Embryology Course 1946

**Staff**

Donald P. Costello, Professor of Zoology, University of North Carolina, in charge of the course.

Howard L. Hamilton, Assistant Professor of Zoology, Iowa State College.

John A. Moore, Assistant Professor of Zoology, Bernard College.

Albert Tyler, Assistant Professor of Embryology, California Institute of Technology.

**Assistant**

Catherine Henley, The Johns Hopkins University.

Eleanor Lerner, Washington University.

**Research Assistant**

Marjorie Hopkins Fox, University of California.
Embryology Course

Woods Hole 1948

Banner, Albert H. - University of Hawaii - (Ph.D., Univ. of Washington)
Barish, Natalie - Goucher College
Brinsley, Bertram - New York University - (B. A., N. Y. U)
Bruch, Paul R. - Wesleyan University
Bucklin, Donald H. - Brown University - (B. A., Brown)
DiDea, Arthur - Washington University - (B. Sc., C. C. N. Y.)
Ebert, James D. - The Johns Hopkins University - (B. A., Washington and Jefferson)

Eisenberg, Norma - Brooklyn College - (B. A., Brooklyn)
Fitch, Naomi - McGill University
Flood, Francis X. S. J. - Canisius College (B. A., M. A., Woodstock College)
Friedman, Florence - Brooklyn College - (B. A., Brooklyn)
Galbreath, Jean - Elmira College
Harrison, John W. - Washington and Jefferson - (B. A., Washington and Jefferson)

Jones, Alberta Thelma - Howard University (B. A., Howard)
Kuff, Edward L. - Johns Hopkins Medical School (B. A., Johns Hopkins)
Leigh, Walter H. - Wright Junior College - (Ph.D., University of Illinois)
Liu, Chien-Kang - McGill University - (B. Sc., Soochow Univ., M. S., McGill University)

Markert, Clement L. - The Johns Hopkins University - (B. A., University of Colorado, M. A., U. C. L. A.)
Moore, Ellen Louise - Wellesley College
Morris, David M. - Indiana University - (B. A., Earlham College)
Mullally, Rev. Walter Bernard - Catholic University of America - (B. A., Boston College)

Pequegnat, Willis E. - Pomona College - (B. A., U. Cal., M. A., Ph. D., U. C. L. A.)
Schecter, David E. - McGill University (B. Sc., McGill)
Seaman, Arlene R. - Cornell University (B. Sc., State Teachers College West Chester, Pa., M. Sc., Cornell)

Shapiro, Esther May - Goucher College
Tatum, Anne - Rosemont College - (B. A., Rosemont)
Tiemeier, Otto W. - University of Illinois (B. A., M. A., Univ. Kansas)

Uber, Virginia M. - Pennsylvania College for Women - (B. Sc., Penn. College Women)
Embyology Class
Woods Hole 1946
## May, 1946 Tentative Schedule

**Embryology Course, Woods Hole, Summer, 1946.**

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**Experimental Period Staff**

- June 19
- June 26
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**Preparation of Reports**

- June 19
- June 26
- June 26
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- June 26

**Seminars 8:00 P.M.**

- June 19: same problems of Jean Brauchet
- June 26: Jean Brauchet
- June 26: Jean Brauchet
- June 26: Jean Brauchet
- June 26: Jean Brauchet
- June 26: Jean Brauchet
- June 26: Jean Brauchet
**June 17, 1946 Revised Schedule**

**EMBRYOLOGY COURSE, WOODS HOLE, SUMMER 1946.**

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**Seminars 8:00 P.M. Embryology Lab.**

- **Wed. July 3.**
- **Wed. July 10.**
- **Wed. July 17.**
EMBRYOLOGY COURSE 1946

Lectures

Friday, June 14, 9:00 A. M. Introductory Remarks
Dr. Costello

Normal Teleost Embryology from Earliest Germ Cells to Gastrulation.
Dr. Hamilton

Saturday, June 15, 9:00 A. M. Gastrulation and Organogenesis in Teleosts.
Dr. Hamilton

Monday, June 17, 9:00 A. M. Mechanics of Teleost Development
Dr. Hamilton

Tuesday, June 18, 9:00 A. M. Principles of Gastrulation and Location of Organ-Forming Areas in Teleosts, and Comparisons with other Vertebrate Forms
Dr. Hamilton
**EMBRYOLOGY COURSE 1946**

**Lectures**

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<td>Wednesday, June 19</td>
<td>9:00 A.M.</td>
<td>Fertilization</td>
<td>Dr. Donald Costello</td>
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<td>Wednesday, June 19</td>
<td>8:00 P.M.</td>
<td>Some Problems of Embryology</td>
<td>Dr. Jane Oppenheimer</td>
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<td>Thursday, June 20</td>
<td>9:00 A.M.</td>
<td>Artificial Parthenogenesis</td>
<td>Dr. Albert Tyler</td>
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<td>Friday, June 21</td>
<td>9:00 A.M.</td>
<td>Normal and Experimental Squid Embryology</td>
<td>Dr. Howard L. Hamilton</td>
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<td>Saturday, June 22</td>
<td>9:00 A.M.</td>
<td>Method of Sperm Transfer and Structure of the Spermatophore in the Squid</td>
<td>Dr. Howard L. Hamilton</td>
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EMBRYOLOGY LECTURES

1946

Monday, June 24  9:00 A.M.  Cell-lineage and Spiral Cleavage
Dr. D. P. Costello

Tuesday, June 25  9:00 A.M.  History of Embryological Problems
Dr. E. G. Conklin
EMBRYOLOGY SEMINAR

June 26
Wednesday - 8:00 P. M.

Embryology Laboratory, Old Main Building

Dr. Jean Brachet, Professor of Experimental Morphology
University of Brussels

The rôle of nucleic acid in the cell and in the embryo
EDMBRYOLOGY LECTURES

Wednesday, June 26, 9 A.M. -- Echinoderm Embryology I
   Dr. Albert Tyler

Thursday, June 27, 9 A.M. -- Echinoderm Embryology II
   Dr. Albert Tyler

Friday, June 28, 9 A.M. -- Echinoderm Embryology III
   Dr. Albert Tyler

Thursday, June 27, 8:00 A.M. -- Embryology Demonstrations

Development of the fish egg: L. E. Bach + L. Godwick

Fertilization: R. Chambers + C. S. Brand
EMBRYOLOGY SEMINAR

MAIN AUDITORIUM
Physiology Lecture Room, Old Main Building

Tuesday, July 3, 9:00 P.M. Function in Development Dr. F. E. Armstrong.
Wednesday, July 3, 9:00 A. M.  Embryology of Annelida
Dr. D. P. Costello

Thursday, July 4  9:00 A. M.  Experimental Embryology of
Annelids and Molluscs
Dr. D. P. Costello

Friday, July 5  9:00 A. M.  Embryology of Mollusca
Dr. D. P. Costello

Saturday, July 6, 9:00 A. M.  Normal and Experimental
Embryology of the Tunicates
Dr. John A. Moore
Monday, July 8 9:00 A.M.  Normal development and metamorphosis of tunicates-- Dr. John A. Moore

Monday, July 8 8:00 P.M.  Fertilization and infection (Embryology Seminar)-- Dr. Albert Tyler
EMBRYOLOGY COURSE 1946

Guest Lecture - Embryology Laboratory

Tuesday, July 9  9:00 A.M.  Genes and Development - with special reference to the development of color patterns and Mendelian characters in fish.

Dr. H. B. Goodrich
EMBRYOLOGY COURSE 1946

LECTURES

Wednesday, July 10 - 9:00 A.M.
Normal and Experimental Embryology of Coelenterates
Dr. John A. Moore

Thursday, July 11 - 9:00 A.M.
Regeneration in Hydrozoa
Dr. John A. Moore
EMBRYOLOGY SEMINAR

July 17, Wednesday - 8:00 P. M.

