factors on the reproductive cycle of *Styela coriacea* and provided that the temperature is the main factor affecting this cycle. The same point of view was provided in another species *Styela rustica* (Lützen,1960) but with a different investigation based on histological observation. Morgan (1942) studied the percentages of normality and abnormality of the development of the tadpole of *Styela* by self-fertilizing and cross-fertilizing the eggs. After about 30 years one species of *Styela* which is *Styela plicata* was used as an experimental animal to provide the effect of other factors on reproduction of ascidians. West and Lambert (1975) provided that the light is another factor controlling the spawning and larval behaviour of this species. They supported their laboratory observations by field collection data and derived statistically from the spawning curves that gamete release occurs in the afternoon, close the sun set. These authors used white light during the experimentation. Previously, Lambert and Brandt (1967) used white light and monochromatic light to study their effect on spawning of *Ciona intestinalis*. Their results were later on confirmed by Yamaguchi (1970). *Ascidia nigra* used in another type of experiment to study the factors affecting the development of embryos (Goodbody and Fisher, 1947) and those affecting the survival of the populations of the juvenile and adult ascidians (Goodbody and Gibson,1974). These co-workers provided that the eggs are available throughout the year and are always capable of successful development, artificial fertilization, rearing of the embryos and juveniles. If successful experiments are done , the salinity, pH and other factors may be involved in the development and survival of *Ascidia nigra*.

Concerning the breeding season and spawning, ascidians spawn sperm and eggs every day during the breeding season. This breeding season differs among ascidian species. Some species have breeding season restricted to the summer or winter months, whereas others breed throughout the year (West and Lambert, 1976), Spawning is triggered by changes in the photoperiod. Whittingham (1967) concluded that in some ascidian species, a short pulse of light following an extended dark period triggers spawning. In some other species, spawning is initiated by a longer light period following darkness (West and Lambert , 1976). These authors added that the former species spawn in the morning while the latter species spawns at dark, moreover sperm and eggs can be obtained for experimental purposes at any time during the breeding season. Ripe gametes can also be obtained by excision of the gonoducts (Reverberi, 1971). In the text-books so far available (Millar, 1971 ; Stern and Holland , 1993 ; Satoh, 1994 ; Burighel and Cloney, 1997, Wolpert , 1999) it was

stated that larvae of sessile marine invertebrates are generally pelagic and respond to ecological factors in species-specific ways by which they reach the substratum. One of the predominabt ecologicasl factors involved in larval settlement is the light

MATERIALS AND METHODS

Animals

Adults of *Molgula manhattensis* (De Kay, 1843) were collected during March to June (2013) along the northern estruarine harbour 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), wood objects and iron for attachment and food suspension are regularly provided. Randomly selected adults were dissected in sea water. Sperm and eggs were sucked from gonoducts and placed separately in suitable Petri dishes containing sea water and antibiotic. Artificial heterologous insemination has been carried out and polyspermy has been avoided. 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 stage were observed. The time schedule for different stages and the measurement of head and tail together with their proportions were tabulated (Tables 1- 2). The different embryonic stages of *Molgula manhattensis* were obtained and tabulated according to the method of Hofmann et al. (2008) and Saad (2002). Four stages of larval development were collected namely newly hatched larvae about17hrs 30min after fertilization, larvae with long tail collected about 19 hrs after fertilization, partially metamorphosed larvae with short tail collected about 22-50 hrs after hatching and metamorphosed young stages 51-78 hrs after hatching. Specimens were then prepared for photomacroscopy, SEM study and immunocytochemical study, respectively.

Macroscopic observation

Tadpoles were prepared for both macroscopic, immunocytochemical techniques and electron microscopy. Firstly, the normal development of *Molgula manhattensis* was studied. Secondly, other larvae were fixed for 24 hr in buffered 2.5% glutaraldehyde and postfixed for 30 min. in 1% osmium tetroxide. Washing was two times in 0.1 M phosphate buffer, followed by four times in 0.4 M glycerol and two times in PPTA (15 min.). For macroscopic observation specimens were placed on glass slides with embedding mixture of PBS / glycerol / DABCO. Some larvae were stained with Evans stain to observe its internal structures. Other larvae were dissected with microneedles and incised longitudinally. 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 larvae are about 0.7µm long and transparent. Evan Blue stain was added to the live stages and described alive while movement. The photos did not clarify all described structures.

Scanning electron microscopy (SEM)

Samples of larvae were dried by means of the critical point method, mounted using carbon paste on an Al-stub and coated with gold up to a thickness of 400 Å in a sputter-coating unit (JFC-1100E). Observations of larvae morphology in the coded specimens were performed in a Jeol JSM-5300 scanning electron microscope operated between 15 and 20 KeV.

Immunocytochemical techniques

In this study a considerable number of larvae with long tail (18hr 40min) were collected alive under a stereomicroscope from the culture and transferred to wells of 24-well plastic tissue culture dishes. Selected larvae were subjected to certain bioactive inducers that enhanced metamorphosis in hydrozoans, echinoderms and other ascidian species. In each well about 30 larvae 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 NH4Cl (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 □ g/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 larvae were tested every 24hrs till 120 hrs and commented on (Table 3). The experiments were carried out in triplicate. The purpose of this experiment was to investigate larval 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 Hydractinia echinata (the neuropeptides LW-amide and RF-amide, Plickert and Schneider, 2004). These substances were screened for putative inductive or inhibitory effects on metamorphosis of Ascidella aspersa 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). To investigate whether NO and HSP 90 play a role in development of the larval and/or metamorphosing nervous system of *Molgula manhattensis*, specimens from developmental stages (larva with long tail collected about 20 hr from fertilization, partially metamorphosed larva with short tail 50-70 hr after hatching, metamorphosed young stage 75-80hr after hatching) were collected in Eppendorff vials and fixed in 4 % paraformaldyde.

These samples were treated for whole-mount immunohistochemistry towards the green fluorescent protein (GFP) (Ignarro,1990 ; Bredt, and Snyder, 1992), according to Kriegel (1996) as follows: fixed in paraformaldyde 4 % for 24 hrs, washed two times in 0.1 M phosphate buffer, washed four times in 0.4 M glycerol ,then two times in PPTA for 15 mins,

incubated in GFP 10.0 µM and exposed directly to blue light for 15 min according to Bishop and Brandhorst (2001). Samples were washed again with 0.1 M phosphate buffer two times, then in 0.4 M glycerol four times, and finally with PPTA 15 min two times. Specimens were placed on glass slide, with embedding mixture of PBS / glycerol / DABCO (90 % glycerol, 10 % PBS with 25 mg 1,4 -Diazabicyclo-(2,2,2) octane (DABCO)/ml), and covered with cover slip. The same procedure was carried out again using another concentration GFP 50.0 µM. Observations of specimens were viewed and photographied immediately under a fluorescence microscope (ZEISS-Axiophot), and all the preparations were preserved and stored at –20°C.

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

GFP as a biological probe can be used to search for certain types of protein localization during embryonic development in living cells. It is a spontaneously fluorescent protein, which was originally from the jellyfish Aequorea victoria that forms a fluorophore by self-catalyzed protein backbone modification. GFP has a typical beta barrel structure, consisting of one β-sheet with alpha helix(s) containing the chromophore running through the centre. Inward facing sidechains of the barrel induce specific cyclization reactions in the tripeptide Ser65–Tyr66–Gly67 within the 238 amino acid polypeptide extensive that lead to chromophore formation. This process of post-translational modification is referred to as maturation. The hydrogen bonding network and electron stacking interactions with these sidechains influence the color of wtGFP and its numerous derivatives. The tightly packed nature of the barrel excludes solvent molecules, protecting the chromophore fluorescence from quenching by water. GFP is spliced into the genome of the organism in the region of the DNA which codes for the target proteins. It has been shown previously that Hsps are differentially expressed in glial and neuronal cells, as well as in the different structures of the brain. Hsps are expressed in different cell classes (neuronal, glial, and vascular).

