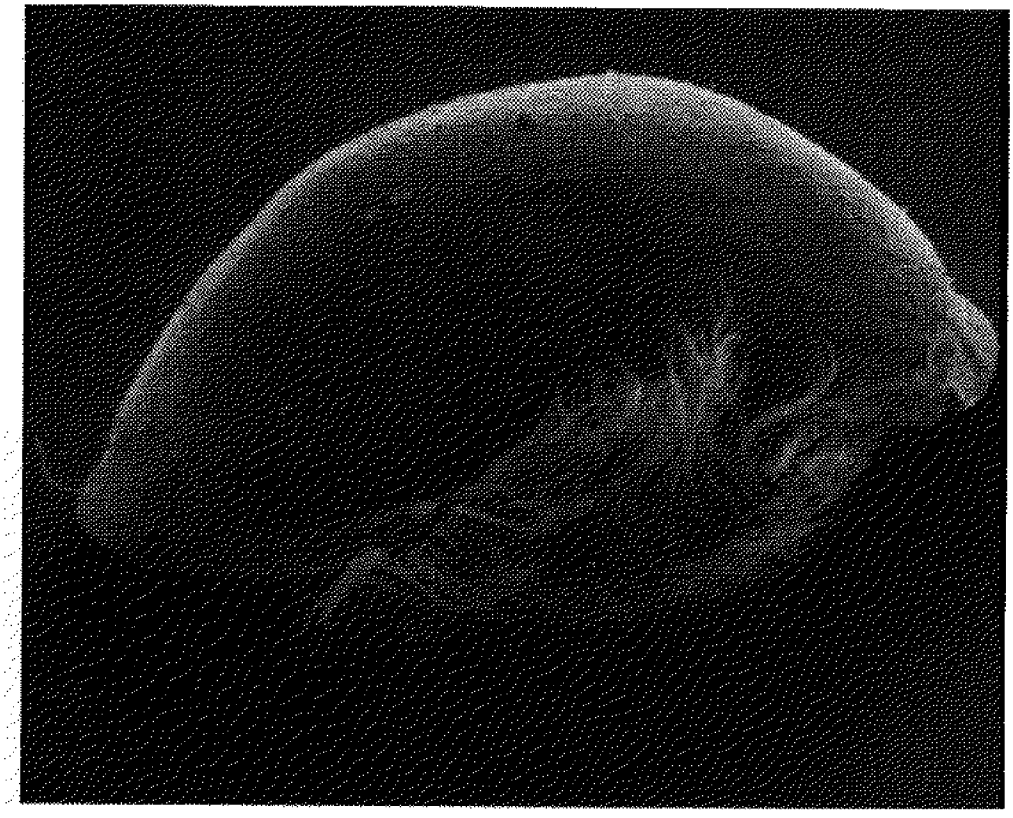
The Illustrated Manual of Hard Clam Reproduction and Development

The Villanova Veliger Group



The Illustrated Manual of Hard Clam Reproduction and Development

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Sea Grant is a unique partnership with public and private sectors combining research, education, and technology transfer for public service. This national network of universities meets changing environmental and economic needs of people in our coastal, ocean and Great Lakes regions.

This manual was produced as part of a cooperative research effort involving Villanova University, New Jersey Sea Grant, and Biosphere Inc., a hatchery in Tuckerton, NJ. Full details of the technical results of this project (*Enhancing Hard Clam Aquaculture Through Manipulations of Larval Culturing Techniques*, R/F-95004) appear in *Deming, C. J. 1998. Relationship between larval and juvenile growth in the hard clam* Mercenaria mercenaria. *MS Thesis, Villanova University.*

The Howard Hughes Medical Institute supported this research through an Undergraduate Biological Sciences Education Programs grant to Villanova University. These funds supported undergraduate fellowships for Jeff Gruber, Rob Meredith, Candace Robertson, and Richard Scafidi. In addition, Natalie Bergren, Sean Byrne, Heather Eshelman, Vincent Giglio, Mike Katsaros, Melissa Logue, Rob Meredith, Vicki Owczarzak, and Steve Smith performed much of the laboratory work. The staff at Biosphere assisted with logistic support and Coley Batey of Little Neck Clam Farms in Charleston, South Carolina provided broodstock for spawning clams to obtain gametes to photograph. Michael Deming drew Figure 1. Jane Morris read a previous draft and Harry Gray and Carolann Weidmayer of Villanova University Graphics Services provided assistance and guidance with the preparation of this document.

Purpose of manual

We intend this manual as an aid for hatchery biologists and aquaculturists who are involved in raising hard clam larvae. The early embryonic and larval phases are the most vulnerable and sensitive periods in the life cycle of most marine species, including *Mercenaria mercenaria*, the hard clam. Raising larval hard clams requires much care, attention, and vigilant observations of the developmental stages. There are a number of publications which cover various aspects of the hard clam fishery from how to set up a hatchery[1] to the history of the industry[2]. In addition, there is a wealth of published information about the biology of hard clams[3]. This manual is not meant to replace these works, or even provide a summary. Instead this manual will compliment these works by providing detailed illustrations and photographs of the difficult-to-observe early embryonic and larval stages. Combining this manual with other sources, e.g., references cited herein, will furnish those interested in the hard clam fishery a resource for successfully rearing the larval stages.

Electron microscopes are not readily available to most workers in the hard clam industry; however, hatchery biologists have light microscopes. While standard compound light microscopes are indispensable tools for assessing larval densities and making gross observations of the general health of larval cultures, they render "flat" or two dimensional images. On the other hand scanning electron micrographs, or SEM's (photographs taken with the electron microscope), reveal structural detail that the light microscope simply cannot. Here we provide SEM's to illustrate the early developmental stages and larvae of the hard clam. These illustrations reveal three-dimensional details of morphology not easily observed with light microscopes. These images will aid in making interpretations of the health and status of larval cultures.

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[1] Castagna, M. and Kraeuter, J.N. 1981. Manual for Growing the Hard Clam Mercenaria. Special Report in Applied Science and Ocean Engineering No. 249, Virginia Institute of Marine Science, Gloucester Point, Virginia. *This is an excellent review of all phases of running an aquaculture facility for rearing hard clams. Unfortunately, it is not in print; however, it is available in the libraries of many academic institutions.*

[2] Rice, M.A. 1992. The Northern Quahog: The Biology of *Mercenaria mercenaria*. Sea Grant Publication No. RIU-B-92-001, Rhode Island Sea Grant. *This is a very readable and thorough review of the fishery. It is available from Rhode Island Sea Grant, Publications Unit, Narragansett, RI 02882-1197*

[3] McHugh, J.L. and Sumner, M.W. 1988. Annotated Bibliography II of the Hard Clam *Mercenaria mercenaria*. NOAA Technical Report NMFS 68.

McHugh, J.L., Sumner, M.W., Flagg, P.J., Lipton, D.W. and Behrens, W.J. 1982. Annotated Bibliography of the Hard Clam *Mercenaria mercenaria*. NOAA Technical Report NMFS SSRF-756. *These volumes contain over 2,500 citations of publications dealing with all aspects of* Mercenaria mercenaria. *They are available through: National Technical Information Service, the US Department of Commerce, Springfield, VA 22161*

A Brief Natural History of Hard Clams, Their Reproductive Biology, and Early Development

Mercenaria mercenaria reside in sandy and muddy shallow coastal waters along the western coast of the Atlantic Ocean, ranging from the Gulf of St. Lawrence in Canada to southern parts of Florida.[4] In the scientific literature published prior to 1960, this species was called *Venus mercenaria* and it is commonly known as the hard clam, northern quahog, littleneck, or cherrystone. Hard clams support a large and valuable commercial shellfishery and aquaculture industry in the eastern United States and they are frequently used in marine invertebrate biological research.

Hard clams are protandric hermaphrodites. This simply means that when individual clams reach sexual maturity they are sperm-producing males and as they grow they turn into egg-producing females.[5] Energetically, sperm are "cheaper" to produce than eggs, i.e., they are smaller and require much less raw material such as proteins and carbohydrates. This type of reproduction allows smaller individuals to achieve reproductive potential by producing sperm whereas eggs are produced in larger, slower growing individuals that can afford to expend relatively more energy on reproduction.[6] Protandric hermaphroditism is a common mode of reproduction among invertebrates.

Most clams, including *M. mercenaria*, are dioecious broadcast spawners – sperm and eggs are released into the surrounding water where fertilization occurs en masse (see Fig. 1 for an illustration of the sequence of all the developmental stages and see Figs. 1 & 2 for photographs of the sperm and egg). The synchronization of spawning events is initially triggered by environmental conditions, e.g., water temperature and phytoplankton (unicellular algae and other microscopic organisms) levels; however, the presence of sperm and eggs in the water also acts as a cue for individuals to spawn.