Embryology Laboratory, Old Main Building

The physical basis of tissue structure

Dr. Paul Weiss
The University of Chicago
Embryology Course
1946
Experimental Period

Group I  Coelenterata: Axial gradients and some aspects of regeneration in Tubularia - Dr. Moore
          Barish, Bruch, DiDea, Tatum

Group II Coelenterata: Isolation of blastomeres in Hydractinia. Vital staining, etc. - Dr. Moore
           Friedman, Tiemeier, Moore

Group III Mollusca: Polar lobe extirpation in Ilyanassa: Experiments on Loligo, etc. - Dr. Hamilton
          Galbreath, Tuttle, Shapiro

Group IV  Teleosts: Production of cyclopia in Fundulus, etc. - Dr. Hamilton
         Mullally, Uber, Markert, Harrison

Group V  Echinodermata: Isolation of blastomeres, operative technique, centrifuging, Li-treatment, etc. Fertilizin. Dr. Tyler
         Pequegnat, Murtland, Banner

Group VI Annelida: Production of Janus-Embryos of Chaetopterus: Differentiation without cleavage. - Dr. Tyler
          Ebert, Kuff, Leigh, Eisenberg

Group VII  Teleosts: Operative technique on Fundulus eggs: Physiological Methods. - Dr. Armstrong
           Fitch, (Schecter), Morris, Liu

Group VIII Annelida: Isolation of Blasomeres of Nereis - Dr. Costello
         Flood, Bucklin, Seaman, Jones

The class will be divided into 8 groups of 3 or 4 students each. Students having special reasons for wishing to be assigned to a particular group should see Dr. Costello before Friday, July 5. Each group will report its work on July 20th.
9:00  (1)  Regeneration in Tubularia  
       Mr. DeDea

(2)  Separation of blastomeres in Hydraoctinia  
       Mr. Tiemeyer

(3)  The results of extirpation of the 3rd polar
     late on the early development of Fascia  
     astalata:  
     Effects of Lithium ions on the development of
     the squid embryo.  
       Miss Galbraith

(4)  The capacity for regulation of the teleost
     blastoderm after destruction of blastomeres
     in early cleavage stages:  Results of chemical
     treatment of teleost embryos;  Hybridization
     experiments on Fundulus.  
       Mr. Markert

(5)  Preliminary investigation of the anti-
     fertilizing factor in sea urchin blood.  
       Mr. Hurland

(6a) Production of double embryos in Chaeopterus
     and Nereis.  
       Mr. Leigh Sbert

(6b) Methods of removal of fertilization membranes
       Mr. Haff

(7)  The elimination of dyes of different molecular
     configurations through the aglomerular pronephros.  
       Mr. Norris

(8)  Isolation of blastomeres of Nereis limbata  
       Miss Seaman

Papers are limited to 15 minutes each.
Development of the Teleost

Two types of eggs will usually be studied: the pelagic egg and the non-pelagic egg.

The non-pelagic egg
Examples: Fundulus heteroclitus
Fundulus majalis
Opsanus tau (the toad fish)

1. CARE OF FISH Fish are usually brought to the laboratory in mixed lots of males and females. After stripping of eggs and sperms they are transferred to a discard tank. If material is limited it is better to keep males and females in separate tanks as the females will then be less likely to shed their eggs.

2. PREPARATION OF CULTURES Two cultures should be started by either student or instructor (announcement will be made) on the first day, one at 10 a.m., and the second as late in the evening as practicable. Arrangements should also be made at each table to start at least one culture for immediate study of fertilization.

a) Strip eggs into a clean bowl which has been moistened by sea water. Strip milt and mix with eggs with barely sufficient salt water to cover the eggs. Eggs should not be allowed to stand more than 5 minutes before fertilizing. After ten minutes add and change sea water and leave in shallow water (not more than 1/4" deep), Keep bowl covered with glass plate. Do not at any time allow eggs to clump or accumulate in one spot. Label each lot with exact time of fertilization. Change water at least once a day.

b) The eggs should first be studied in the condition in which they are spawned but for many purposes it is desirable to prepare the eggs as follows for microscopic study: roll the eggs on a piece of filter paper until the jelly and outer fibres are removed leaving the surface of the outer membrane smooth and clean. Place in sea water in culture slide in which the depth of the depression is slightly less than the diameter of the egg. (These are provided in the laboratory equipment) The egg may now be rotated by moving the cover slip. Another method is to mount on an ordinary glass slide in sea water under a thin flexible piece of mica peeled from the thicker mica sheets that are provided. Draw off the water until capillary attraction causes a pressure on the egg and rotate it as in the preceding method.

3. THE OVUM To study unfertilized eggs they should be obtained from the female that has been kept in fresh water for about 20 minutes. Str and keep the egg in diluted sea water (70% fresh water, 30% seawater) to retain the morphological characteristics observable at time of extrusion. Note details of structure of the unfertilized mature ovum. These include yolk plates, oil drops, protoplasm, membranes, micropyle etc. (The micropyle must be observed before removal of chorionic jelly. If immature ova are present compare these with mature ova).

4. FERTILIZATION Note exact time of fertilization and be prepared to study immediate changes. Note time of change of yolk plates; of formation of perivitelline space. What are your conclusions in regard to the rapidity of the activation of the egg? If practicable find micropyle on unfertilized egg. Inseminate and note spreading of fertilization reaction from lucus to micropyle.
5. FORMATION OF THE BLASTODISC  Note the gradual accumulation of polar and lateral views. Polar bodies may be more advantageously studied in pelagic eggs. What is the relation of the pole of the ovum to gravity? How does this compare with the condition in the frog egg; with the chick egg? Do any processes take place in the unfertilized egg similar to those in the fertilized egg?

6. CLEAVAGE Watch for the appearance of a groove in the surface of the blastodisk— the indication of the first cleavage plane. This usually occurs from 2 to 3 hours after fertilization. The rate of development varies with the temperature. Note the geometric and time relations of the subsequent cleavages. Do the cleavage plane divide the entire ovum? The entire blastodisc? During interkinesis the nuclei are sometimes visible. Distinguish between central and marginal cells. Follow cleavage carefully to the 32 cell stage. Note irregularities. When do horizontal cleavage planes first appear? Does the blastodisc increase in size or alter in form? Note that cleavages continue for a considerable period without much change in form from that of the original blastodisc. This is called the period of the high blastula (See Oppenheimer '36 for chronological terms). When does the change of form to the "flat" blastula occur?

(Note: As developmental rate varies with the temperature no time chronology can be given for stages of gastrula as observed under varying temperature conditions. For list of stages see paper by Oppenheimer '37; for chronology at 25°C see paper by Solberg '38)

7. THE PERIBLAST. (First appearance is from 16 to 24 hours after fertilization). The unleaved protoplasm around the margin of the group of blastomeres is called the marginal periblast; that beneath the blastodisc (not visible except in sections); the central periblast. In the last blastodisc (18-20 hours) observe particularly the behavior of the marginal cells and distinguish between circular and radial cleavages. The large pinkish nuclei of the periblast are easily visible. Note how the nuclei of the marginal row of cells become free from cell outlines, continue their divisions and migrate into the marginal periblast, converting it into a nucleated but non-cellular structure. Note the continuation of the periblast structure in later stages.

8. THE GERM RING AND THE EXTENSION OF THE BLASTODISC (18 to 48 hrs) Subsequent to the nucleation of the periblast note the change in form and size of the blastoderm (blastula). Soon the margin of the disc appears relatively thicker (germ ring) due both to an actual thickening and to the thinning of the central part of the disc. The germ ring can best be observed in F. majalis. During the next few hours the germ ring grows completely over the surface of the yolk mass. The uncovered portion of the egg is the blastopore. The final covering of the yolk or the closure of the blastopore occurs after the first stages of the formation of the embryo. Under favorable conditions the beginning of gastrulation may be observed in the appearance of a slight indentation at the edge of the germ ring at a time when the yolk is about 1/4 covered. Staining with neutral red may help in identification of germ ring. (Use 1 or 2 drops to a syracuse dish of sea water)
9. THE FORMATION OF THE EMBRYO (Beginning 24 to 36 hours)
   a) While the germ ring is extending around the yolk, the formation of the embryo takes place. Its first indication is a cellular thickening known as the embryonic shield, caused by a more active movement of cells in one part of the germ ring. This formation is initiated when the blastoderm has covered from one quarter to one third of the surface of the yolk. By the time that the blastoderm has covered about one half of the yolk the embryonic shield has become a bluntly triangular area extending from the margin of one portion of the blastoderm to near the center of the blastoderm. The embryonic shield can be most readily identified when seen in profile. As the blastoderm spreads over the surface of the yolk the embryo grows rapidly in length.

   b) Observe the whole egg in profile view, so that the embryo is seen in sagittal optical section, when the yolk is 1/3, 1/2, 2/3 and 3/4 covered. What proportion does the length of the embryo bear to the diameter of the blastoderm and to the length of the germ ring in each of these successive stages?

   c) After the yolk is covered 7/8, look for a large clear vesicle near the hind end of the embryo. (do not confuse this with a cluster of small oil drops frequently found in a similar position). This is Kupffer's vesicle.

   d) The embryo has become segmented. This segmentation is confined to the mesoderm which lies on each side of the axis of the embryo forming mesoblastic somites. How many somites do you find at the time of the closure of the blastopore?

   e) Look for the notochord. Study it in longitudinal and transverse optical sections. Where does it terminate anteriorly and posteriorly?