RESULTS

Stereoscopy revealed that the fertilized egg of *Molgula manhattensis* (23min. after fertilization) is spherical in shape measuring 180 – 200 µm in diameter and provided with a wide pervitelline space (Figs. 1-2). It is surrounded by a tear outer and an inner loosen follicular epithelia, a third follicle layer, the test cells, appeared as a discontinuous layer inside the peripheral zone of the cytoplasm. The chorion can be well differentiated from the inner contents of the cell. The yolk granules are equally distributed in the cytoplasm. This study observed that the fertilized egg underwent a bilateral holoblastic cleavage (2.20-3.50 hrs). The first cleavage plane establishes the earliest axis of symmetry in the embryo, separating the embryo into its future right and left sides (Fig. 3). Each successive division orients itself to this plane of symmetry, and the half-embryo formed on one side of the first cleavage plane is the mirror image of the half-embryo on the other side. The second cleavage is meridional, like the first, but unlike the first division, it does not pass through the center of the egg (Fig. 4). Rather, it creates two large anterior blastomeres and two smaller posterior ones. Each side now has a large and a small blastomere. During the next three divisions, differences in cell size and shape highlight the bilateral symmetry of these embryos. At the 32-cell stage, a small blastocoel is formed (Fig. 5), and gastrulation begins (4hr 55 min). Gastrulation is characterized by the invagination of the endoderm, the involution of the mesoderm, and the epiboly of the ectoderm. About 5hrs 39min after fertilization, the vegetal (endoderm) cells assume a wedge shape, expanding their apical margins and contracting near their vegetal margins. Blastomeres appear to lead this invagination into the centre of the embryo. This invagination forms a blastopore whose lips will become the mesodermal cells. The presumptive notochord cells are now on the anterior portion of the blastopore lip, while the presumptive tail muscle cells are on the posterior lip of the blastopore. The lateral lips of the blastopore comprise those cells that will become mesenchyme. The second step of gastrulation (5 hr 45 min) involved the involution of the mesoderm. The presumptive mesoderm cells involute over the lips of the blastopore, and by migrating upon the basal surfaces of the ectodermal cells, move inside the embryo. The ectodermal cells then flatten and epibolize over the mesoderm and endoderm, eventually covering the embryo (Fig. 6). After gastrulation is completed, the embryo elongates along its anterior-posterior axis (6 hr 10 min). The dorsal ectodermal cells that are the precursors of the neural tube invaginate into the embryo and are enclosed by neural folds. This forms the neural tube (Fig. 7), which will form a brain anteriorly and a spinal chord posteriorly (6 hr 25 min). Meanwhile, the presumptive notochord cells on the right and left sides of the embryo migrate to the midline and interdigitate to form the notochord (7hr 30 min). Thus, a tadpole is formed inside the chorion and now became initial tail bud stage (8.10-16.25 hrs) (Fig. 8). This larval stage underwent many morphological changes as follows (Figs. 9-20): 1- first indication of a separation between tail and trunk regions. The tail is not bent and has the same length as the trunk. Any notochord cells not finished intercalation. 2- The tail is clearly separated from the trunk. Tail and trunk have same length. Neuropore still open, a-line neurulation. 3- The tail bends about 40° and is slightly longer than the trunk. A few anterior most notochord cells begin to intercalate and linear. 4- Neuropore closed, tail bent by 60°, neurulation complete. 5- Tail 1 1/2 times longer than trunk and curve ventrally (90°). Intercalation of notochord cells just finished. 6- The body adopts a half circle shape. Tail twice as long as trunk. 7- Initiation of the pigmentation of the otolith. Tail strongly curved with tip close to the anterior end of the trunk. 8- Notochord vacuolation begins, palps start to be visible at the front end of the embryo. Tail straightens. 9- Ocellus melanization. All notochord cells have vacuoles. Tail bent dorsally. 10- Hatching. head adopts an elongated rectangular shape. 11- larva with long tail. 12- larva with short tail. 13- newly metamorphosed young.

This study observed that larval metamorphosis of *Molgula manhattensis* that involved numerous rapid morphogenic movements that are initiated at the moment of settlement. Hatched larva (18 hr 20 min) is entirely surrounded with evaginated test cells from the fertilized oocyte stage. The 19 hr 30 min larva began to secrete adhesives by papillae or the epidermis of the trunk, papillae gradually retract as tail resorption proceedes, the larva shed the outer layer of its tunic, the visceral organs rotated with the expansion of the pharynx and elongation of the juvenile, Phagocytosis of visceral ganglion, sensory organs and cells of the axial complex and finally release of organ rudiments from an arrested state of development.This study tried to describe the structure of the larval stages of live samples dissected with micro-needles and stained with Evan Blue since this larva is transparent and measured about 0.7µm. Several trails have been done to photograph the internal larval structures but all trails failed. After hatching (18 hr 20 min), the larva is completely covered by an extra-cellular tunic which forms the fins of this stage. The fins all run along the long axis of the larval stage. The largest fins consist of a dorsal and a ventral flap of tunic running the entire length of the tadpole. One major fin also runs along each lateral surface of the body. In addition, many tiny fins and short finger-like projections are found along the outer surface of the tunic. The tunic itself is non-cellular. A single-layered epidermis covers the entire larva except where the pharynx and the two atria break through. The combination of a single layered epidermis and an outer tunic-like secretion (probably derived from the test cells around the epidermis). In *Molgula manhattensis* tadpole, the epidermal cells form a simple cuboidal epithelium. Throughout the epidermis, the outer cell surface is generally smooth without extensive ridges. The external surface of the epidermis appears quite active, being studded with coated pits in all stages of formation and contains exocytotic vesicles, especially along the tail epidermis. The boundaries between adjacent epidermal cells tend to be fairly smooth. The inner surfaces of the epidermal cells are fairly smooth and sit upon a basal lamina. larvae of *Molgula manhattensis* are characterized by a prominent notochord, is a rod-shaped tissue, running the length of the tail and extending into the caudal body. Dorsally, the notochord is covered by the deuterencephalon of the nervous system; ventraly, it overlies the endodermal strand; and laterally, it is surrounded by the muscles of the tail. The tail musculature comprises two sets of striated muscle cells. Each set of muscle cells is aligned as three longitudinal bands flanking the notochord laterally. At the beginning of metamorphosis, the adhesive organ serves to attach the larva to a settlement surface. An adhesive organ consists of three cone shaped protrusions at the anterior end of the tadpole. The papillae have a triangular arrangement, with one lying ventrally and two lying dorsally. In the 19 hr 30 min tadpole, papillae axtends about 6 µm from the anterior end of the larva. The central nervous system (CNS) extends most of the length of the larva. Rostrally, it abuts the pharyngeal-gut junction, protruding under the primordial pharynx, i.e., the incurrent siphon rudiment. Caudally, it reaches the end of the tail. The entire CNS is surrounded by a basal lamina. The CNS lumen is greatly expanded rostrally and is closed off entirely near the caudal end of the tadpole trunk, and is opened with a small uniform bore throughout the tail. By a number of anatomical criteria, the CNS can be divided into two parts: a prosencephon rostrally and a deuterencepha!on caudally. A one cell-thick pocket of cuboidal and cells forms the primordial pharynx in the dorsal epidermis of the tadpole body. The primordial pharynx is an oblong tubular body capping the rostral end of the endodermal cavity and then extending caudally over the rostral tip of the CNS. The external opening of the lumen of the primordial pharynx is directly over the CNS. Ventrally, the ventricle of the CNS prosencephalon extends as a small bore ciliated tube under the primordial pharynx. The most rostral end, the neuropore of this ventricle opens into the rostral end of the lumen of the primordial pharynx. The cytoplasm of cells of the primordial pharynx is slightly darker and has a more finely grained texture than that of the surrounding epidermal cells. Pockets of cuboidal cells form the two atrial primordia, one on the left and one on the right, in the epidermis of the body wall. The atrial primordia border the gut primordium at a level just rostral to the head of the notochord. Each atrial primordum is a spherical pocket with walls which are one cell thick. The gut primordium is a closed folded tube, one cell thick, that fills the ventral two-thirds of the body. The gut extends from the pharyngeal primordium rostrally to the head of the notochord caudally. The gut primordium is followed in the tail by the endodermal strand a lumenless set of gut cells, in single file, lying directly under the notochord along its entire length. The prosencephalon lies directly along the top wall of the gut primordium. In the rostral half of the tadpole body, the bottom wall of the gut primordium lies directly along the ventral epidermis. The rostral end of the gut primordium borders an extracellular space behind the adhesive organ. This space is called the preoral lobe; it contains separated spherical cells and will apparently continue to fill with cells before metamorphosis. Cells of the gut primordium and of the endodermal strand are roughly cuboidal and are characterized by large numbers of yolk vesicles. The cytoplasm of these cells appears loose and vacuolated and contains few organelles. The tadpole larva contains a number of mesodermal pockets, two bilateral pairs are found between the ventrolateral walls of the gut primordium and the ventrolateral epidermis.

The present study aimed at studying the effect of defined bioactive chemical inducers on metamorphosis of larvae of *Molgula manhattensis*.raised in the laboratory. Some of the compounds had been applied before in published work cited above, Ammonia induced metamorphosis in an ascidians (Berking and Hermann, 1990), C8, 1,2-dioctanyl-rac-glycerol, an activator of protein kinase C involved in signaling systems (Kriegel 1996), acetylcholine and serotonin acted as neurotransmitters (Bishop et al. 2001) and 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. 2001). A multinational project (Takahashi et al.1997) has systematically isolated and identified peptide and neuropeptide signal molecules from Hydra sp., which regulate development in this genus. The eminent role of neuropeptides in controlling development, molt, metamorphosis, and reproduction is long known from insects, and applies also to metamorphosis of tadpoles of anuran amphibians (Gilbert et al. 1996). In the present study, the number of larvae in each trial in stage II and the number of larvae in stage III together with the newly metamorphosed youngs stage IV were directed analysed with One-Way analysis of variance (ANOVA) (Histogram 1). The present results show that the applied bioactive compounds had different results on larval metamorphosis (Table 3). It was concluded that NH4Cl (2.5 mM) , C8 (1 µ M), NOS (2.5 mM) , seratonin (10 □ g/ml) and Acetyl choline (0.5 mM) accelerated the transition of larval stage from stage II with long tail to a newly metamorphosed sessile youngs stage IV. The other bioactive

compounds did not show a remarkable observation. Heavy metals hindered larval metamorphosis and mortality was obvious among larvae with long tail. The number of larvae in stage II subjected to each bioactive compound in the three experiments and the number of larvae with short tail stage III together with newly metamorphosed youngs stage IV were calculated and the percentage was subjected to Tukey's Multiple Comparison Test P > 0.05 and One-Way analysis of variance (ANOVA). Seratonin (10 □ g/ml) has enhanced and activated larval metamorphosis (Table 4 and Histogram 2). seratonin vs acetyl choline 95% CI of diff -48.21 to 80.46 , NOS vs serotonin 95% CI of diff - -83.08 to 45.59 , C8 vs serotonin 95% CI of diff --87.15 to 41.52, NH4Cl vs seratonin95% CI of diff – -80.15 to 48.53 and antibiotic sea wate vs seratonin95% CI of diff - -76.68 to 52.00.