Fertilization takes place when sperm fuse with an egg cell and the genetic material of a single sperm penetrates the egg membrane. Immediately after fertilization a series of reactions occur to prevent other sperm from penetrating the egg. Within minutes the fertilized egg undergoes repeated cell divisions, i.e., one cell divides into two, two into four, four into eight etc. Early embryonic cell division is sometimes referred to as cleavage. Although the number of cells increases, the size of the developing embryo does not, at least initially. Just prior to the first cleavage, but after fertilization, a small "polar body" buds from the fertilized egg (see Figs. 4 & 5). This represents the remnants of the process (meiosis) that took place in the female clam to produce the viable egg. It is visible through the first few cleavage stages and is eventually lost, e.g., it is visible in the 4-cell stage (Fig. 7).

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[4]Morris 1973. A Field Guide to Shells of the Atlantic and Gulf Coasts and the West Indies: Houghton Mifflin Press.

[5]Mackie, G.L. 1984. Bivalves. In: The Mollusca. Pp. 351-418. Academic Press. New York: Academic Press.

[6]Ghiselin, M.T. 1987. Evolutionary aspects of marine invertebrate reproduction. In: Reproduction of Marine Invertebrates. Vol. IX. Giese, A.C., Pearse, J.S. and Pearse, V.B. (eds.) Pp. 609-665. Blackwell/Boxwood Press. Pacific Grove, California.

There is a characteristic pattern to the early division of the cells in the embryonic stages of clams, i.e., cleavage is *unequal* and *spiral*. Unequal refers to the asymmetric sizes of the two "daughter cells" that result from the early divisions – this is clearly illustrated in the 2-cell stage (Fig. 6) and can be seen in the 4-cell (Fig. 7) and 8-cell (Fig. 8) stages as well. In addition, the position of the cells relative to each other at the 8-cell stage form a *spiral*, i.e., the 4 smallest cells occur in a plane that is parallel to the plane of the four largest cells and these 4 smallest cells sit in the furrows formed between the adjacent largest cells (Fig. 8). In some other groups of organisms the spatial positioning of cells is known as radial: this is when the 4 smallest cells sit directly on top of the 4 largest cells rather than in the furrows.

The cells continue to divide and the first distinct larval stage, the *trochophore*, is reached in approximately one day (Fig. 9). At this point in development the multicellular trochophore is roughly the same size as a fertilized egg (compare Fig. 5 to Fig. 9). The trochophore does not look anything like an adult clam; however, this stage lasts less than one day and quickly transforms into the veliger larval stage. The *veliger* is the most distinctive larval phase of mollusks (the group of organisms that hard clams and all other bivalves belong to).

Externally, veligers resemble adult clams primarily because they display the first traces of the shell (Figs. 12 – 13). Veligers derive their name from a specialized swimming and feeding structure that is unique to this period of the lifecycle, i.e., the *velum* (a Latin word meaning veil or curtain). This structure consists of hundreds of hair-like projections (cilia) extending from a contractile collar that surrounds the mouth. These cilia beat synchronously in a wave-like fashion and the velum is used to both propel the veliger through the water and capture food particles. Initially, veligers lack many internal adult features but will develop most of them during this 10-day to 2-week swimming stage. As the veliger continues to feed it matures and grows larger while developing more and more adult features. Some workers subdivide the veliger phase of the lifecycle into separate stages that are characterized by the appearance of distinct features.

The last larval stage, the *pediveliger*, is recognized by the presence of the muscular foot which at this stage is ciliated. The pediveliger is the transitional phase of the lifecycle when the clam transforms from a swimming larvae into a benthic (bottom-dwelling) juvenile. When the velum is reabsorbed, the clam has completed its swimming larval stage and then spends the rest of its life burrowing into the sediment. It takes a recently "settled" clam at least one year and sometimes two years to reach sexual maturity. After settlement the cilia on the foot are soon lost and a clam uses its foot to burrow into and through the sediment.

The entire larval phase from fertilization to final settlement takes anywhere from one to two weeks and depends on environmental conditions such as water temperature and available food. During this time the larvae are actively feeding on phytoplankton and transported by currents. Although capable of swimming they do not move very far and larvae are transported from areas where they are spawned to sites of final settlement by nearshore currents.

Methods and Materials

To photograph the various stages of hard clam development we spawned clams and reared the developing embryos and larval stages. Details of the techniques and methods we used in this process are contained in the *Manual for Growing the Hard Clam Mercenaria* (see footnote #1). Below we provide an outline of these methods. We obtained sperm and eggs from clams provided by Little Neck Clam Farm in Charleston, South Carolina. These clams were shipped to Villanova University and all the work described herein was conducted in the laboratory at the university.

Spawning Clams, Fertilization, and Larval Rearing

1. All seawater used in the following steps was filtered to remove all the particulate material and as many contaminants as possible. The salinity of the seawater was maintained between 28 and 30 ppt. To further sanitize the seawater we used a UV sterilizer, a 1 micron bag filter, and then boiled it for 10 minutes (in previous trials we found that boiling did not alter the salinity significantly). After this process we held the seawater in two 20-liter reservoirs at two temperatures: 12° C and 28° C.
2. The day before spawning we scrubbed the clams (no soap) in cold fresh water to remove any debris from the shells and placed them in a refrigerator at 5° C where they remained dry overnight.
3. The day we spawned them we isolated each clam in a 1-liter beaker and placed the beakers in a water bath. Initially both the seawater in the beakers and the water bath were maintained at 22° C. It took approximately 45 minutes for half the clams to open up and extend their siphons (this was encouraged by placing a small amount of algal food in the water, i.e., *Isochrysis galbana*).
4. Beginning with a warm cycle, we administered a series of warm and cold temperature shocks every 15 minutes. This was accomplished by using a turkey baster to carefully siphon off as much seawater as possible from each beaker (without disturbing or exposing the clams) and replacing it with either warm (28° C) or cold (12° C) seawater. During these cycles either warm or cold tap water was run through the water bath and a thermometer was used to monitor the temperature in each beaker.
5. When a clam began to spawn, the beaker was removed from the water bath.
6. After 3 cycles, the clams were removed from the beakers and the seawater containing the eggs and sperm were filtered (separately) with a 250-micron sieve to remove mucous and fecal material. Figure 2 of a single sperm and Fig. 3 of an unfertilized egg are from this step in the process.
7. The sperm water was diluted (1:1 sperm water : seawater) and then 5 to 10 ml of the sperm water was mixed into the beakers containing the egg water. Mixing occurred for 5 minutes. The micrograph (Fig. 4) of the sperm attached to the egg is from this step in the process.
8. The fertilized eggs were washed free of sperm by filtering the mixture through a 25-micron sieve. The fertilized eggs were used to set up larval cultures using sterilized beakers with filtered seawater at a density of 5 fertilized eggs/ml. The fertilized egg in Fig. 5 is from this step in the process.
9. Each beaker with a larval culture had a small glass tube (2 ml inside diameter) extending about half way down in the middle. This tube was connected to a small aquarium air pump which was regulated to produce about one bubble/second. This provided gentle aeration and circulation in the culture. The temperature of the larval cultures was held at 22°C.
10. Once the cultures reached the late trochophore – early veliger stage, the developing larvae were fed a mixture of *Isochrysis galbana* (T-iso and C-iso). The remaining figures (6 through 13) are from the last two steps in the process.

Electron Microscopy

The references cited below contain beautiful scanning electron micrographs of clam larvae and provide detailed descriptions of larval clam development and functional anatomy[7]. These references also outline methods for preserving and preparing larvae for examination using an electron microscope.

All stages of development from gametes (the generic term for sperm and eggs) to veligers were preserved and prepared using the methods detailed below. Although the larval cultures were held at 22°C, all other steps occurred at room temperature. We used a Hitachi S-570 scanning electron microscope to view the samples and photographs were taken with Polaroid Type 55 film.