10. LATER DEVELOPMENT Obtain series of embryos of 2, 3, 4, 5, 6 days and make a detailed comparative study covering the following points.

   a) Somites. Note first appearance and determine numbers formed on the successive days.

   b) The brain and sense organs. Find in early stages the optic vesicles, lens, neuromeres, mid, fore, and hind-brain regions and trace the development into cerebrum, optic lobes, cerebellum, medulla, etc. (Drawings at 24 hour intervals will give a good record of development of parts). Study the development of the eye, olfactory pit and otocyst. How is the lens of the eye formed?

   c) The circulatory system: Note extra-embryonic body cavity; formation of pericardium; first blood vessels; (and especially their mode of formation from wandering mesenchyme cells); first action of the heart; form and position of heart. Compare course of circulation on 4th and 6th days. Illustrate by diagrams.

   d) Mesenchymal cells. Note wandering mesenchymal cells, especially abundant beneath posterior end of embryo on 2nd and 3rd days. Can you distinguish various types? By successive observations at brief intervals, the change in form, migration and division of these cells may be noted.
c) Note first appearance of fins, of the urinary vesicle or bladder (a bilobed outgrowth of the hind gut), the liver posterior to left fin bud.

d) The young fish may be studied just after hatching by anesthetizing with chloretone.

Consult paper by Oppenheimer '37 for further details of developmental stages.

Permanent total preparations of Fundulus eggs may be made by fixing in "Stockard's solution" (Formalin 5 parts, glacial acetic 4 parts, glycerine 6 parts, distilled water 85 parts) which turns the protoplasm white but leaves the yolk transparent. The fixative may be used as a preservative or the material may be transferred to 10% formalin after 2 days.

11. TECHNIQUE OF HANDLING THE PELAGIC EGGS When possible, comparisons will be made with pelagic eggs. Those of the cunner, (Tautogolabrus adspersus Cuv.) the scup (Stenotomus chrysops, Linn.) and the mackerel (Scomber acombrus, Linn.) are most likely to be obtained. The scup or mackerel must be stripped as taken fresh from the live car. Cunner may be brought to the Laboratory aquarium. Femal cunners are usually obtained only after 12 M. Obtain and mix egg and sperm in large culture dish. Pour into erlenmeyer flask, filling with sea water to neck. Good eggs will float to the top and may readily be pipetted off from the narrow neck of the flask. Later, keep eggs in dishes surrounded by running water to keep cool. The optimum temperature for mackerel eggs is 16°C. Artificial insemination may be useful. Dead eggs turn white and sink to bottom and may be removed and water changed by pipetting from bottom of dish. In observing the polar body formation, blastodisc, and early cleavage it is advantageous to place the microscope in a horizontal position so that the blastodisc may be observed in profile. It is difficult to see the polar bodies by any other method. They appear from 5 to 10 minutes after fertilization. Metotic cleavage spindles can be outlined by staining with neutral red.
### Stage of Development

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Sea water</th>
<th>1/2 sea</th>
<th>1/2 distil.</th>
<th>Tap water</th>
<th>Dist. water</th>
</tr>
</thead>
<tbody>
<tr>
<td>15°C</td>
<td>1 2 3 4 5 6</td>
<td>1 2 3 4 5 6 7 8</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>20°C</td>
<td>1 2 3</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>25°C</td>
<td>1 2 3</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4 5 6 7 8</td>
</tr>
<tr>
<td>30°C</td>
<td>1 2 3</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4</td>
<td>1 2 3 4 5 6 7 8</td>
</tr>
</tbody>
</table>

A table to show the number of days (24 hrs) required by Fundulus Heteroclitus embryos to reach different stages of development from the blastula to the formation of the swim bladder just before hatching under varying conditions of temperature, salinity, etc.

Daniel Merriman, Osborn Zoological Laboratory and Bingham Oceanographic Laboratory, Yale University.
Fundulus heteroclitus may be readily crossed with Fundulus majalis and the mackerel (Scomber scombrus). Embryos can be obtained from reciprocal crosses of F. heteroclitus x F. majalis which live until about the time of hatching. With the mackerel, however, only one combination is visible, Fundulus female x Scomber male, and these may develop to advanced embryonic stages.

To make Fundulus heteroclitus x F. majalis crosses, one should use the same procedure as in fertilizing F. heteroclitus eggs. The mackerel cross must be made at the fish traps, in as much as mackerel do not live long after being caught. At the time that hybrid crosses are made control cultures from both parents should be started. All cultures must be raised at the same temperature.

In studying the development of the hybrids attention should be paid to comparative rates of development, appearance and shape of organ primordia, morphology and types of pigments cells, etc.

References


Goodrich, H. B. 1935 The development of hereditary color patterns in fish. Am. Nat. 69

Newman, H. H. 1908 The process of heredity as exhibited by the development of Fundulus hybrids. J. E. Z. 5: 503-561

--------------- 1915 Development and heredity in heterogenic Teleost hybrid. J. E. Z. 18: 511-576

--------------- 1918 Hybrids between Fundulus and Mackerel. J. E. Z. 26: 391-421

EXPERIMENTAL PRODUCTION OF CYCLOPIA IN FUNDULUS

Cyclopia and other abnormalities of the head may be obtained by treating Fundulus embryos with alcohol early in development (Stockard 1910). Eye abnormalities alone can be obtained by treatment with MgCl₂.

Consistently good results may be gotten with dilute solutions of alcohol in sea water (3-9%). 60-100 eggs should be placed in a finger bowl containing 3% alcohol, another 60-100 eggs in 4%, etc., during early cleavage stages. Eggs should not be exposed to this treatment for more than 24 or possibly 36 hours. They should then be removed and placed in sea water for further development.

Best results with MgCl₂ are obtained by using solutions of 16-22 cc of 1M. MgCl₂ made up to 60 cc, by adding sea water. Eggs should be placed in MgCl₂ solution shortly after fertilization (8 cell stage) and left in it for 3 days, after which they should be returned to sea water for further development.

It is advised that results be recorded in tabular form listing the various abnormalities obtained with each agent and with each concentration.

References

Adelmann, H. B. (Good bibliography) 1936 The problem of cyclopia Quart. Rev. Biol. 2:161-182 284-304
Child, C. M. 1941 Patterns and Problems of Development. pp. 270, 282
1921 Developmental rate and structural expression. Am. Jour. Anat. 28 (Good Bibliograph)
EMBRYOLOGY OF THE SQUID

If you are not familiar with the anatomy of the adult squid you may find it advantageous to examine the dissected specimens which are placed on demonstration tables. (Consult Williams).

The sexes are distinguishable. The testis of the male shows white through the mantle at the posterior end. The females are usually smaller than the males, and are shorter and broader. The females often have a small pocket of white sperm in the collar between the head and the free edge of the mantle. The accessory nidamental glands of the female are red during the breeding season and can usually be seen through the mantle.

To secure eggs slit open the mantle from the siphon to the tip. (Use large scissors). With forceps tear the thin wall of the ovary and allow the eggs to escape into a dish of sea water. If the eggs are fully mature they separate readily from the ovary and appear beautifully transparent as glass. Immature eggs are not transparent and will not develop.

1. Morphology of the Egg

Study mature eggs taken from the ovary of the squid.

1. The egg is surrounded by a transparent chorion which is closely applied to it.

2. At the pointed end, find a depression and a minute canal extending entirely through it. This is the micropyle.

3. Note the polarity (blunt and pointed poles) and the bilaterality of the egg by turning it over. The more convex side of the egg is the future "anterior" or mouth side of the embryo.

4. Note the thin cap of cytoplasm covering the yolk at the pointed pole. Study the extent of the cytoplasmic cap by rolling the egg. This cytoplasm will give rise to the embryonic structures.

Make a drawing of the egg and the chorion.

Study a string of eggs. The eggs are embedded in a gelatinous matrix which is produced by glands of the oviduct, and covered by a jelly membrane produced by the nidamental glands.

II. FERTILIZATION AND MATURATION

Artificially fertilized eggs are more favorable for the study of the first phases of development than are those laid by the female because they lack the jelly envelopes. Fertilization and cleavage can be observed readily in this way. However, these eggs are very sensitive and must be kept in a large volume of water.

Open a male and a female squid by making a longitudinal section through the mantle along the posterior (funnel) side. Remove the ink sac. In the female cut the ovary open and shake all transparent eggs into a finger bowl. In the male pick up the bundles of spermatothores at the opening of the sperm-duct, transfer them into a
The spermatophores will explode when placed into sea water; a concentrated sperm solution will thus be obtained.