It is well known that NOS activity catalyzes the formation of NO by converting arginine to citrulline with the concomitant release of NO. The production of NO by NOS has been associated with a wide range of important physiological functions including neuronal signaling in early embryonic development, host response to infection. Additionally, sustained synthesis of NO has been implicated in the etiology of endotoxic shock, inflammation-related tissue damage, and neuronal pathology. Bishop and Brandhorst (2001) concluded that Nitric oxide (NO) signaling repressively regulates metamorphosis in two solitary ascidians and a gastropod. evidence for a similar role in the sea urchin Lytechinus pictus was provided. NO commonly signals via soluble guanylyl cyclase (sGC). Nitric oxide synthase (NOS) activity in some mammalian cells, including neurons, depends on the molecular chaperone heat shock protein 90 (HSP90); this may be so in echinoid larvae as well. So, it was another aim in this study to search for the heat shock protein 90 (HSP90) in the tissues of larvae in stage II and III and the metamorphosed youngs and whether this HSP90 played a role in larval metamorphosis. This study concluded that larvae in stage II had a considerable quantity of HSP90 in their neurons in all tissues of the trunk and tail regions.

Testing the response of larvae of *Molgula manhattensis* for GFP

The three stages of larval development, namely larva with long tail collected about 19 hr 30 min from fertilization, partially metamorphosed larva with short tail 50 hr 55 min after hatching and metamorphosed young stage 80hr 15 min after hatching, were examined to test if HSP 90 is present in the nervous complex or any part of the body using green fluorescent protein (GFP). This test revealed that the heat shock protein 90 is present in the brain vesicle, the nerve cord and all neurons supplying the different parts of the larval body of the larvae with long and short tails (stages II and III) (Fig. 21). Gradully this protein diminished from the brain vesicle as the larva lost almost its tail (Fig. 22-24) and if this protein presents, negligible amount. Newly metamorphosed young showed fluorescence without illumination in its external boundary (Fig. 25). This means that the brain vesicle has given a signal to the different body parts to enhance or to activate the process of retrogressive development and metamorphosis of the larva to a newly squirt. In other words, HSP 90 may function in emitting signals necessary for development of the different body organs and to change the brain vesicle of the larval stage to the nervous complex of the adult stage consisting of a nerve ganglion and a neural gland. Meanwhile it may function in apoptosis of the tail region with its structures of notochord and nerve cord. NOS activity was present in neurons of larval tissues. The NADPH diaphorase histochemical assay was used under conditions specific for vertebrate NOS enzymes. Whole larvae were stained and observed; some were fixed and sectioned before examination by microscopy. The brain vesicle, mid- and hindgut were stained for diaphorase activity. These sites of NADPH diaphorase activity most likely represent sites of NOS activity. Larvae having large rudiments resembling those used for the inhibitor assays were also sectioned and stained. Diaphorase activity was found in a variety of structures. Stained cells were found within the larval brain vesicle. Stained cells, often have a neuronal appearance. Larvae were stained with anti-NOS antibodies to see whether sites of NADPH activity were coincident with the location of NOS. The production of NO repressively regulates the initiation of metamorphosis and that a sensory response to environmental cues reduces the production of NO, and consequently cGMP, to initiate metamorphosis. This experiment shows that NO (Nitric Oxide) accelerates larval metamorphosis and cGMP is a second enhancing factor for NO whereas L-NAME arrestes larval metamorphosis.

Discussion

Embryonic development of many ascidian species has traditionally been studied following artificial fertilization (Krohn,1852 for one of the earliest reports). Unfortunately, a non-invasive method to obtain mature eggs and sperm is not available, gametes have to be obtained surgically by dissecting reproductive specimens and collecting them with micropipettes from the oviduct and the vas deferens. *Molgula manhattensis* is known to be self-fertile, but eggs were routinely inseminated with sperm from a different animal. Some species, however, are self-sterile, either strictly or at varying degrees, depending on the population. Self-sterility, a rare case among animal species, has been reported first for *Ciona intestinalis* by Castle (1896) and has been studied for more than 40 years (Morgan, 1942). The observations of the present work, carried out on *Molgula manhattensis*, with no published data. The present study showed that the fertilized egg of *Molgula manhattensis* is spherical in shape measuring 180 – 200 µm in diameter and provided with a wide pervitelline space. It is surrounded by a tear outer and an inner loosen follicular epithelia, a third follicle layer, the test cells, appeared as a discontinuous layer inside the peripheral zone of the cytoplasm. The chorion can be well differentiated from the inner contents of the cell. The yolk granules are equally distributed in the cytoplasm. The fertilized egg underwent a bilateral holoblastic cleavage (2.20-3.50 hrs). At the 32-cell stage, a small blastocoel is formed, and gastrulation begins (4hr 55 min). Gastrulation is characterized by the invagination of the endoderm, the involution of the mesoderm, and the epiboly of the ectoderm. The embryo elongates along its anterior-posterior axis (6 hr 10 min). The dorsal ectodermal cells that are the precursors of the neural tube invaginate into the embryo and are enclosed by neural folds. This forms the neural tube, which will form a brain anteriorly and a spinal chord posteriorly (6 hr 25 min). Meanwhile, the presumptive notochord cells on the right and left sides of the embryo migrate to the midline and interdigitate to form

the notochord (7hr 30 min). Thus, a tadpole is formed inside the chorion and now became initial tail bud stage (8.10-16.25 hrs). This study observed that larval metamorphosis of *Molgula manhattensis* that involved numerous rapid morphogenic movements that are initiated at the moment of settlement. Hatched larva (18 hr 20 min) is entirely surrounded with evaginated test cells from the fertilized oocyte stage. The 19 hr 30 min larva began to secrete adhesives by papillae or the epidermis of the trunk, papillae gradually retract as tail resorption proceedes, the larva shed the outer layer of its tunic, the visceral organs rotated with the expansion of the pharynx and elongation of the juvenile, Phagocytosis of visceral ganglion, sensory organs and cells of the axial complex and finally release of organ rudiments from an arrested state of development. This study tried to describe the structure of the larval stages of live samples dissected with micro-needles and stained with Evan Blue since this larva is transparent and measured about 0.7µm. Several trails have been done to photograph the internal larval structures but all trails failed. Meticulous observation of ascidian embryogenesis dates back to Krohn (1852), Kowalewski (1866) and particularly to Conklin (1905 a &b) who established the cell lineage and fate map of *Styela plicata*. More recent studies of early development under constant temperature conditions and along a precise time scale are available for *Ascidella aspersa* (Niermann-Kerkenberg and Hofmann, 1989, Saad, 2002 , Hofmann, et al. 2008) and for *Phallusia mammilata* (Wahl und Lafargue, 1992, vid Ibid). The previous pioneers confirmed that embryonic development is very similar in most ascidians, both morphologically and with regard to the time course, so their publications served well as a prerequisite for further experimental studies. Embryogenesis, which incurs enclosed in an envelope consisting of two cellular and one non-cellular layers, ends with releasing of a fully developed larva. The size of newly hatched larvae varies from one species to another. They range in length from 0.7 mm (*Molgula manhattensis*), 0.6 mm (*Ciona intestinalis*), 1.5 mm (*Halocynthia roseria*), 11 mm (*Eudistoma digitatum*), to 12.5 mm in the giant tadpole of *Ecteinascidia turbinata*. The tadpoles of *A. aspersa* and of *P. mammilata* are somewhat smaller than those of *C. intestinalis*. An extensive morphological and ultrastructural analysis of the tadpole of the latter species has been performed by Katz (1983). The length of the swimming period of the larvae in oviparous species can range from a few minutes to several hours, or even a few days as shown in this study. Grave (1944) studied activities and duration of larval life in detail in *Styela partita*. In ovoviviporous species it may extend from minutes to several days. The free-swimming larva of sea squirts react to light and gravity in a manner that ensures their dispersal. The receptors to these stimuli are situated within the cerebral vesicle of the tadpole (Dilly, 1961). It was until (1986) Holmberg stated that these receptor cells are innervated by only one neuron and not by two as previously believed. Olsson et al. (1990) recorded that receptors in the larvae of *Ciona intestinalis*, *Phallusia mammilata* and *Ascidia nigra* consist of a unicellular otolith and an ocellus made up from about ten cells. Jang et al. (2005), using wild type and mutant larvae of *Ciona savignyi*, demonstrated that pigmentation in the tadpole's sensory organs is essential for normal behavior. The activities of most marine invertebrate larvae consist basically of three phases: active movement, settlement, and metamorphosis. Larvae of sessile marine invertebrates, either thrifting pelagically or creepring epibenthically, respond species-specific to ecological factors directing them towards the substratum. In the two subsequent phases the larvae become transformed into juveniles with an entirely different mode of life. Settlement mostly includes attachment to a suitable substratum, metamorphosis denotes profound morphological and physiological chances, with both processes occurring usually within a very short time (Marshall et al.2003). Tadpole larvae prepare for settlement and metamorphosis during the swimming period e.g. by altering the response to light and gravity. They are first negatively geotactic and positively phototactic, but then reverse the reactions. Shortly before settlement they tend to avoid light and to settle on dark or shaded surfaces. As shown in the present Work, larvae of *Molgula manhattensis* undergo metamorphosis even under sterile culture conditions spontaneously and fairly rapidly. Other species however need to become "competent" until the larvae will be able to respond to external physical or other environmental cues. Competence, which can be defined so far only with biological terms, may be aquired only after several hours (Bishop et al. 2001).