Sample Preparation

1. All the seawater in these steps was filtered (1 micron bag filter) and buffered with sodium cacodylate (25 mM). Samples were drawn from cultures with a pipette (1 – 2 ml for eggs and sperm; 5 – 10 ml for later stages). These were preserved in a 2% glutaraldehyde solution made with seawater. Samples remained in this solution for at least 24 hours.
2. The samples were placed on poly-L-lysine coated cover slips (the specimens stuck to the surface of the cover slips and in all subsequent steps the specimens remained on the cover slips).
3. Cover slips were rinsed in seawater three times (at least 15 minutes each rinse).
4. Samples were then post-fixed in 1% Osmium tetroxide made with seawater (30 minutes).
5. The samples were rinsed again in seawater three times (at least 15 minutes each rinse).
6. Specimens were dehydrated in a graded series of ethanol solutions, i.e., 15 minutes in each: 25%, 50%, 75%, and 95%. The 25% and 50% ethanol solutions were diluted with a 3.5% NaCl aqueous solution. The 75% and 95% ethanol solutions were diluted with distilled water.
7. The samples were then placed in 3 changes of 100% ethanol, 15 minutes each.
8. The ethanol was exchanged with Amyl acetate in 3 rinses, 15 minutes each.
9. Prior to critical point drying amyl acetate was substituted with liquid CO_2 by purging the sample chamber 3 times at 30 minute intervals.
10. The critical point was attained by warming the water jacket so that the internal temperature and pressure reached 35°C and 1200 psi respectively. The pressure was then bled off. The critical point drier is a Polaron E3100.
11. The slides containing the samples were mounted on aluminum specimen stubs with double sided conductive carbon tape. Silver paint was applied in a ring around the sample to provide contact to the metal stub prior to sputter coating with Gold/Paladium for 60 seconds in a Polaron SC7640 sputter coater.
12. The stubs were placed in the electron microscope and the samples photographed.

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[7]*Two papers containing both excellent scanning electron micrographs of clam larvae and instructions for preparing specimens are:*

Waller, T.R. 1981. Functional morphology and development of veliger larvae of the European oyster, *Ostrea edulis* Linne. Smithsonian Contributions to Zoology. 328: 1-70.

Gros, O., Frenkiel, L. and Moueza, M. 1997. Embryonic, larval, and post-larval development in the symbiotic clam *Codakia orbicularis* (Bivalvia: Lucinidae). Invertebrate Biology 116: 86-101.

Figures

All the scale bars in the SEM's are in units of microns (μm). There are 1,000 μm in one millimeter (there are approximately 25 millimeters in an inch so there are roughly 25,000 μm per inch).

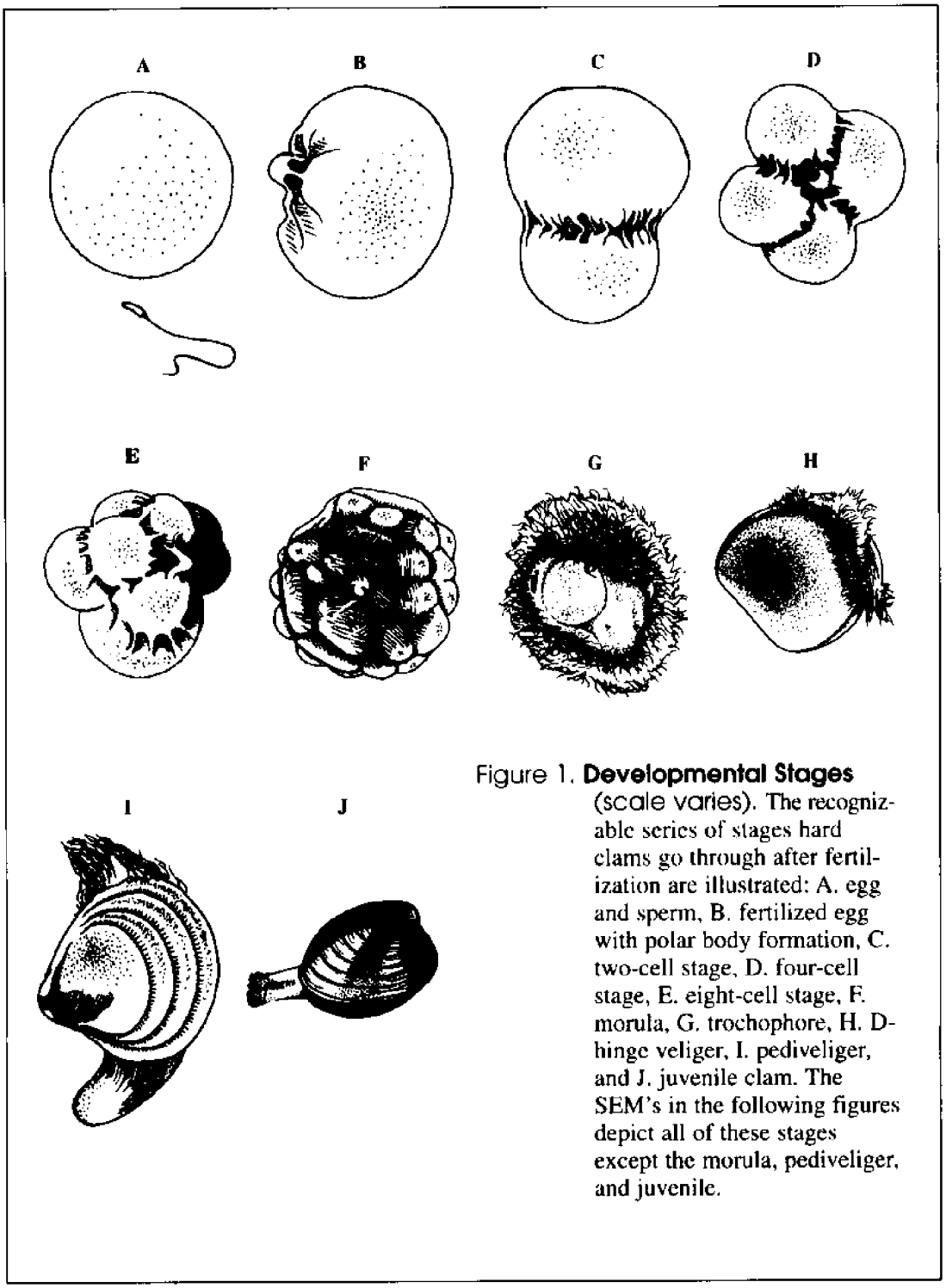


Figure 1. **Developmental Stages** (scale varies). The recognizable series of stages hard clams go through after fertilization are illustrated: A. egg and sperm, B. fertilized egg with polar body formation, C. two-cell stage, D. four-cell stage, E. eight-cell stage, F. morula, G. trochophore, H. D-hinge veliger, I. pediveliger, and J. juvenile clam. The SEM's in the following figures depict all of these stages except the morula, pediveliger, and juvenile.

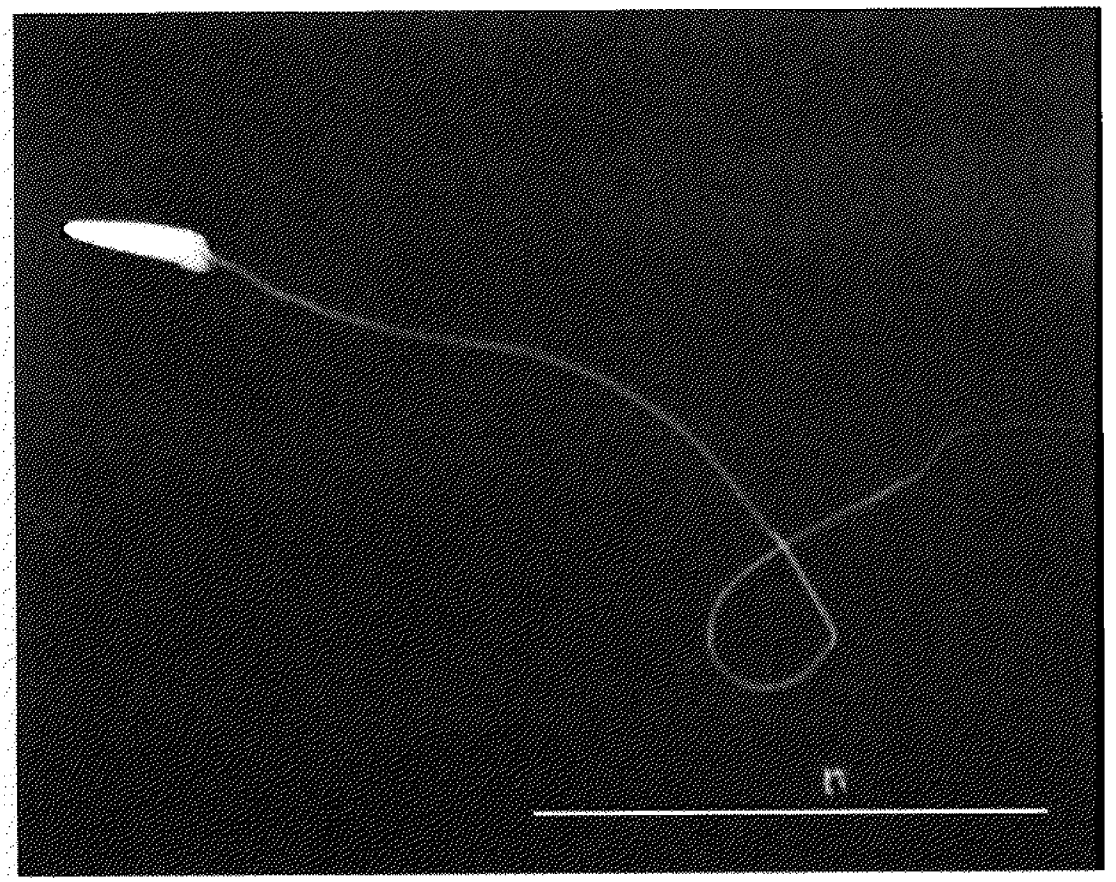


Figure 2. **Sperm** (scale bar = 20μm).