Place the eggs in a watch glass and add a drop of sperm solution. Mix thoroughly. Transfer a few eggs immediately into a depression slide and observe the fertilization under high power:

1. Penetration of a sperm through the micropyle.

2. After a short time, the cytoplasmic cap will withdraw from the chorion, and a clear perivitelline space will appear. This is an indication of successful fertilization.

3. The first polar body appears about 20 minutes after fertilization. Observe the appearance of the second polar body and further divisions of the polar bodies (see Hoadley, 1930).

Place the greater part of the fertilized eggs into a large dish filled with clean sea water and do not disturb them for about 2½-3 hrs. They are set aside for the observation of cleavage.

III.
CLEAVAGE

In order to obtain a polar view of the cytoplasmic cap which alone will undergo cleavage it is necessary to mount the eggs in upright position. Place a small portion of vaseline on a dry depression slide, fill it with water and mount the eggs with a hair loop so that they stand up. Mount 6-12 eggs which have both polar bodies formed, and watch them at short intervals.

Observe the first cleavages and note their relation to the axes of symmetry of the egg. The first cleavage plane coincides with the median plane of the future embryo. (Consult the figs. of "Stas", 1891). The cleavage is meroblastic, and not spiral, in contrast to other Mollusc eggs.

IV. TIME TABLE OF DEVELOPMENT

There is considerable variation due to temperature differences, and the following schedule gives a rough approximation of the times at which certain stages are reached:

<table>
<thead>
<tr>
<th>Time after fertilization</th>
<th>Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 minutes</td>
<td>1st polar body</td>
</tr>
<tr>
<td>1 hour</td>
<td>2nd polar body</td>
</tr>
<tr>
<td>3 hours</td>
<td>1st cleavage</td>
</tr>
<tr>
<td>12 hours</td>
<td>Blastoderm over top of egg</td>
</tr>
<tr>
<td>24 hours</td>
<td>&quot;Gastrula&quot;-thickened peripheral ring</td>
</tr>
<tr>
<td>2 days</td>
<td>Blastoderm half way over egg</td>
</tr>
<tr>
<td>3 days</td>
<td>Blastoderm nearly covering the egg</td>
</tr>
<tr>
<td>3½ days</td>
<td>Appearance of shell gland and eye stalks</td>
</tr>
<tr>
<td>5½ days</td>
<td>Siphonal folds and arms appear; eyes project</td>
</tr>
<tr>
<td>6½ days</td>
<td>Siphonal folds fused into a tube. Eye stalks prominent</td>
</tr>
<tr>
<td>11-12 days</td>
<td>Hatching.</td>
</tr>
</tbody>
</table>
V.
SPREADING OF THE BLASTODERM

Study eggs about 24 hours after fertilization. Later blastoderm stages will be supplied. Note the gradual extension of the blastoderm about the yolk. The "blastocones" which are supposed to give rise to the yolk epithelium are not very distinct in Loligo. Note the thickening of the margin of the blastoderm (formation of the entomesoderm - "gastrulation").

VI
ORGAN FORMATION

It is convenient to call the pole where the shell gland and mantle appear "dorsal" and the yolk pole "ventral", the siphon side "posterior" and the mouth side "anterior", although those designations are not correct from the comparative anatomical point of view. Study a sequence of at least 6 stages as represented on the chart. Study the embryos from all sides. Take drawings of different stages.

Early stages. (Chart figs. 1 and 2) cf. also the text books of McBride and Korschelt).

1) Shell gland at dorsal pole
2) Mantle primordium, an ectodermal concentric fold beneath the shell gland. (fig. 2)

On the anterior side:
3) Mouth
4) Eye primordia - ectodermal invaginations

On the posterior side:
5) Anterior and posterior siphonal folds. The former are the primordia of the siphon, the latter will form its retractor muscles.
6) Statocysts
7) Gill Primordia.

At the boundary of blastoderm and yolk note:  
8) the primordia of the anus
9) Note the rhythmical contractions of the yolk epithelium. They serve the purpose of circulating the liquified yolk material in the yolk sac vessels. The material is carried into the embryonic tissues in this way. (See Hertmann. 1926)

Medium Stages (Chart figs. 3 and 4). Note the gradual constriction of the yolk sac. The latter continues into the embryo which is thus formed around a core of yolk mass.

Observe:
1) Growth of mantle and of fins. (The shell gland is meanwhile completely invaginated and not visible).
2) The eye stalks are prominent.

On the posterior side:
3) The formation of the siphon by concrescence of the anterior siphonal folds. The posterior siphonal folds continue as ridges to the anterior side.
4) The anus, between the gill primordia
Note the further growth of other primordia; contractions of the yolk sac, etc.

Old stages (before hatching: Chart figs, 5 and 6)

1) The eye stalks are very prominent. They contain the primordia of the optic and cercoral ganglia, the so-called "white bodies", also a central mass of yolk.

2) The lens. The inner sector which is formed by the outer part of the optic vesicle (different from Vertebrates) is clearly visible as a club shaped rod extending into the eye vesicle.

3) The mantle has overgrown the anus and gills. It is contractile. In oldest stages it is beset with

4) The mantle has grown the anus and gills. It is contractile. In oldest stages it is beset with

5) Contractions of the yolk sac, etc.

6) Old stages (before hatching: Chart figs, 5 and 6)

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5) Chromatophores. Note different types and colors; observe their contraction and expansion. They are equipped with muscle and are innervated.

6) The statocysts lie close together.

7) The feather-like gills can be observed through the mantle.

8) The rectum and ink bag.

9) Trace the outline of the internal yolk mass and notice the gradual decrease in size of the external yolk sac.

10) Observe the locomotion of an old embryo after it has hatched

VII

The Spermatophore.

The excellent papers by Drew (1911 and 1919) and their illustrations should be consulted for all details. Open living male squid in the usual way by cutting through the mantle from the sipunc to the tip. With corcep transfer a large number of spermatophores to a shallow dish of water. Watch their explosion and the ejaculation of sperm.

Study the intact spermatophore

1) Transfer some unexploded spermatophores quickly into conc. (40%) formaldehyde; fix for 10 minutes. (They will explode in a weaker solution)

2) Rinse with distilled water several minutes

3) Stain with Ehrlich's Triacid for 5-10 minutes. The stain is made by diluting stock solution: 6 drops to 8 cc of distilled water (8 cc fills a syracuse dish about half full).

4) Rinse stain off with distilled water and put the spermatophores on slide under cover slip

OBSERVATIONS

1) The opaque sperm mass in the center

2) The flask shaped cement body in front of it

3) The spiral filament in front of the cement body

4) The outer tunic is the outermost layer of the entire envelope

5) The cap and cap thread at the smaller tip end.

6) The middle tunic may be slightly swollen.

7) A liquid space around the sperm mass, lined by middle and inner tunics.

8) Of the three "membranes" which are formed around the ejaculatory apparatus and inside of the "tunics", the middle membrane
can be most easily identified. It is relatively thick and extends from the cement body to the cap. It is fastened to the outer tunic at the cap end. This fusion will never break during the process of explosion; but the entire contents of the capsule will evaginate at this point. (cf. diagrams in Drew, 1919)

10) The outer membrane begins also at the cement body. It is so closely applied to the inner tunic that it is difficult to distinguish between them. The oral end of the inner tunic and outer membrane can be easily identified as a thickened ring around the middle membrane, at a short distance from the cap.

The evaginated inner tunic and outer membrane will form the sperm reservoir after the explosion. The sperm reservoir is closed at one end by cement from the cement body and open at the other end (see under 10). The sperm, mixed with a gelatinous mass, will ooze out slowly in a cloud; this will continue for hours or days. All other structures are left behind after explosion.
Use only the pipettes at the stock dishes to obtain gametes. Carelessness will result in contaminating the stock of unfertilized eggs with spermatozoa. The stock dish of eggs will be kept at the front of the room, the stock dish of spermatozoa, at the rear. Care must be taken to avoid contaminating the stock of unfertilized eggs with spermatozoa. Too many spermatozoa often cause polyspermy which results in abnormal cleavage and development. Polyspermic eggs of some forms develop more rapidly than normally fertilized eggs. The polyspermic eggs of Nereis, however, usually fail to cleave.

Use the ordinary low power of the compound microscope (approximately 100 diameters magnification, i.e., 10X eye-piece and 10X objective) for observation and study. This permits maintaining the eggs in a considerable volume of water in a Syracuse watch glass so that concentration of sea water by evaporation is not rapid. Most phenomena can be seen readily with this magnification, which affords excellent definition. Higher powers may be used if one desires to observe spermatozoa in detail under a coverslip. After sperm penetration in Nereis has been followed by observing the eggs in the watch glass for at least 95 minutes, it will be instructive to inseminate a second batch, and periodically mount some of these under a coverslip to observe under higher power. Remember that they remain normal but a short time under these conditions.