Metamorphosis involves numerous rapid and coordinated morphogenetic movements, physiological changes, cellular rearrangements, and also significant degradation processes, which appear to be initiated by the time of settlement. Cloney (1975, 1982 & 1990) reviewed ascidian metamorphosis extensively and listed 10 principal progressive and regressive metamorphic events. The following ones are most salient: secretion of adhesives by anterior trunk papillae, resorption of the tail, loss of the outer cuticular layer of the tunic, rotation of the visceral organs through an arc of about 90°, expansion of the branchial basket with concomitant elongation of the juvenile, retraction of the sensory vesicles, phagocytosis of visceral ganglion, sensory organs, and cells of the axial complex, release of organ rudiments from arrested developmental state. The in vitro metamorphosis experiments of the present work excluded many components of the natural environment e.g. there was no noticeable water column available in the tissue culture plates, no other organisms, and no natural substratum. Thus it is important to note that settlement on a natural substrate was not a prerequisite for metamorphosis. Resorption of the tail was observed in free floating individuals and transformation into the juvenile was seen to occur in specimens loosely adhering upside-down to the surface pellicle. The latter can be easily transferred onto microscope slides with depression to demonstrate metamorphic stages to student classes. Impact of altered culture media, supplemented chemicals, and compounds extracted from ascidians on larval metamorphosis: Vázquez et al. (2000) studied the effect of salinity on larval metamorphosis of three colonial ascidians Eudistoma olivaceum, *Eudistoma hepaticum*, and *Ecteinascidia turbinata*. Transformation of the larvae proceeded more quickly at 33 ppt than at lower salinities. The thresholds for successful metamorphosis differed among species in a manner that corresponded to the adult distributions. A multitude of studies was devoted to the search for chemicals which fasten metamorphosis and which might be involved in the physiology of this process. For example Whittaker (1964) stated that metamorphosis of certain species of ascidian larvæ can be initiated by increasing the copper content of seawater. However, Glaser and Anslow (1949) observed that various materials that cause an acceleration of the onset of ascidian metamorphosis have an unusually high copper content; they concluded that the copper content of seawater is causally

related to the onset of natural metamorphosis. This hypothesis has not been generally accepted, it has never been experimentally disproved. Berking and Hermann (1990) found ammonia and dicraprylolyl glycerol to stimulate metamorphosis of *Ciona intestinalis* larvae. Degnan et al. (1997) reported elevated potassium concentrations bearing on an anterior signaling center accelerated the transition in Herdmania momus. Addition of iodine and also of L-thyroxine (T4), a vertebrate thyroid hormone, was also reported to speed up metamorphosis in C. intestinalis larvae (Patricolo et al. 1981 & 2001). Functions of the ascidian endostyle have since long been homologized with those of the vertebrate thyroid gland. D'Agati and Cammarata (2006), studying metamophosis in *Ascidia malaca, Ascidiella aspersa, Phallusia mammilata*, and *Ciona intestinalis*, emphasized that the endostyle is a mucus-secreting pharyngeal organ that has iodine-concentrating activity and shows biosynthesis of thyroid hormones. T4 was found to be localized in mesodermal cells and, interestingly, thiourea, an inhibitor of thyroid hormone synthesis, was shown to reversibly inhibit tail resorption. Coniglio et al. (1998) demonstrated dose-dependent cholinergic enhancement of settlement and shortening of the explorative phase of *C. intestinalis* larvae by acetylcholine (ACh). Furthermore they noted that α-bungarotoxin, an inhibitor of ACh, immobilized the larvae, prevented settlement and attachment by papillar secretions, but did not block tail resorption. These findings show that the complex control mechanism can be pharmacologically dissected and that acetylcholine is presumably involved only in the very first steps of metamorphosis. Kimura et al. (2003), on the other hand, reported that interaction of adrenalin or noradrenalin interaction with the □ (1)adrenergic receptor in the larval nervous system triggers early metamorphosis in *Ciona savignyi*.

The present work aimed at studying the impact of defined chemical compounds on metamorphosis of larvae of *Molgula manhattensis* raised in the laboratory. Some of the compounds had been applied before in published work cited above, the neuropeptides LW-amide and RF-amide were tested for the first time. These neuropeptides play an important morphogenetic role in controlling planula larva metamorphosis in the hydroid Hydractinia echinata (Plickert and Schneider, 2004). A multinational project (Takahashi et al.1997) has systematically isolated and identified peptide and neuropeptide signal molecules from Hydra sp., which regulate development in this genus. The eminent role of neuropeptides in controlling development, molt, metamorphosis, and reproduction is long known from insects, and applies also to metamorphosis of tadpoles of anuran amphibians (Gilbert et al. 1996). The present results show that the applied bioactive compounds had deleterious effects and that metamorphosis to stage IV occurs over time at all conditions chosen. This study concluded that NH4Cl 2.5 mM , C81 µ M, Serotonine10 □ g/ml and Acetyl choline 0.5 mM accelerated larval metamorphosis. None of the compounds exerted inhibitory activity except heavy metals. It was found that the differences in efficiency on altering time course and proportion of larva-to-juvenile transition are gradual. In no case was metamorphosis dramatically enhanced and protracted as would be expected in case of a highly specific cue. Nevertheless the proportion of transformed larvae was somewhat higher throughout the different treatments than in the controls. Also a proportion of at least 50% metamorphosed individuals occurred earlier in all treated samples. It appears that the array of chemical treatments in the chosen range of concentrations provide conditions which are permissive for entering metamorphosis and facilitate the transition to some extent but NH4Cl 2.5 mM , C81 µ M, Serotonine10 □ g/ml and Acetyl choline 0.5 mM represent specifically enhancing inductive signals, either directed to singular events or to the integrated process of metamorphosis. Despite the progress of studies reviewed above bearing on various factors affecting ascidian metamorphosis, the statement by Bishop et al. (2001) holds true also for the present study that no unifying hypothesis integrating the effects of seemingly unrelated substances and putative endogenous signaling molecules is available to date. Studying larval metamorphosis, and tail regression in particular, in Boltenia villosa and Cnemidocarpa finnmarkensis Bishop et al. (2001) found that regulation of the process involves nitric oxide/cyclic guanosine monophosphate (NO/cGMP) signaling and the heatshock protein HSP90. When the activity of the enzyme nitric oxide synthase (NOS), which is known to interact with HSP90, was blocked with the specific inhibitor L-NAME (N□-nitro-L-arginine), the frequency of metamorphosis was increased. The authors suggested that NO represses metamorphosis and that interference of various chemical agents with NOS may reduce NO to sub-threshold levels and consequently, suspend the repression and alow entering tail regression. Significant promotion of larva-to-juvenile transformation by the NOS inhibitor L-NAME has now been confirmed in Phallusia mammilata. Comes et al. (2006) addressed control of tail resorption with regard to caspase-dependent apoptosis in C. intestinalis. They reported that programmed cell death in tail regression is governed dose-dependent by nitric oxide. An increase of NO levels delays while a decrease accelerates regression. The process could be hastened also by inhibition of cGMP-dependent NO signaling, thus confirming the observations reported by Bishop et al. (2001). As caspase-linked apoptosis occurs also at other sites of the larva during transformation (Comes et al. 2006). The pattern and timing of NOS activation, and the freely diffusing product NO have become important players at least in the degenerative events during metamorphosis. As other morphogenetic processes appear to remain unaffected by NO signalling, but possibly are subject to thyroid hormone action, and as settlement and attachment have been shown to be under cholinergic control. The detection of a control system is still warranted that integrates the functions within the diversified complex of metamorposis of a pelagic larva into a benthic sessile juvenile ascidian.

ISSN 2347-6893

Legends

Fig. 1

Phase contrast photomicrograph of a whole mount of a fertilized egg of *Molgula manhattensis*. at lower magnification to provide better overview of ovular envelopes. Note, the fertilized egg is spherical in shape measuring 180 – 200 µm in diameter.

Fig. 2

Phase contrast photomicrograph of a whole mount of a fertilized egg of *Molgula manhattensis*. Note, the fertilized egg is provided with a wide pervitelline space. It is surrounded by a tear outer and an inner loosen follicular epithelia, a third follicle layer, the test cells, appeared as a discontinuous layer inside the peripheral zone of the cytoplasm. The chorion can be well differentiated from the inner contents of the cell. The yolk granules are equally distributed in the cytoplasm.

Fig. 3

Phase contrast photomicrograph of the onset of cleavage division in *Molgula manhattensis*. The first cleavage plane established the earliest axis of symmetry in the embryo, separating the embryo into its future right and left sides.

Fig. 4

Phase contrast photomicrograph of a whole mount of alive 4-cell stage of *Molgula manhattensis*. (background and contrast modified). The first division completed, it created two large anterior blastomeres and two smaller posterior ones.

Fig. 5

Phase contrast photomicrograph of a whole mount of alive 64-cell stage of *Molgula manhattensis*. Note, differences in cell size and shape highlight the bilateral symmetry of the embryos.

Fig. 6

Phase contrast photomicrograph of a whole mount of alive late gastrula stage of *Molgula manhattensis*. Note, invagination of the endoderm, the involution of the mesoderm, and the epiboly of the ectoderm.

Fig. 7

Phase contrast photomicrograph of a whole mount of alive neurula stage of *Molgula manhattensis*.

Fig. 8

Phase contrast photomicrograph of a whole mount of alive tailbud stage of *Molgula manhattensis*.

Fig. 9

Phase contrast photomicrograph of a whole mount of alive developing larval stage of *Molgula manhattensis*.

Fig. 10

Phase contrast photomicrograph of a whole mount of alive larval stage inside the chorion of *Molgula manhattensis*.

Fig. 11

SEM of a whole mount of hatched larval stage of *Molgula manhattensis*.

Fig. 12

Phase contrast photomicrograph of a whole mount of alive trunk region of *Molgula manhattensis*.

Fig. 13

SEM of a whole mount of trunk region of hatched larval stage of *Molgula manhattensis* showing test cells and brain vesicle with statolith and oolith.

ISSN 2347-6893

Fig. 14

SEM of a whole mount of trunk region of hatched larval stage of *Molgula manhattensis* showing distribution of test cells.

Fig. 15

Phase contrast photomicrograph of a whole mount of a hatching larval stage of *Molgula manhattensis* (beginning of differentiation of the trunk and tail regions).

Fig. 16

Phase contrast photomicrograph of a whole mount of a larval stage of *Molgula manhattensis* (Early tail resorption is indicated by an arrow).

Fig. 17

Phase contrast photomicrograph of a whole mount of a larval stage of *Molgula manhattensis* (advanced tail resorption). The direction of tail phagocytosis is indicated by an arrow.

Fig. 18

Phase contrast photomicrograph of a whole mount of a late metamorphosed larval stage of Molgula manhattensis (note, the test cells surrounding the larva on the periphery).