The long tapering tail (~60μm) is approximately equal to the diameter of an egg (see Fig. 2) and is 7.5 x the length of the short triangular shaped head. Sperm are motile and the tail beats to propel the sperm through the water. When a clam spawns the sperm (or eggs if it is a female) are released through the exhalant siphon.

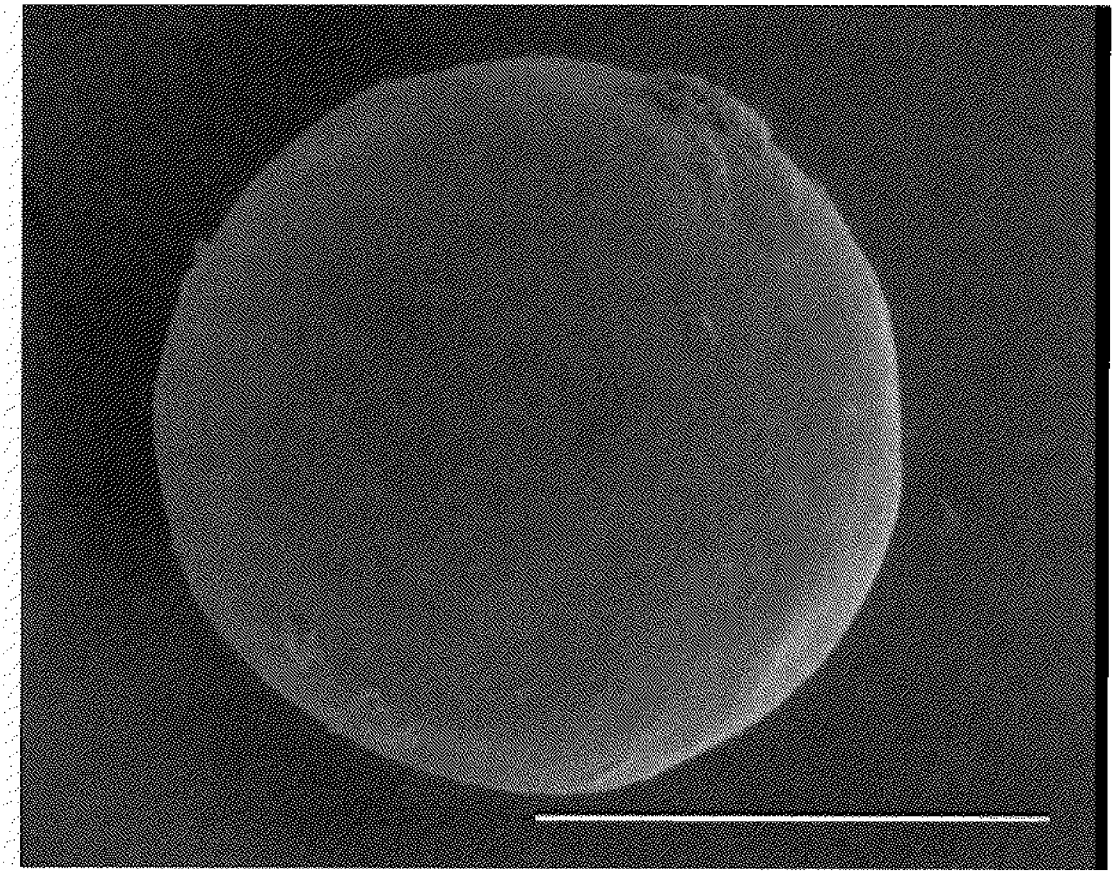


Figure 3. **Unfertilized Egg** (scale bar = 40μm).

These are nearly spherical and range in diameter from 60 to 70 μm. Eggs are negatively buoyant and will sink to the bottom in calm water. When a female spawns the collection of eggs on the bottom of the container appears pink. If left undisturbed on the bottom for more than 10 or 15 minutes the eggs will begin to stick together and will not develop normally; however, too much agitation will rupture the membranes.

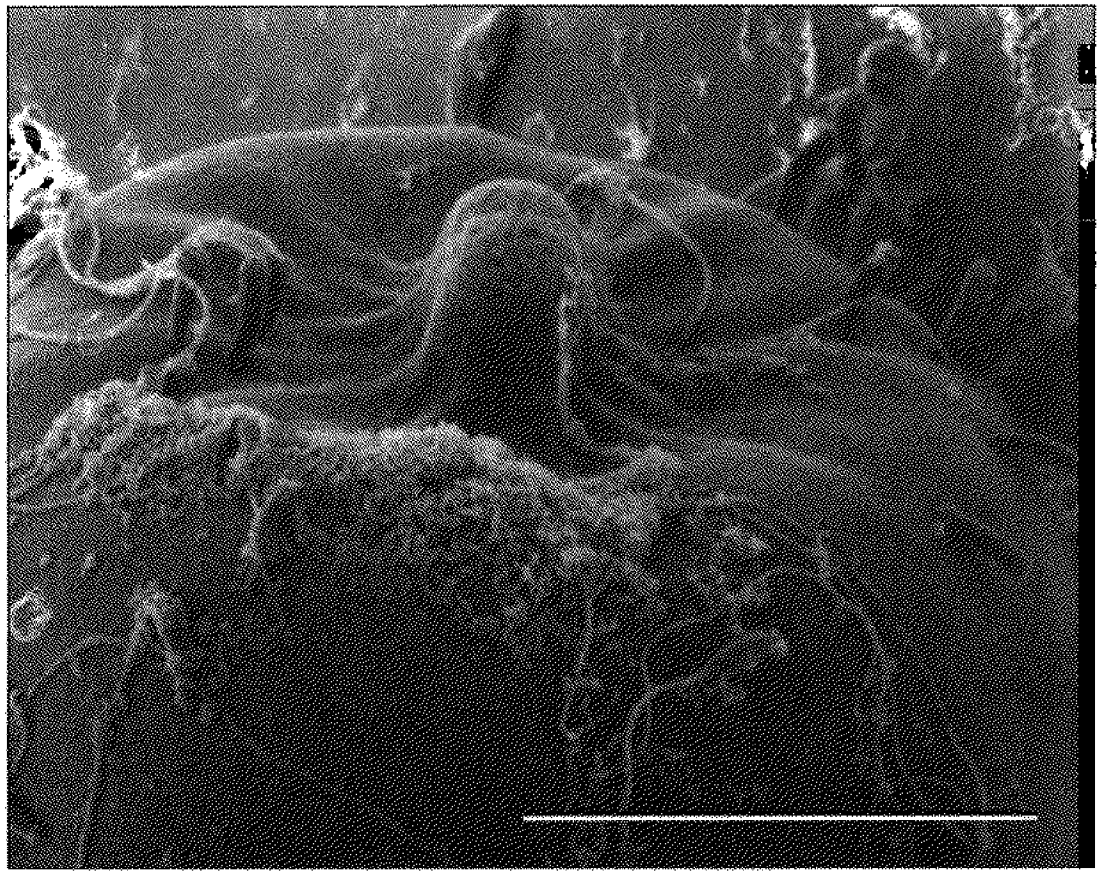


Figure 4. **Sperm Fertilizing Egg** (scale bar = 20μm).

Several sperm can be seen with the tips of their heads attached to the membrane of the egg. Although the genetic material from only one sperm will combine with the genetic material from the egg, several sperm are usually required to render the egg susceptible to fertilization. Once the genetic material from a sperm successfully penetrates the egg, a reaction occurs along the membrane of the fertilized egg that prevents other sperm from penetrating. This is critical if normal development is to occur. If the genetic material from more than one sperm penetrates the egg then normal development is unlikely; therefore, it is possible to "over-fertilize" eggs.