NEREIS:

Obtain a few unfertilized eggs in sea water in a Syracuse dish. They are approximately 140 microns in diameter as seen from above, and 100 microns high in side view. Because of their shape, they tend to orient on a flat surface with the animal pole either above or below, rarely to the side. Observe the large immature nucleus (germinal vesicle or nucleus of the primary oocyte), and the oil droplets and yolk spheres in the cytoplasm surrounding the nucleus. Note also the thick cortex of the egg.

After becoming familiar with the unfertilized egg, inseminate by adding a drop of freshly prepared sperm suspension. Stir the eggs at once by a circular movement of the dish and observe changes. These first changes will begin a few seconds after insemination. At 21°C., the following schedule applies to 50% of a population of eggs (time after fertilization). 1st. polar body, 45 minutes; 2nd polar body, 60 minutes; 1st cleavage 95 minutes (ref. #22). The laboratory will probably be warmer than 21°C., so that development will proceed more rapidly. There are many changes in the egg to observe before the first polar body forms. Observe and record the following: laboratory temperature, time of breakdown of the germinal vesicle, time of final penetration of sperm head through membrane, time of first polar body formation, time of second polar body formation, time of cleavage. The time schedule in the descriptive text below should hold approximately if the air temperature of the laboratory is about 24°C.

If possible, arrange to observe two or more eggs which are touching immediately after fertilization. 2 - 3 minutes after fertilization they will begin to be pushed apart by transparent jelly secreted by
the eggs external to the vitelline membrane. By 20 minutes, the zone of jelly around each egg will be as wide as the egg diameter. The margin of the jelly can often be made out by observing super-numerary spermatozoa and other particles at the edge of the jelly. 5 or 6 minutes after fertilization the vitelline (fertilization) membrane will be noticeable due to the formation of the narrow perivitelline space upon jelly extrusion. At 7 or 8 minutes, the entrance cone begins visibly to form. Find an egg showing a profile view of the entrance cone and the sperm which is to enter. Form 8 to 12 minutes or longer, the sperm is clearly visible outside the vitelline (fertilization) membrane at the tip of the conspicuous entrance cone. In the course of the next 8 to 10 minutes, the vitelline membrane is indented slightly at its point of contact with the entrance cone. This tends to obscure the sperm from view to some extent, and at about 20 minutes after fertilization the egg wrinkles, becoming distorted and quite irregular in somewhat amoeboid fashion. The entrance cone has already flattened considerably, but is still present, and although the sperm is partially obscured from view, the entrance of its head into the egg is not completed until some time later (ref. #12,15,16). The final penetration of the sperm head thru the membrane, leaving the middle piece and tail outside, may also be observed. At about 30 minutes, the egg rounds up again, but as the time approaches for 1st polar body formation, the egg elongates in a direction perpendicular to the polar axis. If no eggs lie so that the forming polar body is on the horizon, the dish should be shaken. The 1st polar body may form at about 36-40 minutes, and it lies in the space between the egg and the vitelline membrane. This space is wider in the region of the animal pole than elsewhere. The second polar body often forms at about 50 minutes and commonly does so immediately under the first polar body, which is thus lifted into perivitelline space. At perhaps 80 minutes the eggs will begin to divide into two unequal blastomeres. Observe 2nd and 3rd cleavages also, if time permits. The 3rd division, from 4 to 8 cells, produces 4 micromeres by spiral cleavage (ref. #24.). 

Place some very recently fertilized eggs of Nereis in a drop of frosh, thick chinese ink suspension (made up by rubbing a piece of ink on a Syracuse dish moistened with sea water) in the center of a Syracuse dish. As the jelly is secreted, the attached sperm causes a canal to form in the secreted jelly into which particles of ink will penetrate. This is due to inhibition of jelly outflow at the point of sperm attachment. The ink thus marks the entrance point of the sperm. After the canal has filled with ink, add sea water and if time permits, observe and record for a number of eggs the relation of the first cleavage plane to the polar bodies and the entrance point of the sperm as marked by the ink (ref. "12,16,19"). (Caution: do not leave the piece of chinese ink in a dish of sea water; it will disintegrate).

NEREIS: Exaggerated Entrance Cones:

Place some Nereis eggs inseminated 5 to 8 minutes earlier in a Syracuse watch glass containing alkaline NaCl (pH 10.3-10.5. Observe immediately. The vitelline membranes will elevate due to a sudden inhibition of jelly release through the membrane and a subsequent accumulation of the jelly in the perivitelline space (ref. #9).
The vitelline membrane remains permeable to water which enters the perivitelline space as the jelly swells. The elevation of the membrane stretches out the sperm entrance cone between membrane and egg surface, forming a long filament which frequently causes marked indentation of the membrane. If the eggs have been kept in an ice box they may become polyspermic upon insemination and show numerous exaggerated entrance cones upon treatment with alkaline NaCl. About ten minutes after treatment the sperm head may be seen moving across the perivitelline space to fuse with the egg surface, at which time the membrane indentation is relaxed. If these eggs are carefully removed from the alkaline NaCl to sea water, and washed, some will develop normally within the raised membranes. If left in alkaline NaCl the optimum length of time before washing, and if the alkaline NaCl has been changed once or twice to remove most of the sea water, the eggs may be completely freed of their membranes. These "naked" eggs have been used for experiments on the development of isolated blastomeres (ref. #8).

Nereis: Centrifuged eggs:

If time permits, centrifuge some unfertilized Nereis eggs in the Emerson electric centrifuge (cover off) for 60 minutes. A layer of 0.95 molar sucrose (indistilled water) at the bottom of the centrifuge tubes prevents injury to the eggs. This is somewhat hypertonic, but provides an adequate support for the Nereis eggs without injuring them in any way. This amount of centrifuging separates the various formed components of the egg into several strata (ref. #7). Insominate the centrifuged eggs after washing off the sucrose with sea water, and observe asymmetrical jelly-extrusion. Is more jelly extruded at the centripetal or centrifugal pole?

If eggs stratified in an ultracentrifuge are available, compare these with the Nereis eggs centrifuged in the Emerson electric centrifuge at about 10,000 X gravity (ref. #10).

Breeding habits of Nereis limbata

Nereis swarm in Eel Pond about an hour after dark at certain phases of the lunar cycle. (See Lillie and Just, 1913). On each of two appropriate evenings, about an hour after sunset, half of the class will gather on the floating stage behind the Supply Department to observe this interesting phenomenon.

CHAETOPTERUS:

The chaetopterus egg is rather dark and granular. It is slightly more than 100 microns in diameter, but before fertilization the eggs are often not quite spherical. The polar bodies are larger than in Nereis, and the egg divides to form two unequal cells by means of a polar lobe at the vegetal pole (ref. #14, 18).

When the egg is taken from the female it contains a large immature nucleus (germinal vesicle), as does the Nereis egg, but unlike the egg of Nereis, it spontaneously undergoes partial maturation when placed in sea water, even if not fertilized. A number of species of eggs partly mature when they enter sea water and Pasteels (ref. #21) has shown that this is dependent upon the presence of Calcium in the sea water.
Chætopterus eggs develop quite rapidly. If eggs are fertilized just after the partial maturation in sea water has been completed, they develop as rapidly as eggs inseminated 12-15 minutes earlier when first placed in sea water (ref. 223). (Time counted from fertilization): 1st polar body 14.5 minutes; 2nd polar body, 27 minutes; "pear" shaped stage, 46 minutes; polar lobe bulge, 52 minutes; cleavage with polar lobe attached, 58 minutes; completed cleavage with polar lobe resorbed into one blastomere, 62 minutes; 4 cell stage, 82 minutes. If the laboratory air temperature is about 24°C, the development will be more rapid, and about at the rate indicated in the descriptive text below.

When the eggs are taken from the female, the large immature nucleus is in the center of the egg. After about 15 minutes in sea water, maturation will have proceeded to the metaphase of the first polar spindle at which stage development is arrested unless the egg is inseminated or artificially activated. The spindle cannot be distinguished as much in the living egg, but it will be observed that the relatively clear region of the nucleus and spindle is now located quite eccentrically. It reaches the surface of the egg in the polar region, where the polar bodies will be given off.