Fig. 19

Phase contrast photomicrograph of a whole mount of a metamorphosed stage of *Molgula manhattensis*. Note, the tail is completelyresorbed.

Fig. 20

SEM of a whole mount of trunk region of hatched larval stage of *Molgula manhattensis* showing brain vesicle with statolith & oolith and adhesive papillae.

Fig. 21

Phase contrast photomicrograph of a whole mount of a hatched larval stage of *Molgula manhattensis* GFP staining. Note, brain vesicle and the nerve cord showed illumination.

Fig. 22

Phase contrast photomicrograph of a whole mount of partially metamorphosed larval stage *Molgula manhattensis* GFP staining. Note, brain vesicle showed more illumination than the nerve cord.

Fig. 23

Phase contrast photomicrograph of a whole mount of advanced metamorphosed larval stage *Molgula manhattensis* GFP staining. Note, illumination of brain vesicle and the nerve cord gradually decreased.

Fig. 24

Phase contrast photomicrograph of a whole mount of more advanced metamorphosed larval stage *Molgula manhattensis* GFP staining. Note, brain vesicle showed no illumination and the nerve cord was minimally illuminated.

Fig. 25

Phase contrast photomicrograph of a whole mount of metamorphosed stage of *Molgula manhattensis* GFP staining. Note, no illumination.

ISSN 2347-6893

Abbreviations

A P	Adhesive Papilla
AS	Atrial Siphon
B	Blastocoel
BV	Brain Vesicle
Ch	Chorion
FO	Fertilized Oocyte
I F E	Inner Follicular Epithelium
GP	Gut Primordium
N D	Nerve Cord
NC	Notochord
OS	Oral Siphon
OT	Otolith
O F E	Outer Follicular Epithelium
PV S	Perivitelline space
ST	Statolith
T	Tail
TC	Tunic
TK	Trunk
TS	Test Cells
Y	Yolk material

ISSN 2347-6893

(Table 1)

Stages of early embryonic development of *Molgula manhattensis*. A total of 26 stages are divided into six periods. Parentheses in each period mean the start-time and endtime of each period at 20°C.

Stage	**Time duration after fertilization**	**% of hatching**
One cell	23 min	8%
Cleavage period (2.20-3.50 hrs)		
44-cel	2 hr 20min	20%
64-cell	3 hr	25%
76-cell	3 hr 50 min	27%
Gastrula Period (4.55-6.10 hrs)		
110-cell, initial gastrula	4hr 55 min	29%
early gastrula	5 hr 15 min	30%
mid gastrula	5 hr 45 min	35%
late gastrula	6 hr 10 min	37%
Neurula Period (6.3-8.5 hrs)		
early neurula	6 hr 25 min	39%
mid neurula	6hr 50 min	41%
late neurula	7hr 30 min	45%
Tailbud Period (8.10-16.25 hrs)		
initial tailbud I	8 hr 10 min	47%
initial tailbud II	8 hr 40 min	49%
early tailbud I	9hr 10 min	52%
early tailbud II	9hr 50 min	56%
mid tailbud I	10hr 15 min	59%
mid tailbud II	10hr 55 min	64%
late tailbud I	12 hr 30min	70%
late tailbud II	13hr 15 min	79%
late tailbud III	16hr 25 min	95%
Larva Period (18.20 – 50.55 hrs)		
hatching larva	18 hr 20 min	98%
larva with long tail	19 hr 30 min	98%
larva with short tail	50 hr 55 min	70%
newly metamorphosed young	80hr 15 min	50%

ISSN 2347-6893

(Table 2)

Measurement of embryos of *Molgula manhattensis*. T, Time after fertilization (average at 20°C), % hatch = rate of T (min), head length, tail length and ratio of tail / head length

Stage	Measurement of embryos		
	Head length (µm)	**Tail length (µm)**	**Ratio tail/head**
mid neurula	80.2	80.0	0.99
late neurula	84.6	83.1	1.01
initial tailbud I	90.2	89.6	0.99
initial tailbud II	98.9	106.3	1.07
early tailbud I	101.2	128.4	1.26
early tailbud II	117.5	150.4	1.28
mid tailbud I	117.1	186.1	1.58
mid tailbud II	122.3	233.8	1.91
late tailbud I	135.3	260.7	1.92
late tailbud II	145.2	456.9	2.92
late tailbud III	156.8	601.4	3.83
hatching larva	165.4	676.9	4.09
larva with long tail	172,1	804.3	4.67
larva with short tail	180,8	293.7	1.62
newly young	190.8	--	--

ISSN 2347-6893

(Table3) Effect of bioactive substances on larval metamorphosis in *Molgula manhattensis*

Chemical substance	ITS CONC :	No. tested larvae	Obtained stage	after 24 hrs			after 48 hrs			after 72 hrs			after 96 hrs			after 120 hrs		
				1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail	1st trail	2nd trail	3rd trail
Sea water	--	29*	l	29	27	28	24	22	21	12	10	11	9	8	8	6	4	5
		27**	s t				4	5	7	16	17	17	12	14	15	11	12	14
		28***	m				-	-	-	-	-	-	7	5	5	11	11	11
			d				1	-	-	1	-	-	-	-	-	-	-	-
Antibiotic sea water	--	30*	l	30	25	24	24	18	17	13	12	11	10	9	8	5	4	3
		25**	s t				5	7	7	16	13	13	11	9	4	9	10	11
		24***	m				-	-	-	-	-	-	8	6	11	15	10	9
			d				1	-	-	1	-	-	-	-	-	-	-	-
Sea water with heavy metals	--	29*	l	29	31	26	14	16	12	11	10	10	6	4	4	2	3	1
		31**	s t				3	2	1	1	1	1	1	2	2	2	2	3
		26***	m				-	-	-	-	-	-	1	-	1	1	-	2
			d				12	13	13	17	20	15	21	26	19	24	27	20
NH4Cl	**2.5 mM**	27*	l	27	28	29	15	14	12	9	10	11	4	3	6	1	1	2
		28**	s t				12	13	15	17	17	16	10	11	8	5	6	2
		29***	m				-	-	-	-	-	-	12	13	13	20	20	23
			d				-	1	2	1	1	2	1	1	2	1	1	2
	300 µM	32*	l	32	30	26	27	21	19	20	17	10	16	12	6	10	8	2
		30**	s t				2	1	1	9	3	6	6	3	2	9	3	6
		25***	m				-	-	-	-	-	-	6	5	8	9	9	8
			d				3	8	5	3	10	10	4	10	10	4	10	10
C8	10 µ M	26*	l	26	27	29	18	17	21	16	15	18	12	10	12	7	5	6
		27**	s t				2	1	1	4	3	4	5	3	6	6	6	9
		29***	m				-	-	-	-	-	-	3	5	4	11	7	7
			d				6	9	7	6	9	7	6	9	7	6	9	7
	1 µ M	31*	l	31	28	29	15	16	14	8	13	10	10	9	5	1	2	1
		28**	s t				13	11	12	21	14	15	7	8	5	2	4	3
		29***	m				-	-	-	-	-	-	12	10	15	24	21	21
			d				3	1	3	2	1	4	2	1	4	2	1	4
	0.1 µ M	29*	l	29	28	25	21	22	22	19	16	18	16	13	15	10	10	10
		28**	s t				1	2	2	4	8	6	4	7	5	5	6	3
		25***	m				-	-	-	-	-	-	3	4	4	8	8	11
			d				7	4	1	7	4	1	7	4	7	7	4	7
NOS		27*	l	27	23	20	23	19	17	17	15	15	14	12	11	9	8	7
	1 mM	23**	s t				1	1	2	7	5	4	4	5	3	5	2	5
		20***	m				-	-	-	-	-	-	4	3	4	8	10	6
			d				3	3	1	3	3	1	3	3	2	3	3	2

ISSN 2347-6893

	1.5 mM	24* 25** 29***	l s t m d	24	25	29	22 1 - 1	23 1 - 1	25 2 - 2	17 7 - 1	18 8 - 1	20 7 - 2	13 6 5 1	14 6 6 2	16 6 5 1	9 5 10 1	10 4 12 2	9 7 11 1
	2.5 mM	21* 24** 27***	l s t m d	21	24	27	12 8 - 1	13 9 - 2	15 12 - -	8 12 - 1	10 12 - 2	9 18 - -	3 8 9 1	4 6 12 2	4 6 17 -	- 2 18 1	1 3 18 2	1 1 25 -
seratonin	10 µg/ml	28* 25** 27***	l s t m d	28	25	27	15 13 - 1	14 11 - -	13 14 - 1	8 18 - 1	9 16 - -	10 16 - 1	3 7 16 1	4 10 11 -	4 8 14 1	1 1 24 1	- 2 23 -	- 1 25 1
Acetyl choline	1 mM	24* 28** 30***	l s t m d	24	28	30	21 1 - 2	25 1 - 2	27 1 - 2	18 4 - 2	19 7 - 2	20 8 - 2	15 4 3 2	17 5 4 2	17 8 3 2	10 5 7 2	11 6 9 2	9 10 9 2
	0.5 mM	33* 26** 28***	l s t m d	33	28	28	15 16 - 1	14 12 - 2	13 14 - 1	11 21 - 1	10 16 - 2	8 19 - 1	4 12 16 1	3 1 23 2	4 2 21 1	- 1 31 1	- 2 25 2	- 1 26 1
	30 µ g/ml	28* 29** 32***	l s t m d	28	29	32	27 1 - 1	27 2 - -	28 2 - 2	19 8 - 1	18 10 - 1	21 9 - 2	16 7 5 1	16 6 6 1	19 5 7 1	10 9 9 1	11 7 11 1	12 6 13 1

* = No. of tested larvae in the first trial l = larva with long tail s t = larva with shortened tail