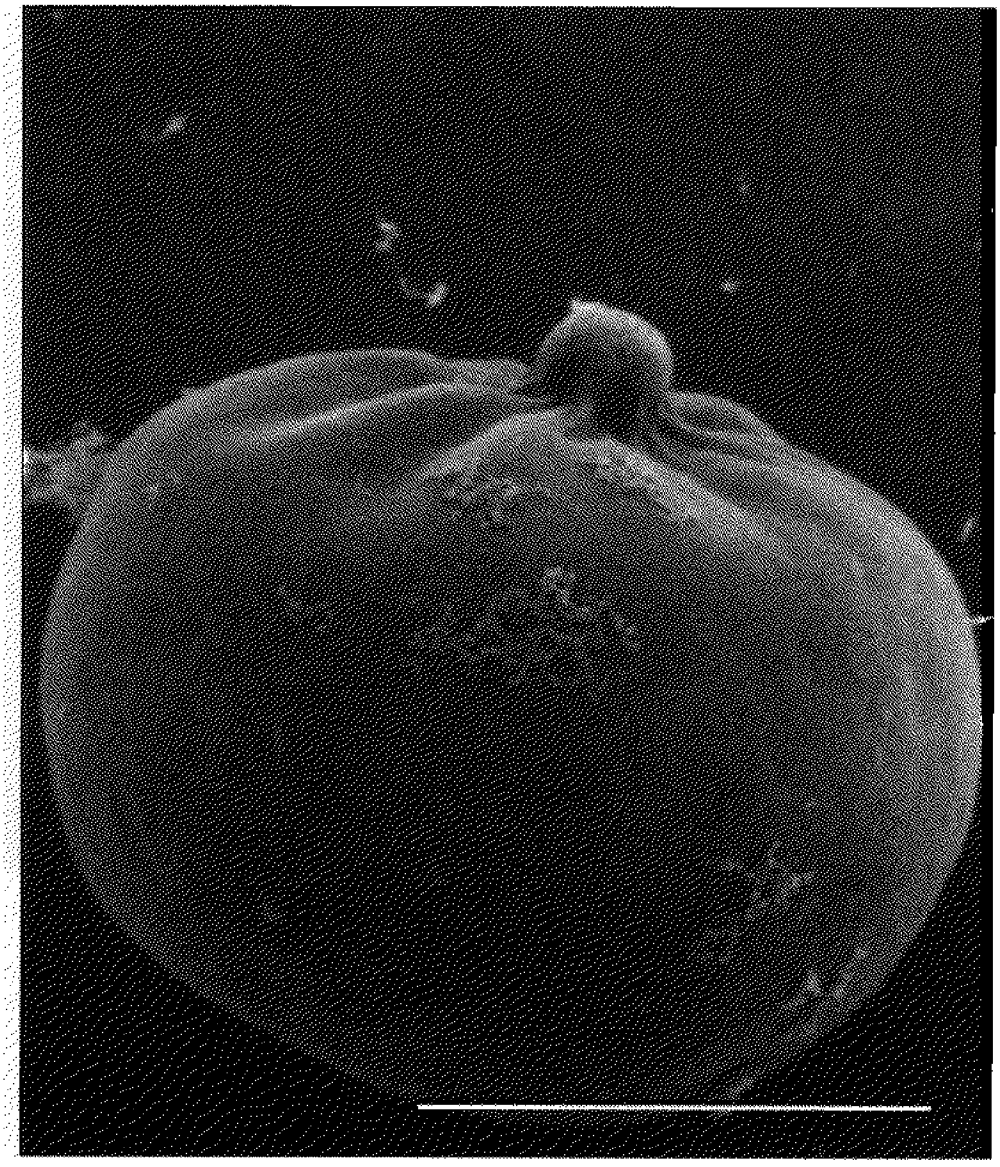


Figure 5. **Fertilized Egg** (scale bar = 30μm).

The sperm have been washed from the surface of the egg (see step #8 under *Spawning Clams, Fertilization and Larval Rearing* above). The *polar body* is obvious in this and the previous figure. It is the "stem" of this "pumpkin shaped" cell. Each sperm and egg have half the genetic material of the clam so when the two fuse the fertilized egg gets half its genes from its mother and half from its father. During the production and development of gametes the genetic material is first doubled in an immature gamete cell. This cell then divides the genetic material in half and splits into two cells. In sperm these two cells divide again (each halving the genetic material) and produce 4 functional sperm with half the complement of the genetic information. However, in females, after the first division of the immature egg cell, only one of the two cells is viable. The halving of the genetic material occurs *inside* this viable cell (the other cell is non-functional). Once fertilization occurs the other half of the maternal genetic material that is not used is "budded off" as a polar body.

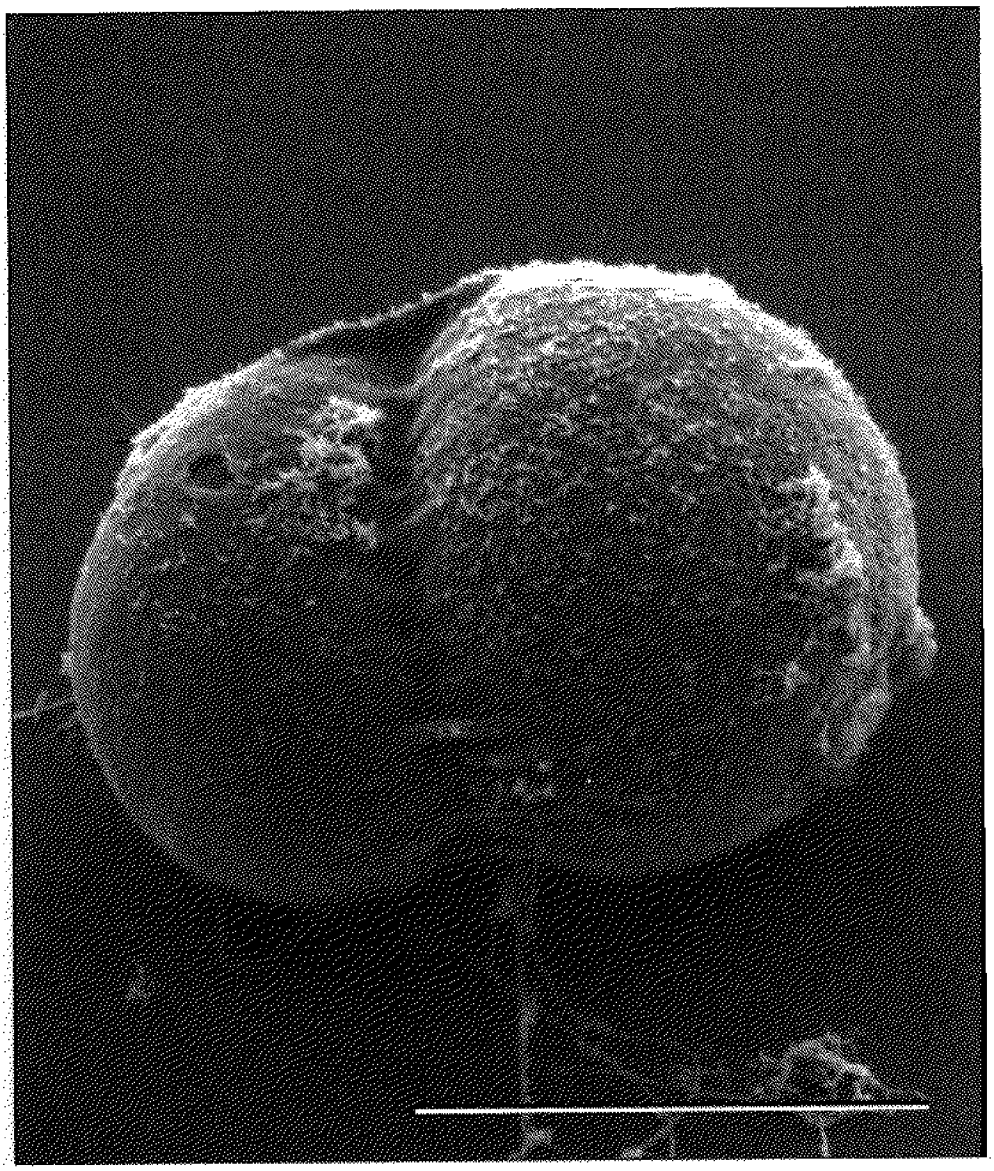


Figure 6. **Two-cell Stage** (scale bar = 40μm).