If eggs are now lightly inseminated and stirred, a few sperm may be seen adhering to the eggs almost immediately. Within 5 or 6 minutes, the vitelline membrane may be seen to separate from the egg surface, after which time it is called the fertilization membrane. It is not conspicuous and does not elevate much above the egg surface. By 10 or 12 minutes, the oocytes, which had become almost spherical after fertilization, are seen to elongate in an axis perpendicular to the polar axis. This is preparatory to formation of the 1st polar body at about 12 minutes. In this division the egg thus assumes approximately the shape of a blastomere, although the polar body is a vestigial cell. After the 1st polar body forms, the egg again rounds up (16-19 minutes), but it elongates again in the same manner to form the 2nd polar body at perhaps 23 minutes. The 2nd polar body often forms under the first, which is thus pushed away from the surface of the egg. The egg again rounds up (26-30 minutes); The egg pronucleus may now sometimes be seen migrating toward the center of the egg, and occasionally the sperm pronucleus may also be detected. By 35 minutes, the clear zone has extended from the polar region toward the equator of the egg, and at 37-40 minutes a typical "pear shaped" stage is reached. The polar bodies lie at a position corresponding to where the stem attaches to a pear, and the bulge which forms the polar lobe begins quite suddenly at the anti-polar end of the egg (40-43 minutes). When the polar lobe is fully developed, however, it corresponds to the stem end of the "pear", and the polar bodies are opposite.

Observe the extrusion of the polar lobe which contains coarse globular material. By 47-48 minutes the cleavage furrow begins at the animal pole and passes to one side of the polar lobe, which thus comes to be attached to one of the two blastomeres. The resorption of the polar lobe into this blastomere causes it to become larger than its mate, and after about 51 minutes two smooth unequal blastomeres lie against each other. Polyspermic eggs will now often be in an abnormal 3 cell stage. By 60 minutes the two blastomeres are quite fused together. At 67-70 minutes the 2nd cleavage takes place. The large blastomere again forms a polar lobe, and a 4 cell stage results with one blastomere larger than the other three. By 90 minutes, or
earlier, the clear nuclei in the 4 cells may readily be made out. At 93-97 minutes the 3rd division takes place, forming 4 relatively large micromeres. A profile view will reveal the macromeres, micromeres, and polar bodies. A polar view will show the rotated displacement of the micromeres, resulting from spiral cleavage, although the displacement is not great or conspicuous in Chaetopterus.

CONCENTRATION AND ACTIVITY OF SPERMATOZOA:

Place a small shallow drop of "dry" spermatozoa directly from the testis on a slide. The sperm must not be diluted with any sea water whatsoever. Sea urchin spermatozoa are good for this because it is especially easy to obtain them "dry" directly from the testis. Place a drop of sea water nearby on the slide so that it does not touch the drop of spermatozoa. With ordinary low power (approx. 100X) examine the spermatozoa and note the degree of activity especially at the edge of the drop where they may be seen more readily. Now take a small glass rod, or a match stick, or other object and drag its tip from the drop of sea water into the drop of spermatozoa so that a connecting bridge is established. As the sperm diffuses into the sea water a gradient of concentration is established. Note swimming activity in relation to concentration.

CUMINGIA:

If Cumingia eggs are available, observe the migration and fusion of the pronuclei. While Cumingia eggs are small (about 60 microns in diameter), they are clear and show the pronuclei in the living state especially well. They cytoplasmic constituents of these eggs are very readily stratified into four zones in the centrifuge.

SPECIAL PROJECTS:

During spare time students may undertake special projects relating to fertilization or cell-lineage. The following problems are suggested, but students may formulate their own projects subject to the approval of the instructor.

1. Fertilization of Platynereis. Collect platynereis from the Cayadetta Wharf floating stage at the right phase of the moon. Consult E. E. Just's papers on breeding habits and fertilization. Compare your observations with those on Nereis libata.

2. Development of Isolated Blastomeres of Nereis. Remove membranes of inseminated Nereis eggs by the alkaline NaCl method previously described. Use semi-sterile technique with all dishes and instruments. Make Spemann glass needles after directions of Horstadius in McClung's Microscopical Technique. Use small watch glasses (preferably Columbia) with a thin layer of filtered agar made up in sea water, as operating and isolation dishes. Separate the blastomeres with the fine tips of the glass needles immediately after the cleavage furrow is complete. Transfer to separate isolation dishes with mouth pipette and permit isolated blastomeres to develop in cool moist chambers. Observe at frequent intervals.

3. Development of Centrifuged Nereis Eggs. Centrifuge Nereis eggs for 60 to 90 minutes in Emerson electric centrifuge with cover off (or in air turbine, if available), with sucrose, as previously
described. Wash off sucrose in sea water, inseminate and study cleavage. Statistics as to the number of A5 and CD blastomeres forming from centripetal or centrifugal ends of the centrifuged eggs would be of interest. Position of micromeres may also be noted in relation to stratification and in relation to egg polarity.
General References:

3. Lillie, F. R. and E. L. Just 1924 Chapter 8 in Cowdry, General Cytology, Univ. of Chicago Press

Further references relating to laboratory work:

8. Costello, D. P. and R. A. Young 1939 The mechanism of membrane elevation in the egg of Nereis limbata Collecting Net, 14, 210
12. Just, E. E. 1930 The present status of the fertilizin theory of fertilization. Protoplasma, 10, 300
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Lunar Periodicity


3. Hempelmann, Fr., 1911 (Nereis dumerilii) Zoologica, Bd. 25


CELL LINEAGE

Maturation, Fertilization and Cleavage in Crepidula

Due to the opacity of the living eggs, the details of maturation, fusion of germ nuclei, and cleavage can best be studied from prepared slides. The eggs are not sectioned but are mounted whole. A lower power eyepiece and high power objective are useful. Refer to Conklin, 1897, Embryology of Crepidula.

Crepidula is a dioecious genus with the males fewer in number and smaller than the adult females. The spermatozoa mingle with the ova before the egg capsules are formed within the oviduct of the female. The mature females are sedentary, the males locomotive, and at the breeding season, or perhaps once for all, the females are visited and fertilized by these motile males. All the ova produced by one individual are laid at about the same time.

Make a careful study of various stages in the two maturation divisions (slide A). In the same eggs find the sperm nucleus and note its approach to the egg nucleus.

Study the 1st and 2nd cleavages, noting the direction of the axes of the mitotic figures in the latter stage.

Details of the formation of the first three quartettes of microsomes and the cells derived from the 1st and 2nd quartettes can be found on slide B. Find examples of the 8, 12, 16, 20, 24 and 25 cell stages. (Slides A & B contain all stages up to 25 cells).

On slides C and D find stages in the development of the gastrula. Note the blastopore, and the increase in number of endoderm cells. (Slides C & D contain most stages, through gastrulae).

With a heavy knife, loosen a Crepidula shell from its attachment and find the egg capsules. Those that are small and light yellow contain eggs in the earlier stages of development; the larger, deep yellow or mud colored capsules contain older embryos and larvae. Examine the stages removed and obtain samples from the other members of the class for comparison.

A method of preparing whole mounts of cleavage stages of Crepidula.

1. Obtaining the material:

From the place of their attachment to the substrate, remove the egg clusters to a Syracuse dish containing a small amount of water, and free the eggs from their enveloping membranes with dissecting needles. Having freed the eggs, agitate them by gentle rotary rinsing with a pipette in order to wash them and concentrate them in the center of the dish. Change the water two or three times. Remove the stripped capsules with a pipette, concentrate the eggs, take them up into a pipette, and drop them, with the few drops of water in which they are suspended, into a vial 3/4 filled with Kleinemberg's pi-rosulphuric fixative. Fix the eggs for 15 minutes.
Remove the fixative using a pipette of small diameter equipped with a syringe bulb, and fill the vial with 70% alcohol. Wash in 70% until the eggs are white. It is advisable to avoid washing too long in 70%, since the stain employed is best when it does not penetrate the macromeres. These latter should therefore be left slightly acid. Thus the eggs are removed from 70% immediately after the last wash which removes no picric from them, hydrated in 50%, 35%, and washed thoroughly in 2-3 changes of water.

II. Staining:

After washing with water, fill the vial with undiluted Mayer's haemalum, and stain for 5-10 minutes. For the polar body stages, 5-7 minutes is usually sufficient. After staining, wash thoroughly in water, dehydrate, and clear in xylol. Remove the xylol used in clearing and replace it with a small amount of thin damar.