** = No. of tested larvae in the second trial m = newly metamorphosed ascidian d = died larva of any stage

*** = No. of tested larvae in the third trial

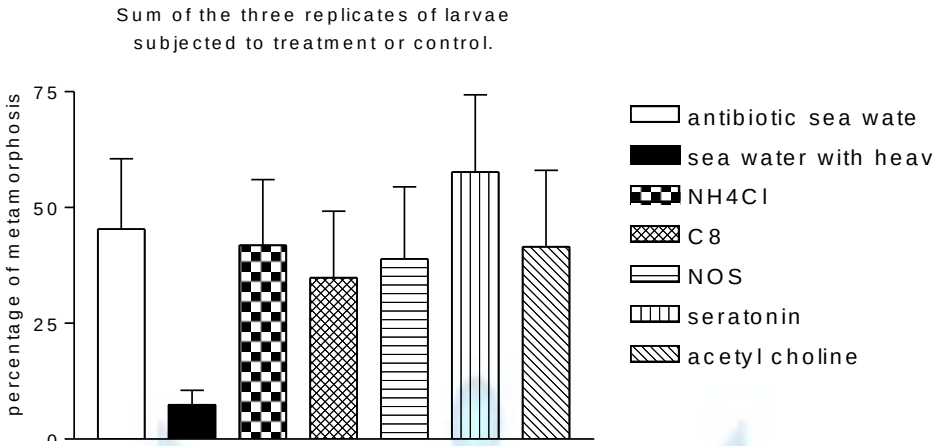
(Table 4)

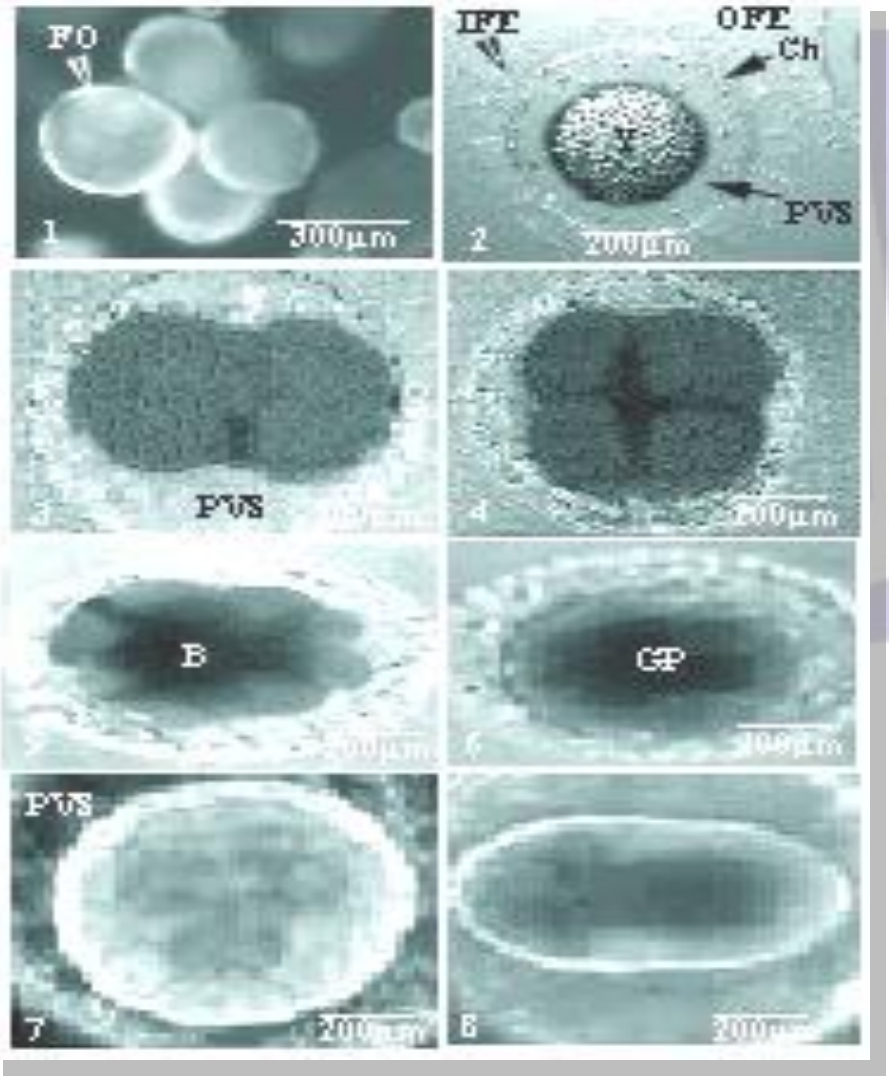
Tukey's Multiple Comparison Test of bioactive chemicals on metamorphosis of *Molgula manhattensis.*

Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff
antibiotic sea wate vs sea water with heav.	37,94	2,648	P > 0.05	-26.40 to 102.3
antibiotic sea wate vs NH4Cl	3,47	0,2422	P > 0.05	-60.87 to 67.81
antibiotic sea wate vs C8	10,47	0,7309	P > 0.05	-53.86 to 74.81
antibiotic sea wate vs NOS	6,407	0,4471	P > 0.05	-57.93 to 70.74
antibiotic sea wate vs seratonin	-12,34	0,8611	P > 0.05	-76.68 to 52.00
antibiotic sea wate vs acetyl choline	3,787	0,2642	P > 0.05	-60.55 to 68.12
sea water with heav vs NH4Cl	-34,47	2,405	P > 0.05	-98.81 to 29.87
sea water with heav vs C8	-27,47	1,917	P > 0.05	-91.80 to 36.87
sea water with heav vs NOS	-31,53	2,201	P > 0.05	-95.87 to 32.80
sea water with heav vs seratonin	-50,28	3,509	P > 0.05	-114.6 to 14.06
sea water with heav vs acetyl choline	-34,15	2,383	P > 0.05	-98.49 to 30.18
NH4Cl vs C8	7,003	0,4887	P > 0.05	-57.33 to 71.34
NH4Cl vs NOS	2,937	0,2049	P > 0.05	-61.40 to 67.27
NH4Cl vs seratonin	-15,81	1,103	P > 0.05	-80.15 to 48.53
NH4Cl vs acetyl choline	0,3167	0,0221	P > 0.05	-64.02 to 64.65
C8 vs NOS	-4,067	0,2838	P > 0.05	-68.40 to 60.27
C8 vs seratonin	-22,81	1,592	P > 0.05	-87.15 to 41.52
C8 vs acetyl choline	-6,687	0,4666	P > 0.05	-71.02 to 57.65
NOS vs seratonin	-18,75	1,308	P > 0.05	-83.08 to 45.59
NOS vs acetyl choline	-2,62	0,1828	P > 0.05	-66.96 to 61.72
seratonin vs acetyl choline	16,13	1,125	P > 0.05	-48.21 to 80.46