This sample was preserved 20 - 30 minutes after fertilization. The single fertilized egg cell has divided into two cells. Now each time a cell divides the genetic material is doubled prior to division and a full complement of genes is apportioned to the 2 cells resulting from the division. These early cell divisions are also known as "cleavage," i.e., the cells are cleaving in two. Notice that there is an asymmetry to the material in each of the two cells (the right cell is bigger and has more material than the left cell). This type of cleavage is known as *unequal*. Although it is present, the polar body is not visible in this sample.

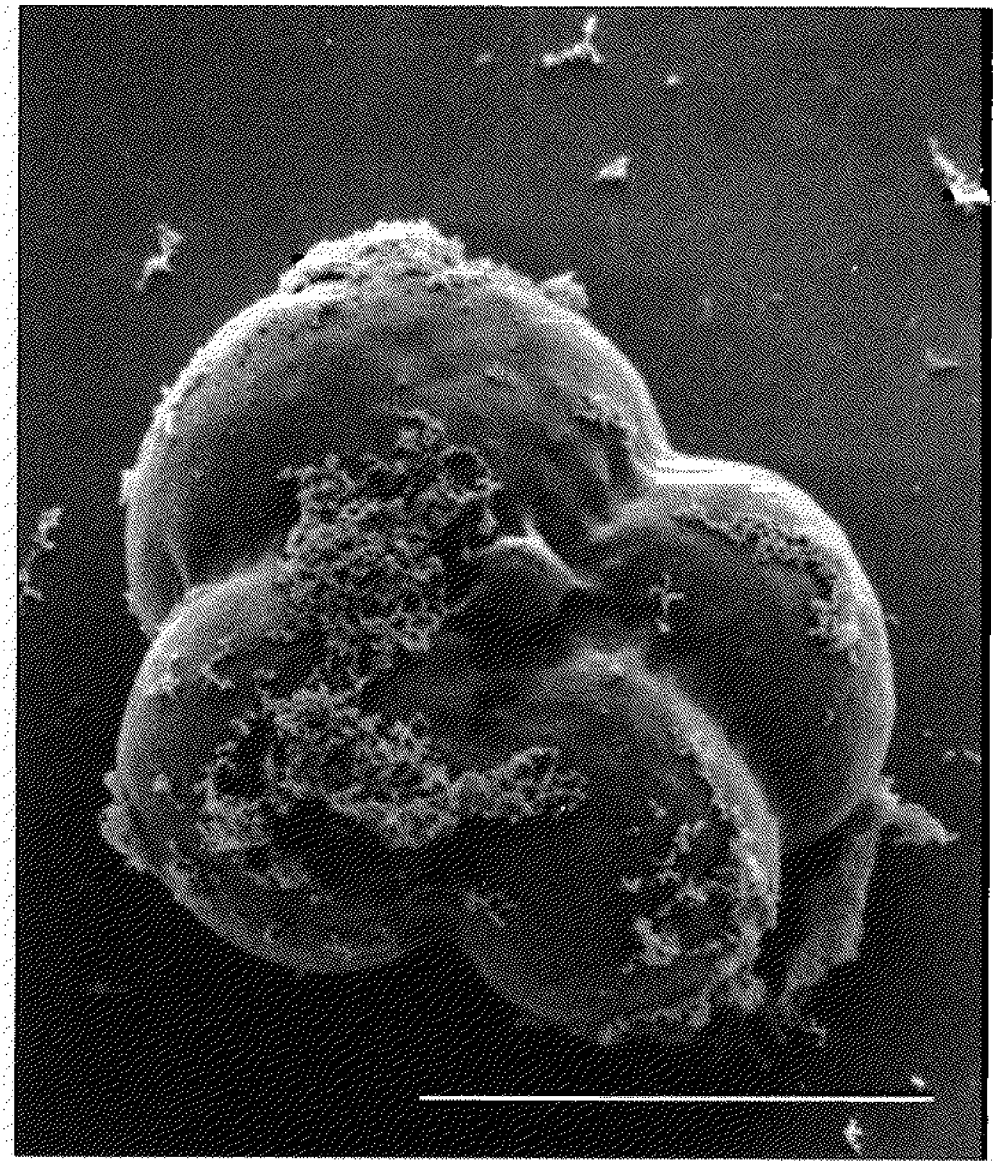


Figure 7. **Four-cell Stage** (scale bar = 40μm).

This sample was preserved 2.5 hours after fertilization. The polar body is visible at the juncture of all four cells. The unequal size of the four cells is obvious. To visualize how the next stage is produced, envision a plane that passes through the center of each cell. Each cell will divide in a direction perpendicular to this plane.

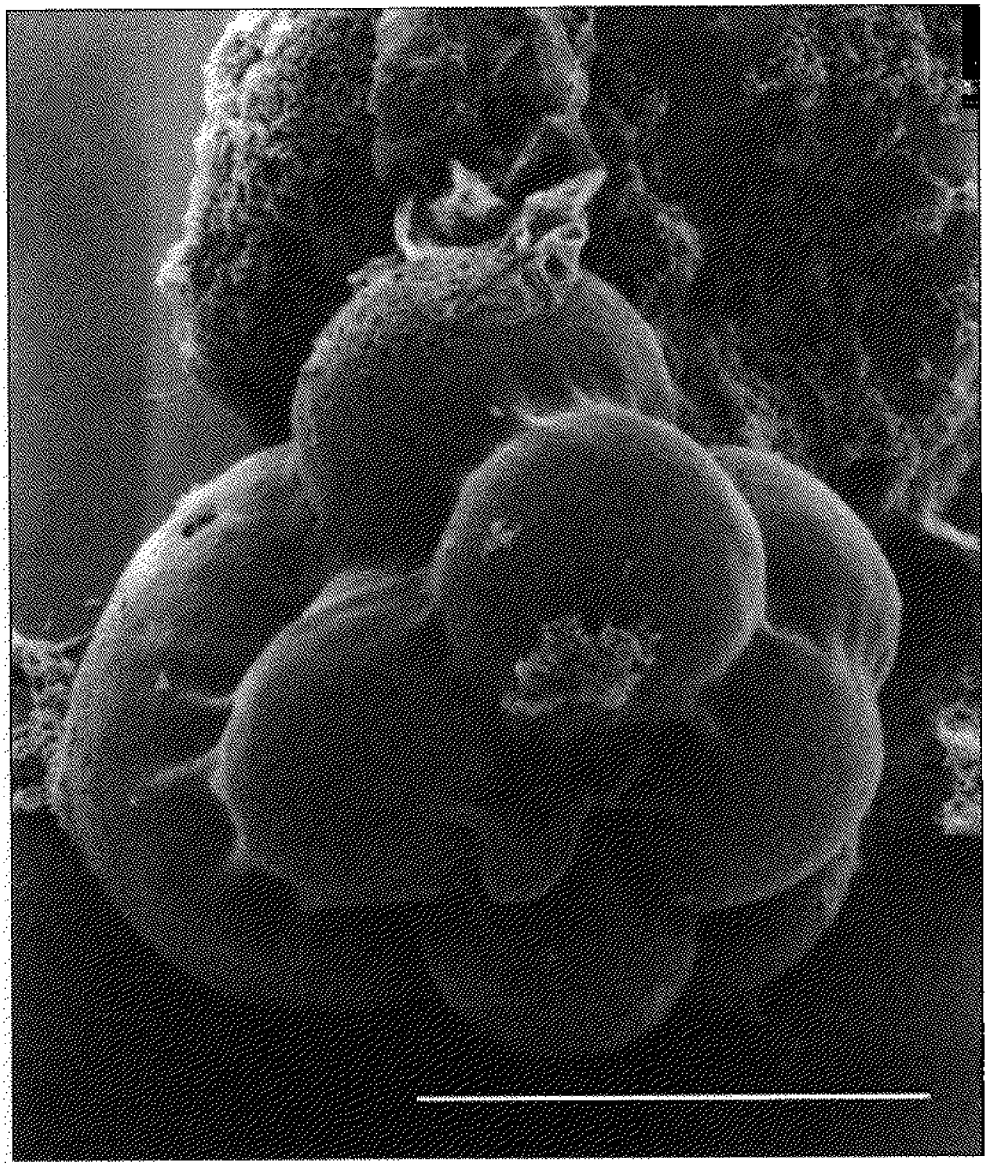


Figure 8. **Eight-cell Stage** (scale bar = 40μm).

This sample was preserved 6 hours after fertilization. The four cells from the previous stage all divided in the same direction thus producing two parallel planes of four cells each, i.e., a total of 8 cells. From this perspective, the plane of four closest to the camera lens contains the 4 smallest cells. The plane of four furthest form the camera lens contains the four largest cells. The cleavage event that produced this stage occurred between these two planes. This type of cleavage is known as *spiral* because the 4 cells in one plane are positioned between the furrows formed by the 4 cells in the adjacent plane. Although the polar body is still visible at this stage it will soon be lost.