III. Mounting:

Cover glasses must be supported. For this purpose it has been convenient to use paper squares the size of 7/8” cover glasses. A hole is punched in the center of each square with a paper punch. In mounting the squares are cleared in xylol, and fixed to the centers of slides by adding three or four drops of thin damar before the evaporation of the xylol. When the paper mounts have dried, the eggs are removed from the vial in which they are stored by the use of a pipette drawn out to a long taper and having a small diameter at its tip. The eggs are allowed to settle toward the tip of the pipette, and one drop of the egg-damar suspension is placed in the center depression of each paper mount. The damar is allowed to dry to the point of formation of a thin film in order that the eggs may remain dispersed and with the macromere quartette adjacent to the slide when mounted. Apply thick damar to the edge of the paper mount, immerse a #0 cover glass in xylol and apply it to the slide over the paper mount.

Alternative Method.

The above technique was used successfully by Mr. John Milford in 1939. An older method sometimes yielding good results is as follows:

1. Fix for 30-120 minutes in Mayer’s Picro-sulphuric.

2. Wash in 35, 50, 70% alcohol. Leave in latter until yellow color ceases to come out.

3. 50, 35% alcohol, to water (5 min. each).

4. Stain in Conklin’s haematoxylin (1 part Delafield’s to 4-5 volumes dist. water, to which 1 drop of picro-sulphuric fixative is added for each 10 cc. of the diluted stain.) for 5-10 minutes.

5. Wash in water, dehydrate 5 min. in each alcohol; 10 min. in 95%; 2 changes of absolute alcohol; xylol.

6. Mount in thick balsam with supported cover glass.
Cell-lineage References

1. General:

Huxley and DeBeer, 1934. Elements of Experimental Embryology, Chap. 5. Cambridge Univ. Press.

2. Special:

EMBRYOLOGY OF MOLLUSCA

Gastropoda

1. The Veliger Larva.

Study the typical Veliger larva of Crepidula fornicate. Obtain material by breaking the animal from the substrate to which it is attached. You will find the yellowish eggs (enclosed in transparent capsules) attached to the substrate or in the shell of the mother. Tease the embryos out of the capsules. Obtain swimming larvae in different stages, particularly old ones with the yolk resorbed. They are transparent and show the inner organs. Consult Conklin ('97) figs. 80-82 and the textbooks of Parker-Haswell vol. 1 (1928) fig. 657 on p. 734, or MacBride figs. 263 ff. on pages 301 ff., or Korschelt, 1936, vol. 2, pp. 881-891. Study dorsal, ventral and lateral views. Mouth and foot are on the ventral side, the anus is on the right side.

Note: 1. Velum with powerful cilia.
2. Head vesicle (dorsal)
3. Eyes with lens (dorsal)
4. Foot (ventral), study it in lateral view.
5. Statocysts (ventral, on basis of foot).
6. Mouth with powerful cilia (ventral, above the foot).
7. Oesophagus (dorsal), stomach, liver. The different parts of the intestine can be distinguished only in older embryos which have resorbed the yolk.
8. Anus (on right side).
9. External kidneys (lateral to foot.)
10. Heart (dorsal). Observe the heart beat in older embryos.
11. Transparent shell.

II. EARLY STAGES OF THE VELIGER LARVA.

There is no typical trochophore stage in Crepidula. Study stages of direct transformation of the gastrula into the Veliger larva. (Conklin, '92, figs. 77-79,) Observe the gradual development of shell gland, shell, velum and foot.

Literature:
Lamellibranchia (Pelecypoda)

III. The Veliger Stage.

Study the Veliger larva of Cumingia. Study larvae 2, 3, 5, and 8 days old; mount them with a few shreds of lens paper to entangle them or use Chloralhydrate as an anesthetic. It will be necessary to find more or less quiet individuals with velum extended. Contracted individuals will not do.

Those which are expanding and contracting the velum are best for study since this moves the digestive tract. Use the low power first and after a good specimen is found turn on the high power. Make out the following:

1. The general shape of the body with straight hinge line and semicircular shell.

2. The ciliated velum and apical tuft.

3. The digestive tract consisting of oesophagus, stomach and intestine. Is the latter coiled or straight?

4. Position of mouth with reference to the velum. Also position of anus.

5. Ciliation of the digestive tract. How much of it is ciliated? What is the mode of feeding? Add Chinese ink to the water.

6. The primary body cavity is a rather large space seen above and below the digestive tract. It is not a true coelom but perhaps a persistent cleavage cavity.

7. Extending from the hinge line to the velum are several strands of muscle fibres.

IV. The Trochophore Stage.

Study the Trochophore stage of Cumingia (12-17 hrs. after fertilization) and its transformation into the Veliger larva. (18-24 hrs. after fertilization).

The trochophore is of short duration, (less than ten hours.) It is best studied 12 to 17 hrs. after insemination. Mount some trochophores in a light suspension of India ink, and entangle them in shreds of lens paper.

1. Observe the general shape in side and polar views.

2. Position of the band of cilia.

3. Apical tuft?

None of the internal organs can be seen because they are obscured by yolk.

Draw side and polar views in outline, showing ciliation.

1944

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Embryology of Annelida

Nos. 1, 2 and 3 are required, #4 should be done if time permits.

The three forms to be studied are *Hydroides* (Eupomatus) *hexagonus*, *Nereis* and *Sabellaria*.

Cultures of advanced stages will be prepared. If you wish to prepare your own cultures proceed as follows: *Hydroides* both males and females will spawn immediately after being removed from their calcareous tubes. Remove several and place them in finger bowls (one worm per dish to keep the sexes separate). Remove the animals after they have spawned. Let sperm stand for about ½ hour. Add a few drops of sperm suspension to a dish of eggs. (Extrusion of polar bodies and cleavage may be easily studied). The blastula stage is reached after 5-6 hrs, gastrulation after 8-12 hrs; the trophophore stage lasts from 20 hrs to 2 weeks. The trophophores are best for study when 2-5 days old. *Sabellaria* may be treated in the same way. Fertilization of *Nereis* has been studied in a previous lab. period

1. The Trophophore of Hydroides.

The trophophore is a typical Annelid trophophore. Consult the excellent figs. in Hatschek ('86) and Shearer ('11). The larvae show positive phototaxis and gather at the window side of the dish. Mount trophophores, 3-5 days old, on a slide on which a few shreds of lens paper have been placed to entangle them and hold them quiet. Narcotics, e.g. a few drops of chloretone or of MgSO4, are not very effective but may be tried. Vital staining obscures rather than clarifies the structures. The larvae are transparent, and proper adjustment of the illumination by moving the mirror and the Abbe condenser will bring out all structures. Study animals in lateral and in polar views (both from animal and from vegetal pole). The apical tuft and the anal vesicle are landmarks for the poles, the mouth is on the ventral side; the eye is on the right side. Observe the locomotion first.

Observe:

1) **Shape of the trophophore**
2) **Apical tuft** (several long cilia, probably functioning as a sense organ).
3) **Apical organ**, a thickening of ectoderm at the animal pole; a nerve center and probably the primordium of the cerebral ganglia.
4) The **Prototroch**, an equatorial band of large cilia. In older trophophores, two rows of cilia will be found; a row of short cilia anterior to the large cilia. The prototroch is the most characteristic structure of the larva, and gave it its name. It is always anterior to the mouth (prooral). It consists of a few large prototroch cells which become pigmented in older stages.
5) The **meta troch** (paratroch), a circular band of cilia in the middle of the posttrochal hemisphere.

6) A **ciliated groove** on the midventral line connecting the mouth and anus. This groove is interesting in that it marks the line of closure of the blastopore. The mouth is the remnant of the blastopore; the anus is a secondary opening at the lower end of the original blastopore slit.

7) One eye on the right side of the pretrochal hemisphere. Note the red eye pigment.

8) Two **statocysts** on the ventral side.

9) The digestive tract, consisting of: mouth opening, *stomadacum* (oesophagus; ectodermal), **enlarged stomach** (entodermal), narrow intestine (entodermal except for the end portion which is invaginated ectoderm & proctodacum), and the **anus**, an opening behind the vegetal pole. All parts are beset with cilia. Feed india ink and study the mechanism of food intake.

10) The **anal vesicle**, a large vacuolated cell at the posterior end, not found in other trochophores.

11) The cavity between intestine and outer body wall is not a true coelom but a **primary body cavity**, the persisting blastocoele.

12) The **larval kidneys** (paired) are typical protonephridia with flame cells; they open near the anus. They appear as slender cords near the statocysts, extending between oesophagus and anus. They are best identified in animals with vegetal pole up (consult figs. in Batschek and Schrader).

13) **Muscles.** Two fine strands will be seen bifurcating at the upper end of the larval kidney. One of them can be traced to its insertion at the apical plate, the other inserts at the oesophagus. These are **longitudinal muscles**. Other longitudinal muscles extend from the stomach to points of the upper hemisphere. A strong **circular muscle** is near the metatroch; the constriction of the larva caused by its contraction will be frequently observed. Note also **circular (sphincter) muscles** in the digestive tract.