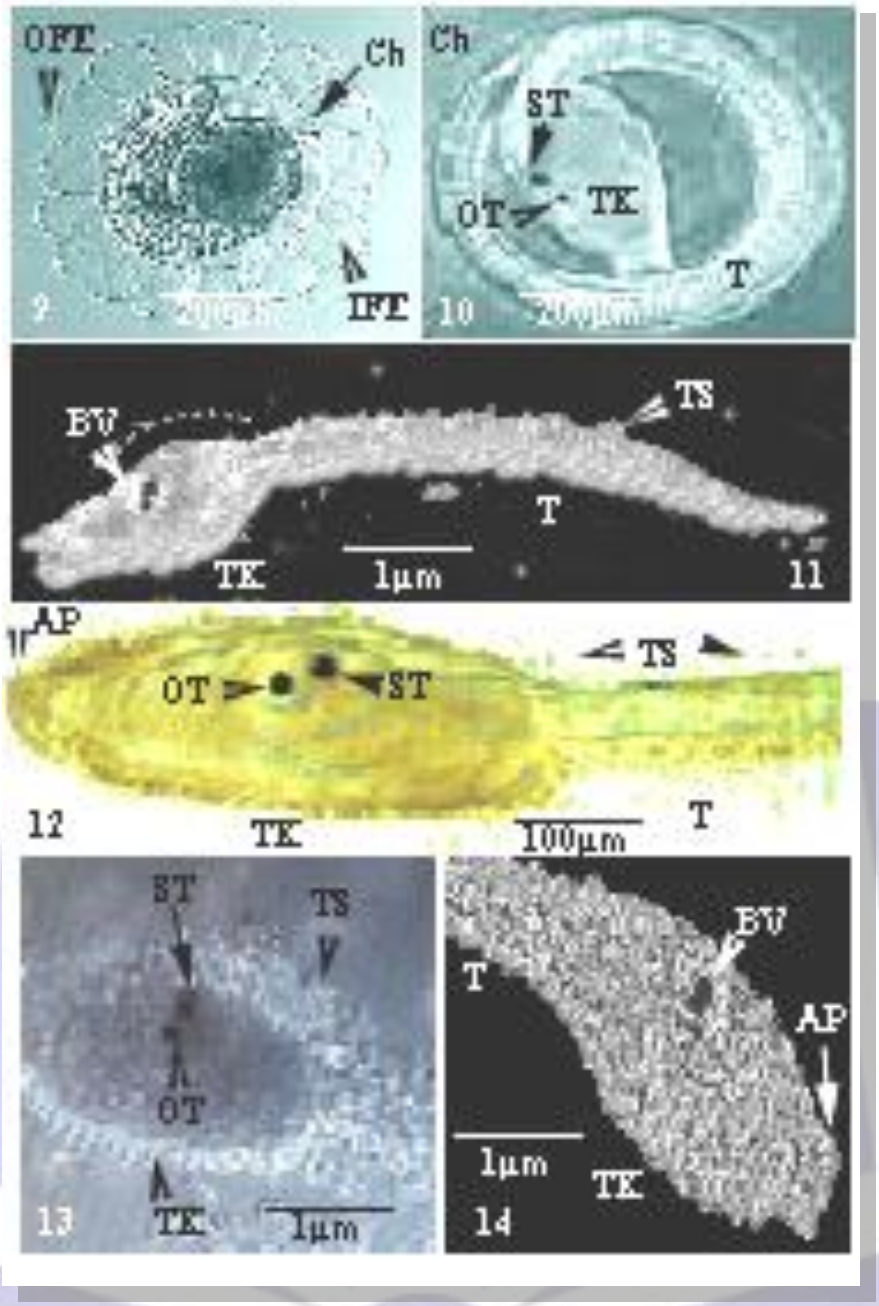
ISSN 2347-6893

Histogram 1

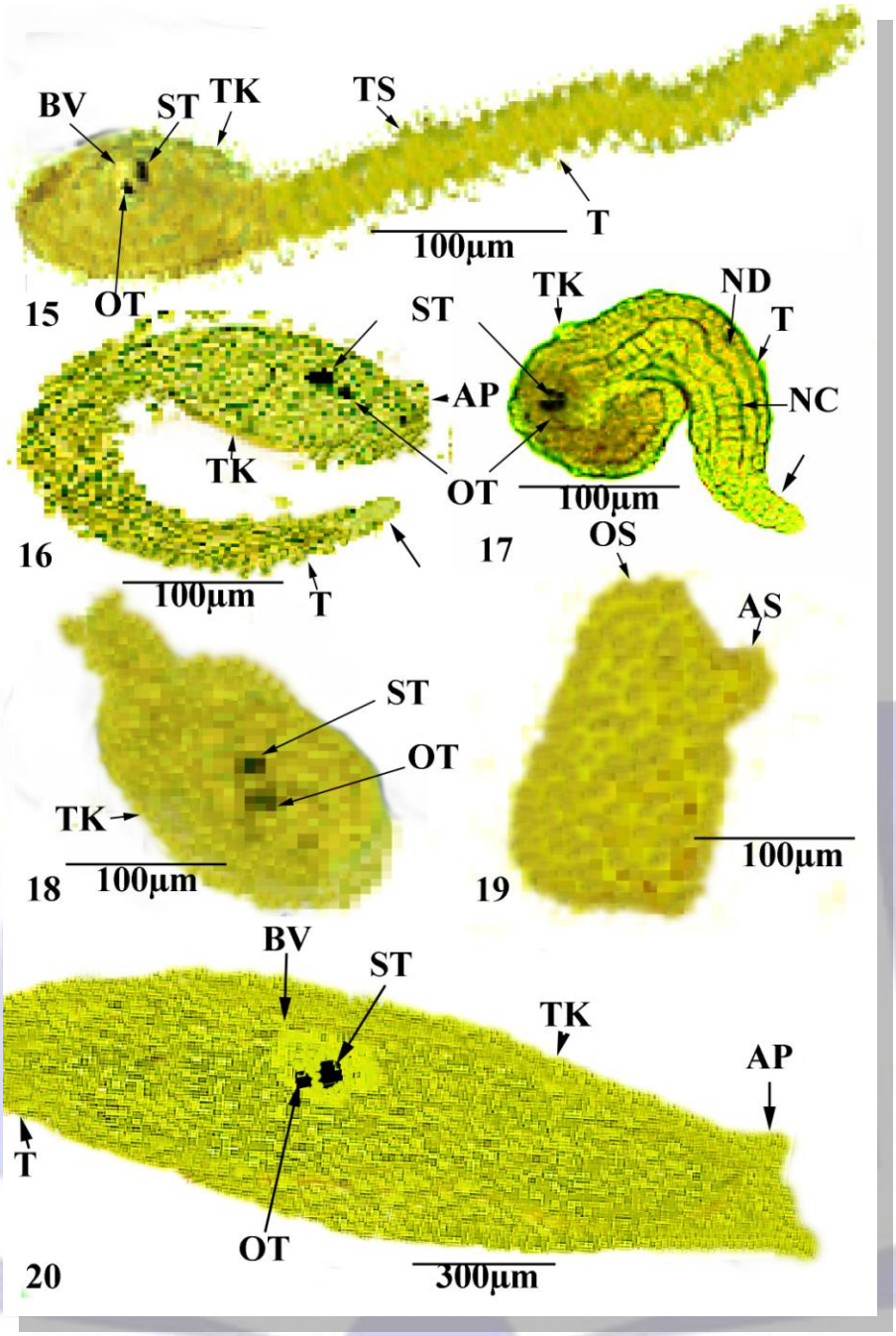




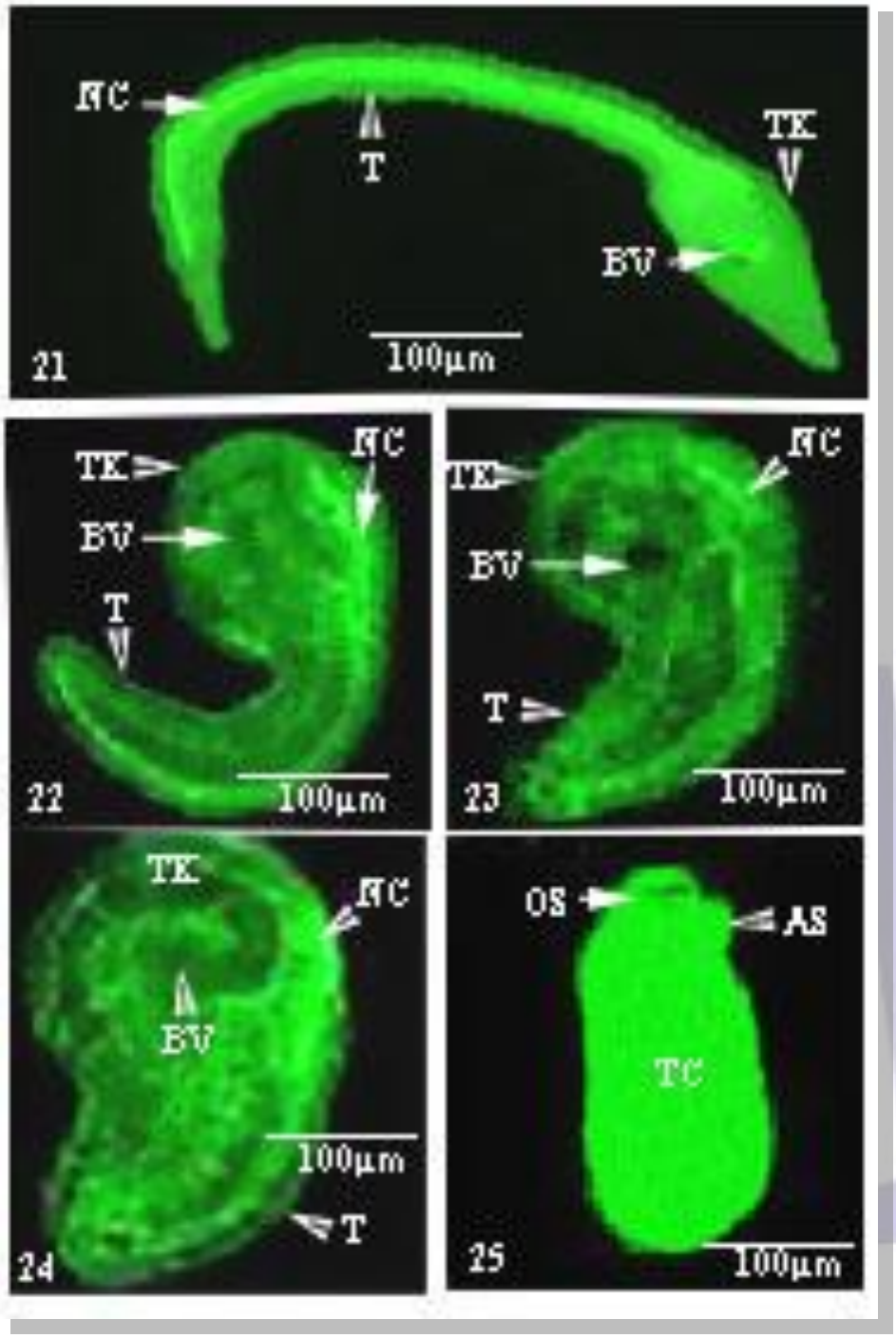
ISSN 2347-6893



ISSN 2347-6893



ISSN 2347-6893



ISSN 2347-6893

REFERENCES

{1} Berking, S. and Herrmann, K. 1990. Dicapryloyl glycerol and ammonium ions induce metamorphosis of ascidian larvae. Roux's Arch. Dev. Biol.198:430–432.

{2} Berrill, N. J. 1947a. Metamorphosis in ascidians. J. Morph. 80: 249-267

{3} Berrill, N. J. 1947b. The development and growth of *Ciona*. J. Mar. Biol. Ass. U. K. 26: 616-625

{4} Bishop, C.D., and Brandhorst, B.P. 2001. NO/cGMP signaling and HSP90 activity represses metamorphosis in the sea urchin *Lytechinus pictus*. Biol. Bull. (Woods Hole), 201: 394–404

{5} Bishop, C.D.; Bates, W.R. and Brandhorst, B.P. 2001. Regulation of metamorphosis in ascidians involves NO/cGMP signaling and HSP90. J. Exp. Zool. 289: 374–384.

{6} 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 *Phestilla sibogae* Bergh (Gastropoda: Opisthobranchia).Evolution and Development, 10(3): 288–299

{7} Bone, Q. 1992. On the locomotion of ascidian tadpole larvae. J. Mar. Biolog. Assoc., U. K. 72: 161-186.

{8} Bredt, D. S. and Snyder, S. H. 1992. Nitric oxide, a novel neuronal messenger. Neuron, 8 : 3 - 11

{9} Burighel, P. and Cloney, R. A. 1997. The invertebrate Chordates'.In Microscopic Anatomy of Invertebrates .Vol. 15 Hemichordata, Chaetognatha and the Invertebrate Chordates. Harris F. W. and Ruppert E. E. eds. A John Willy and sons INC Publication New York .