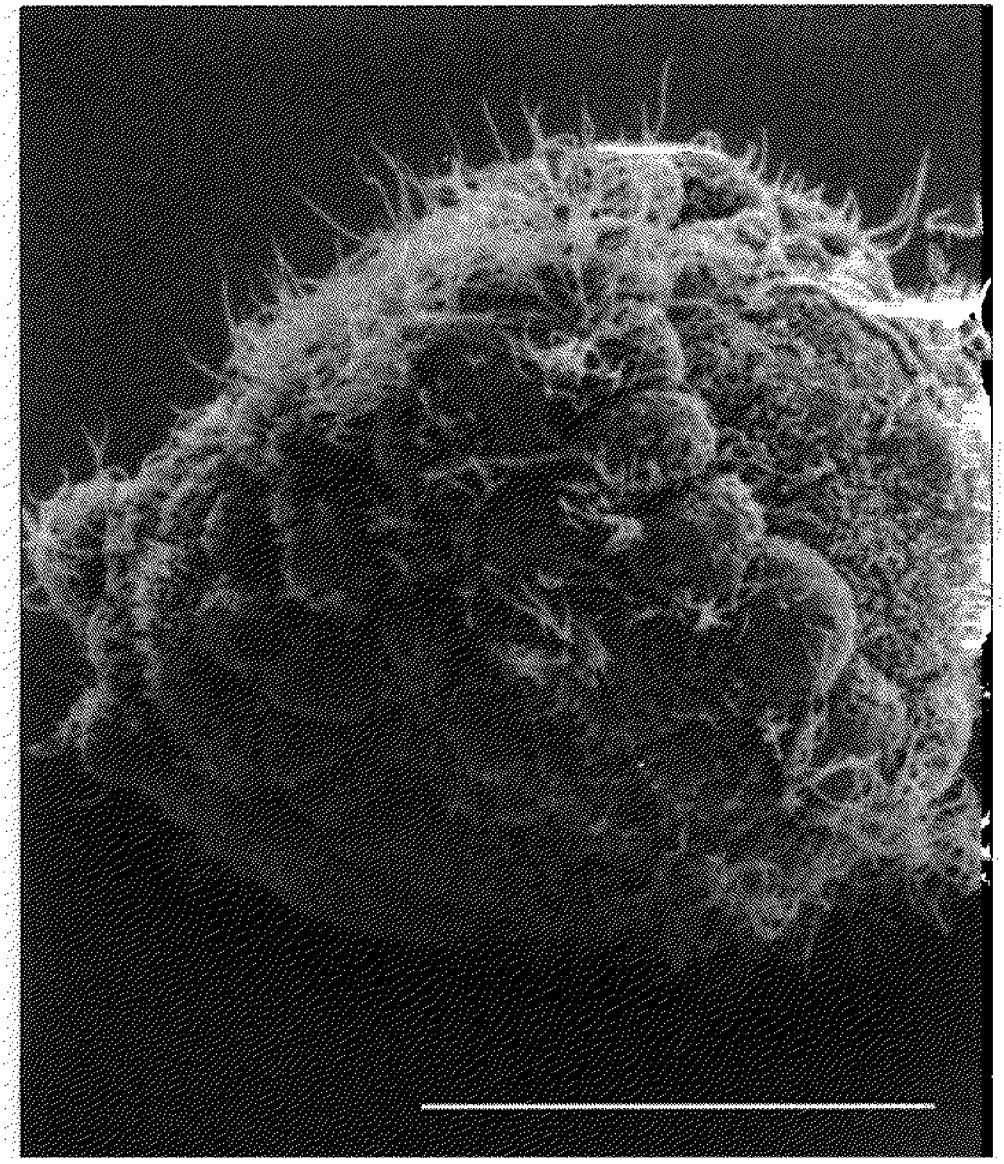
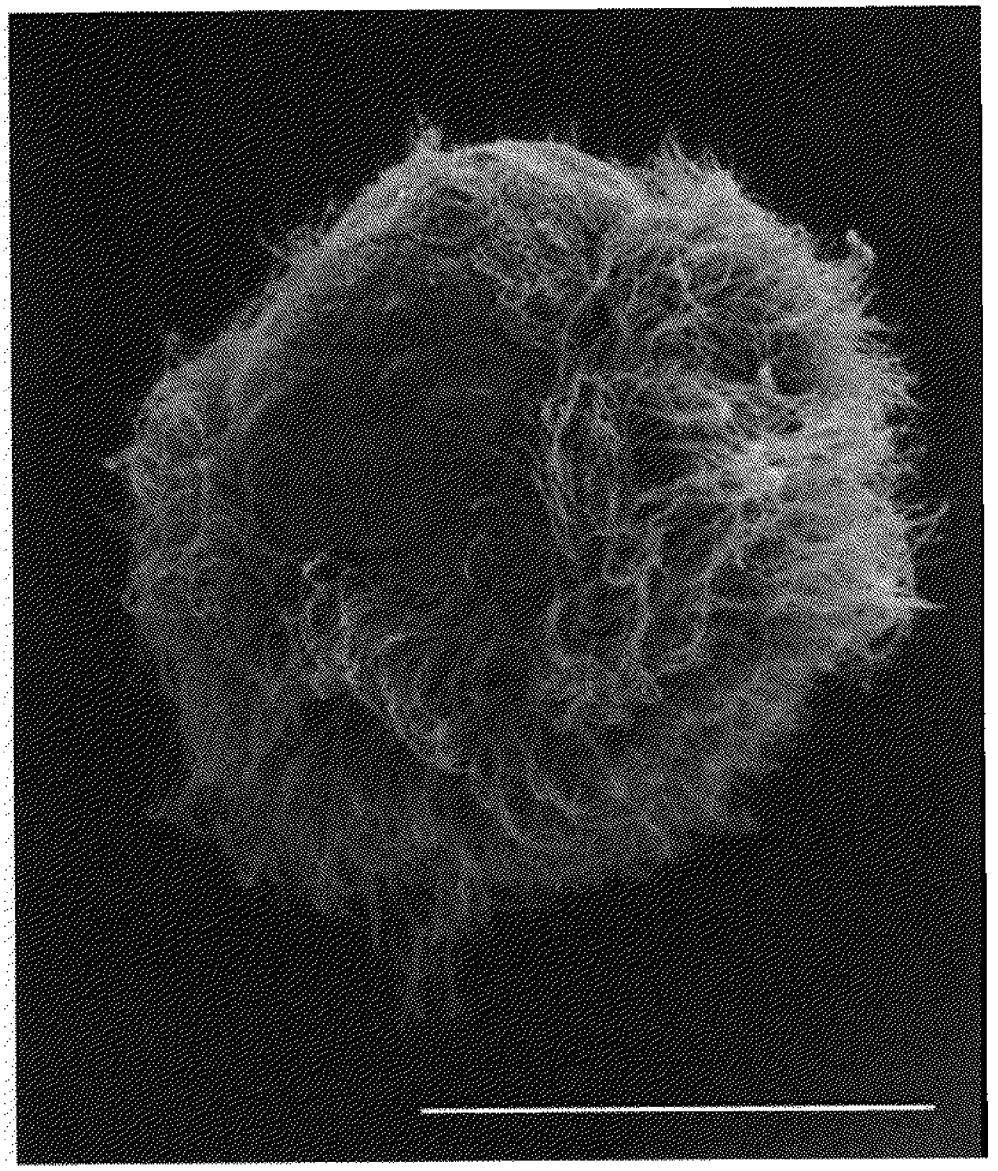