{10} Castle, W.E. 1896. The early embryology of *Ciona intestinalis* Flemming (L.). Bull. Mus. Comp. Zool. 27: 203-280

{11} Cloney , R. A . 1961.: Observation on the mechanism of the resorption of the tail in ascidians. Am. Zool. 1:67-87

{12} Cloney, R. A. 1964. Development of the ascidian notochord. Acta Embryol.Morphol. Exp., 7: 111-130.

{13} Cloney, R. A. 1975. Ascidian metamorphosis . Review and analysis. In China E. S. and Rice M.(eds.). Amsterdam, Elsevier-North Holland Biomedical Press pp 255-282

{14} Cloney, R.A. 1982. Ascidian larvae and the events of metamorphosis. Amer. Zool. 22: 817-826

{15} Cloney, R. A. 1990. Urochordata: Ascidiacea. In K. G. Adiyodi and R. G. Adiyodi (eds.). Reproduction Biology of Urochordata. Vol. IV B. Fertilization, Development and Parental care. New York, Oxford 391- 451

{16} Comes, S.; Locascio, A.; Silvestre, F.; d'Ischia, M.; Russo, G. L., Tosti, E.; Branno, M., and Palumbo, A. 2006. Regulatory role of nitric oxide during larval development and metamorphosis in *Ciona intestinalis*. Devel. Biol. 306: 772-784

{17} Coniglio, L.; Morale, A.; Angelini, C. and Falugi, C. 1998. Cholinergic activation of settlement in *Ciona intestinalis* metamorphosing larvae. J. exp. Zool. 280: 314-320

{18} Conklin, E.G. 1905a. The organization and cell lineage of the ascidian egg. J. Acad. Sci. (Philadelphia) 13: 1-119

{19} Conklin, E.G. 1905b. Organ forming substances in the egg of ascidians. Biol. Bull. 8: 205-230

{20} Conklin, E. 0.. 1931. The development of centrifuged eggs of ascidians. J. Exp. Zool., 60: 1-119.

{21} D'Agati, P. and Cammarata, M. 2006. Comparative analysis of thyroxine distribution in ascidian larvae. Cell Tissue Res 323: 529-535

{22} Degnan, B.M., Sauter; D._;Degnan, S.N., and Long, S. 1997. Induction of metamorphosis with potassium ions requires development of competence and an anterior signalling centre. Dev Genes Evol 206: 370-376

{23} Diehl , M. 1957. Die Ökologie der Ascidie *Styela coriacea*. Kieler Meeresforschungen. 13: 59-68

{24} Dilly, P. N. 1961. Electron microscope obsevations of the. receptors in the sensory vesicle of the ascidian tadpole. Nature 191:786–787

{25} Dybern I. B. 1965.: The life cycle of *Ciona intestinallis* (L.) typica in relation to the environmental temperature. Oikos 16: 109-131

{26} Gilbert, L. I.; Tata, J.R. and Atkinson, B.G. 1996. Metamorphosis- Postembryonic reprogramming of gene expression in amphibian and insect cells. Academic Press, San Diego, New York, Pp687

{27} Gilbert, S. F. and Raunio, A. M. 1997. Embryology: Constructing the Organism. Sinauer Associates Inc. publishers, Sunderland.

{28} Glaser, O. and Anslow, G. A. 1949. Copper and Ascidian metamorphosis. J. Exp., Zool. 111(1):117-139

{29} Goodbody, I. and Fisher, E. 1974. The biology of *Ascidia nigra* (Savigny). IV. Seasonal and spatial patterns of embryonic development and hatching success. J. Biol. Bull. 146: 206-216

{30} Goodbody, I. and Gibson, J. 1974. The biology of *Ascidia nigra* (Savigny). V. Survival in populations settled at different times of the year. Biol. Bull. 140: 217-237

{31} Grave, C. 1944. The larva of *Styela* (*Cynthia*) *partita*: Structure, activities and duration of life. J. Morph. 75:173-188

{32} 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 immunocytochemical approach. Proc. 5th Int.Conf. Biol.(Zool.), 5:235-248.

{33} Jang, D.; Tresser, J.W., Horie, T., Tsuda, M. and Smith, W.C. 2005. Pigmentation in the sensory organs of the ascidian larva is essential for normal behavior. J. exp. Biol. 208: 433-438

{34} Jia,W.G. 1987. Investigations on the larval nervous system and its development in the ascidian, *Ciona intestinalis*. Z. Zellforsch., 112: 287-312

{35} Ignarro, L J. 1990. Biosynthesis and metabolism of endothelium derived nitric oxide. Annu. Rev. Pharmacol. Toxicol., 30:535 - 560

{36} Katz, M.J. 1983. Comparative anatomy of the tunicate tadpole, *Ciona intestinalis*. Biol. Bull. (Woods Hole) 164:1-27

{37} Kowalevski, A. 1866. Entwickelungsgeschichte der einfachen Ascidien. Mémoires de l'Académie Impériale Sci. St. Petersbourg 10: 1-19, plates I-III

{38} 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.

{39} Krohn, A. 1852. Über die Entwicklung der Ascidien. Virchows Archiv f. pathologische Anatomie und Physiologie und klinische Medizin 6: 312-333, and plate 8

{40} Lambert, C. C. and Brandt, C. L. 1967. The effect of light on the spawning of *Ciona intestinalis*. Biol. Bull. 132: 222-228

{41} Lambert ,G. 1968. The general Ecology and growth of a solitary ascidian *Corella willimeriana*. Biol. Bull. 135 : 290-307

{42} Lützen ,J. 1960. The reproductive cycle and larval anatomy of the ascidian *Styela rustica*. J. Dansk Naturhistorisk Forening 123: 227-235

{43} Marshall, D.J.; Pechenik, J.A. and Keough, M.J. 2003. Larval activity levels and delayed metamorphosis affect post-larval performance in the colonial ascidian *Diplosoma listerianum*. Mar. Ecol. Prog. Ser. 246:53–162

{44} Mast, S. O. 1921. Reactions to light in the larvae of the ascidians, *Amarocium constellatum* and *Amarocium pellucidum* with special reference to their photic orientation. J. Exp. Zool., 34: 149-187

{45} Millar R.H. 1971. The Biology of Ascidians. Adv. mar. Biol., 9: 1-100 Morgan, T. H. 1942. Cross and self-fertilization in the ascidian *Styela.* Biol. Bull. 82: 161-171

{46} Nakayama, A. Satoh, N. and Sasakura, Y. 2005. Tissue specific profile of DNA replication in the swimming laravae of *Ciona intestinalis*. Zool. Sci., 22: 301-309.

{47} Niermann-Kerkenberg, E. and Hofmann, D.K. 1989. Fertilization and normal development in *Ascidiella aspersa* (Tunicata) studied with Nomarski-optics. Helgoländer Meeresunters. 43: 245-258

{48} Olsson R., Holmberg K. and Lilliemarck Y. 1990. Fine structure of the brain nerves of *Oikopleura dioica* (Urochordata-Appendicularia) Zoomorphology 110:1-7

{49} Reverberi , G. 1971. Ascidians . In Experimental Embryology of marine and fresh water invertebrates. Ed. G. Reverberi- North Holland Publ. Co. Amesterdam pp 507-550

{50} 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

{51} Sabbadin , A. 1957. Cyclobiologico di *Ciona intestinalis* (L.), *Molgula manhattensis* (Dekay) e *Styela plicata* (Lesueur). Nella laguna veneta. Archivio di Oceanografia a Limnologia.11:1-30

{52} Satoh , N.1994. Developmental Biology of Ascidians Kyoto University Published by the Press Syndicate of the University of Cambridge. Printed in the united states of America ISBN 0-521-35221-5 hardback

{53} Stern C.D. and Holland P.W.H. 1993. Essential developmental biology.A practical approach. The practical approach series editors : Rickwood D. and Hames B.D. Printed in Great Britain by iInformation Press Ltd, Eynsham, Oxford

{54} Takahashi, T.; Muneoka, Y.; Lohmann, M.S.; Lopez de Haro, G.; Solleder, T.; Bosch, T.C.G.; David, C.N.; Bode, H.R.; Koizumi, O.; Shimizu, H.; Hatta, M.; Fujisawa, T. and Sugijama, T. 1997. Systematic isolation of Peptide signal molecules regulating development in *Hydra*: *Lwamide* and PW families. PNAS (USA) 94: 1241-1246

{55} Vázquez, E.; Craig, M. and Young, J.Z. 2000. Effects of low salinity on metamorphosis in estuarine colonial ascidians. Invertebrate Biology 119(4):433 – 444

{56} 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

{57} West, A. B. and Lambert, C. C. 1975. Control of spawning in the tunicate *Styela plicata* by variations in a natural light regime. J. Exp. Zool. 195:203-270

{58} West, A.B. and Lambert, C. C. 1976. Control of spawning in the tunicate *Styela plicata* by variations in the natural light regime. J. Exp. Zool., 195:263 - 270.

{59} Whittaker, J.R. 1964. Copper as a factor in the onset of ascidian metamorphosis. Nature (Lond.) 202: 1024-1025.

{60} Whittingham, D.G. 1967. Light induction of shedding of gametes in *Ciona intestinalis* and *Molgula manhattensis*. Biol. Bull., (Woods Hole), 132: 292 – 296

{61} Yamaguchi, M. 1970. Spawning periodicity and settling time in ascidians, *Ciona intestinalis* and *Styela plicata*. Records of Oceanographic Works in Japan N.S. 10: 147-155

{62} Yamaguchi M. 1975. Growth and reproductive cycles of the marine fouling ascidians *Ciona intestinalis, Styela placata,Botrylloides violaceus* and *Leptoclinum mitsukurii* at Aburatsubo- Moeroiso Inlet (Central Japan). Marine Biol. 29,253-259