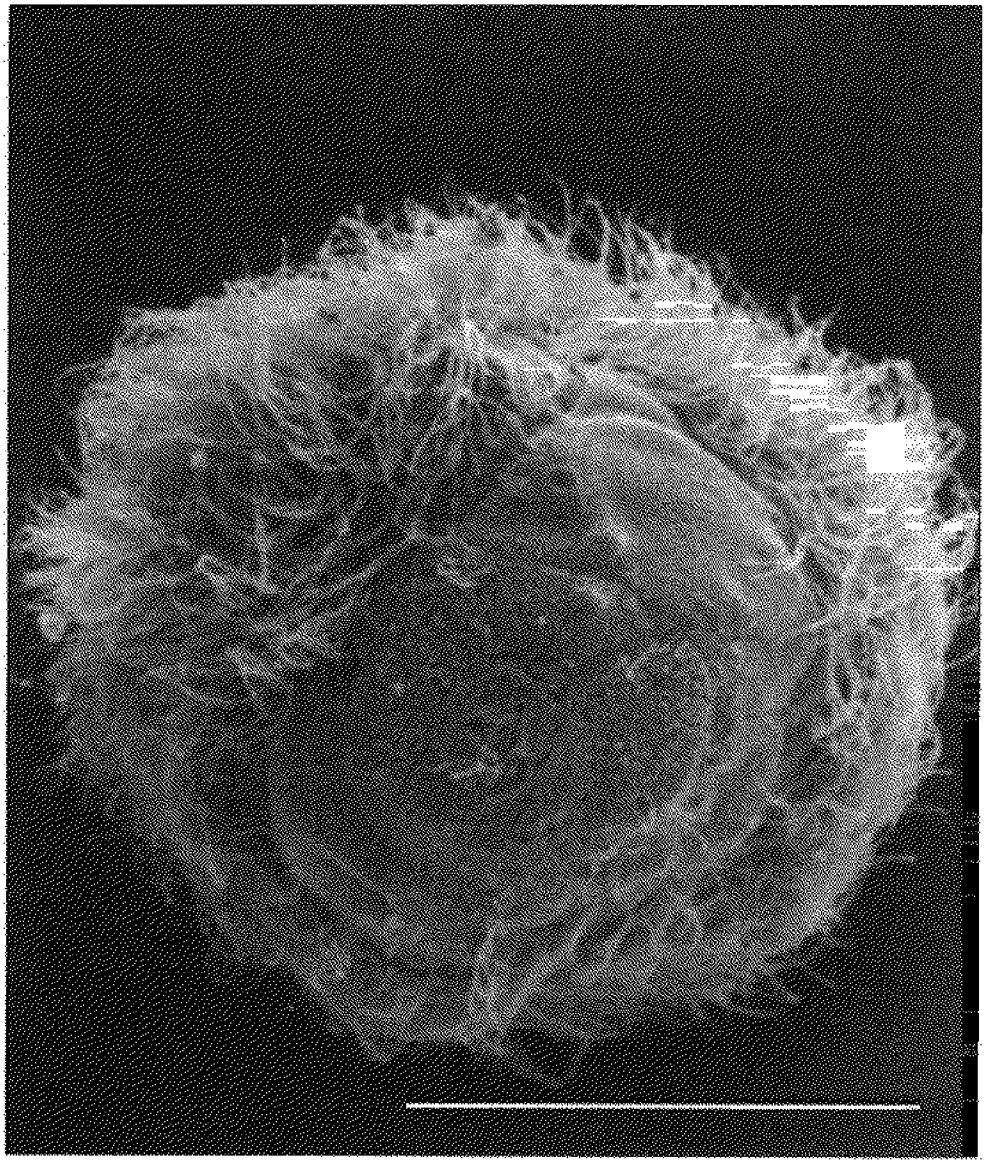
Figure 9. **Early Trochophore** (scale bar = 35μm).

This sample was preserved 24 hours after fertilization. The multicellular early trochophore is about the same size as the fertilized egg. The long axis of this larva is about 60μm and the diameter is 50μm. The embryo has not been ingesting material so far during development therefore all the energy it used to carry out cell divisions and respiration up to this point was contained in the egg.



Figures 10. **Late Trochophore** (scale bar = 50μm).

This sample was preserved 36 hours after fertilization. The cilia are very well developed in the late trochophore as is evidenced by this and the next figure. Trochophores actively swim using their cilia and develop a complete digestive tract. The larvae will begin to feed when they metamorphose into veligers.

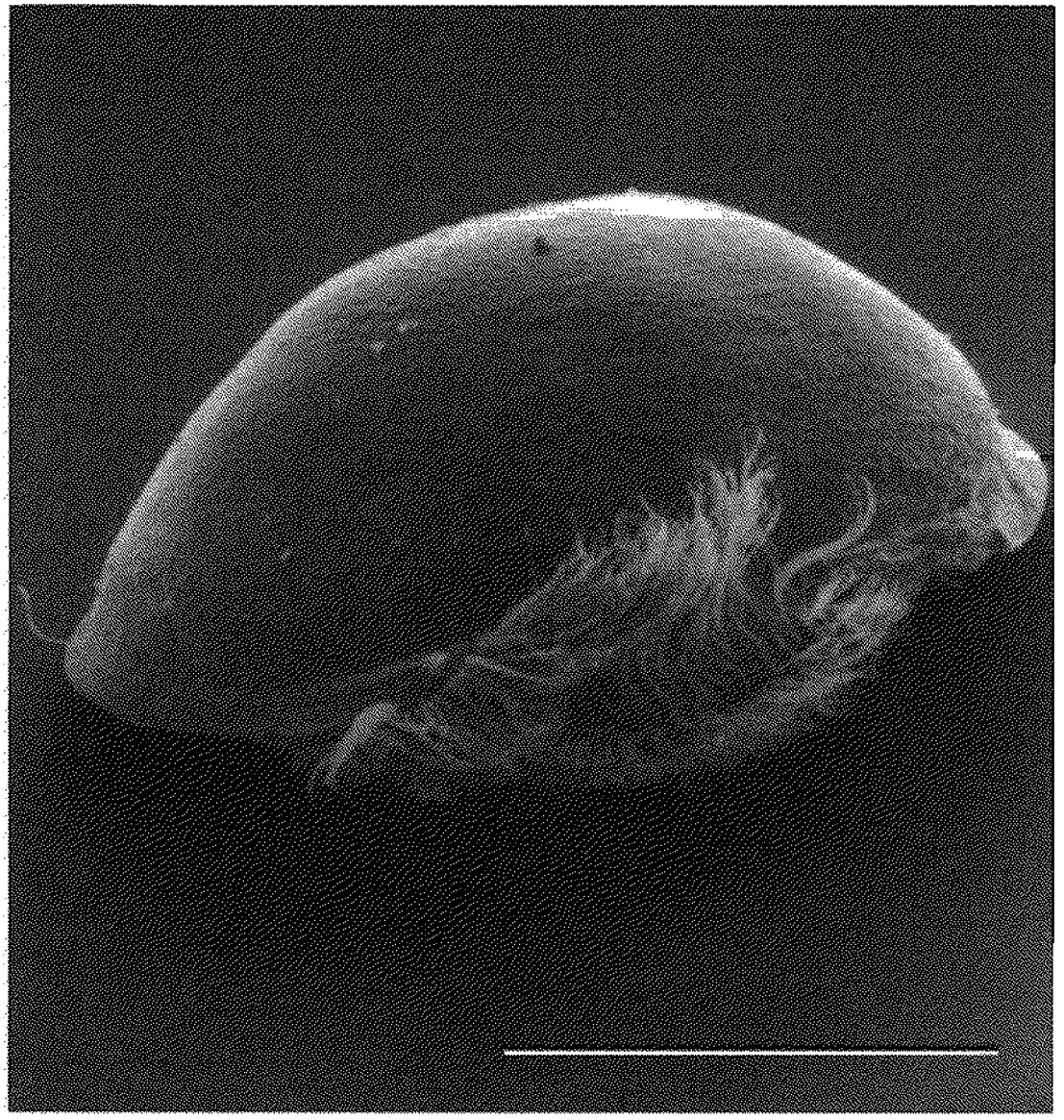


Figures 11. **Late Trochophore** (scale bar = 40μm).

This sample was also preserved 36 hours after fertilization. The shell is beginning to form – this is the smooth, non-ciliated surface visible in this and the previous figure.

Figures 12. **Veliger** (scale bar = 55μm).

This sample was preserved 48 hours after fertilization. The early veliger stage is known as the *D-hinge veliger* because the larval shell is shaped like the capital letter D. The vertical axis of the D is the hinge where the two shells are attached (in this sample it is on the left side of the figure). The velum protrudes out of the opposite side from between the two shells. The hinge is the dorsal edge of the clam and where the velum protrudes is the ventral edge. As the clam continues to develop the shell will grow circumferentially, i.e., radiating out from the hinge to form concentric rings. In this sample the velum is partially retracted inside the shells. When the velum is extended the cilia beat and propel the larva ventrally, i.e., in the direction of the velum (in this view from left to right).



Figures 13. **Veliger** (scale bar = 50μm).

This is a ventral view of the velum of the veliger from the previous figure. The concentric rings of the shell can be seen starting to form on the edge just to the left of the velum. The length of the shell at this point is approximately 100 μm. The cilia of the velum form a ring that is adjacent to the mouth. When a food particle is captured, the cilia stop beating and contract inward towards the center of the mouth forcing the food particle into the digestive tract.