14) Undifferentiated **octomesoderm cells**, single or in small groups, will be seen attached to the stomach, to the inner body wall, near the apical organ, etc.

15) The **important entomesodermal cells** (derivatives of 4d*Teloblasts*) which will give rise to the mesodermal structures of the worm body are difficult to distinguish. They are small groups of cells near the lower end of the head kidney.

Draw lateral and polar views.
Metamorphosis of the Nereis Larva

The metamorphosis of an Annelid larva into a segmented worm can be studied best in Nereis, 1 to 7 days old. Nereis has no typical trochophore but an abbreviated, telescoped larval development. The first signs of the adult, segmental organization appear very early. Prepare slides as under 1. Study larvae from all sides. Consult the figs. in E. B. Wilson (92).

A. Trochophore-like stage 24 hrs. (Wilson, fig. 84)

Observe:

1) The prototroch, composed of 12 very large ciliated cells.
2) The paratroch, near the vegetal pole.
3) A pigmented area at the anal pole: the anal pigment.
4) The mouth and stomodaeum; the latter is a short ectodermal invagination.
5) The large macromeres have not yet differentiated into the entodermal parts of the intestine; their cell boundaries may be seen. No anus is formed as yet.
6) Several "frontal bodies" near the upper end. Circular disc-like structures of unknown function.
7) Two pairs of seta sacs, spherical structures in the posttroch hemisphere. The setae (chaetae) will be differentiated inside of them. These sacs are the first indication of the first two segments of the worm.
8) Observe the trochophore in locomotion.

B. Advanced trochophore, 2 days old (Wilson, figs. 85-89)

Identify all structures found in A. In addition observe:

1) Eye spots
2) A third pair of seta sacs has appeared behind the first two. All three are lined up in a row indicating the first 3 segments. Setae can be seen in the process of formation, inside of these sacs; in slightly older stages, they will be seen projecting from the sacs.
3) Pigment appears in the prototroch cell.

C. Metamorphosis. 2½-3 days (Wilson, figs. 90-91)

Notice the change of shape and the gradual demarcation of the first three segments.
Observe the following new features:

1) **Sense hairs** at the apical pole.
2) **Additional eye spots**; number variable
3) **Mesotrochal ciliary** bands at the boundaries of the segments.
4) Parapodia with **parapodial cirrhi** appearing on segments 2 and 3.
5) **Tentacular cirrhi** on the head.
6) **Stomadaeum, stomach, intestine** become distinct.

This stage combines larval (trochophore) and adult structures in a peculiar way.

D. **Late Stages of Metamorphosis.** 4 days and older.

(Wilson, fig. 92)

The head has lost its balloon shape and develops the appendages characteristic for the adult. Locomotion is still by means of cilia. The end of metamorphosis is marked by the shedding of the prototroch and the mesotrochs, and the change from swimming to crawling.

Observe:

1) **Frontal antennae, tentacular cirrhi, palpi,** all on the head.
2) **Anal cirrhi.**
3) **Two large and several small eyes.**
4) **Jaws inside of the oesophagus; they can be protruded.** The animal is carnivorous and rapacious.
5) **Study the finer structure of the setae, and their motility.** (muscles at their bases).

III.

**The Trochophore of Sabellaria.**

This larva shows very long bristles which have probably both a suspensory and a protective function. Study trochophores, 2 days and older (consult D. P. Wilson '29, and Novikoff '38).

Observe:

1) **Stiff apical cilia** in the place of the apical tuft.
2) The **prototroch** consists of 3 rows of cilia, and shows a gap on the dorsal side.
3) **The neurotroch** in the midventral line.
4) One **eye** on the left side. More eye spots develop later.
5) The **hood**, a fold overhanging the mouth

6) Very long bristles develop in seta sacs. Study their fine structure. They appear one pair after another, eventually 10 pairs. They will be replaced at metamorphosis by ordinary setae.

**IV. Gastrulation and Formation of the Trochophore in Hydroides**

Prepare your own cultures (see p. 1). Gastrulation by invagination occurs approximately 7-10 hrs. after fertilization. Consult the figs. in Shearer ('11) and Hatschek ('86).
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This species is usually ripe from mid-June to mid-September in the Woods Hole region. When stored in laboratory aquaria they may maintain their ripe condition even beyond the breeding season and supply apparently normal eggs and sperm. In general, however, it is safest to use animals within a few days after they are collected.

While in some species of echinoids the sexes may be distinguished externally (see Marx, 1931), no differentiating characteristics have, as yet, been described for A. punctulata. The sexes are readily identified after the animals are opened by the deep-red or purple ovaries and the yellowish-gray testes, or, if unopened animals shed spontaneously, by the red eggs and the white sperm.

Obtaining the gametes:—Any of the following methods may be used.

a) Cut around the peristome and remove the Aristotle's lantern. Pour out the body fluid and place the animal, aboral side down in a dish containing a small amount of sea water. The animals then frequently shed through the gonopores. After 10 minutes remove any eggs that have been shed to a finger bowl (or other large flat dish) containing ca. 200 ml of sea water. Sperm should be kept in concentrated suspension or "dry" (i.e., as it exudes from the testes).

b) Cut around the test about half way between the mouth and the equator and proceed as in a. Shedding is more frequently obtained by this method, but there is also more likelihood of cutting the gonads.

c) Cut as in b, pour out body fluid and remove gonads (at gonoduct end) with blunt forceps, spatula or spoon. The ovaries should be placed in about 200 ml of sea water in a finger bowl and allowed to shed. If undisturbed the eggs are extruded in compact clumps or strings and may be readily removed to a fresh dish without ovarian tissue by means of a wide-mouth pipette. If large quantities of eggs are desired the ovaries should be allowed to shed for about 1 hour with occasional stirring, then poured gently through washed (and sea water soaked) cheesecloth or bolting silk.

d) Inject about 0.2 ml of 0.5M KCl into the peristomial cavity. Ripe animals will begin to shed within 2 minutes. The eggs can be collected by inverting the animal in a dish of sea water or by washing the eggs gently from the surface of the animal with a pipette. The sperm should be removed "dry" or in concentrated suspension.

Spermatozoa:—Upon dilution with sea water the sperm become temporarily intensely active. They lose their motility sooner in concentrated than in dilute suspension, due presumably to the more rapid accumulation of CO₂. On the other hand their ability to fertilize eggs is lost more rapidly in dilute than in concentrated suspension. (See F. R. Lillie, 1915; Cohn 1918; Hayashi 1945.) When kept in the cold 2°C.) "dry" sperm may remain good for several days. At room temperature dilute sperm suspensions may lose their fertilizing power in an hour or less. It is advisable, then, to use freshly diluted sperm for fertilization.

The head of the sperm is comprised of acrosome, nucleus and midpiece that are roughly 0.3 and 1 microns respectively in length and 0.3, 1.3 and 1.2 microns in greatest width. The tail is about 45 microns long and 0.1 micron in greatest width. Its axial filament protrudes a short distance beyond the end of the sheath. Examine under oil immersion and sketch a spermatozoon. Examine moderately active spermatozoa under 'high-dry' and describe their mode of swimming.
Unfertilized Eggs:— Arbacia eggs complete both meiotic divisions while still in the ovary and the polar bodies very seldom remain attached when the eggs are shed. Occasionally, especially from relatively unripe animals or after macerating ovaries, eggs may be found that are in the germinal vesicle (diakinesis of primary oocyte) stage recognizable by the large clear nucleus (about one-half egg diameter) and nucleolus. Such eggs may exhibit some surface response to sperm but they do not develop upon insemination. The ripe egg (75 microns diameter) has a small clear nucleus. It contains uniformly dispersed pale yolk granules and slightly larger red granules containing a pigment called echinochrome which is a substituted naphthoquinone related to the K. vitaminds. (Ball, 1936; Hartmann et al. 1959; Tyler 1939). Upon centrifugations mitochondria and oil spherules are also distinguishable. The nucleus is generally located excentrically. Since the polar bodies are not usually present the position of the nucleus with respect to the polar axis is not readily determined. Occasionally, however, batches of eggs are obtained in which the polar bodies are attached. In those, observations (Hodgley, 1934) have shown that the nucleus may lie in any part of the cytoplasm between the cortex and the center. In the transparent gellatinous coat (about 30 microns wide) of the egg there is a funnel-shaped space which generally lies in the polar axis. The funnel is rendered visible by staining the jelly with Janus green or by placing the eggs in a suspension of Chinese ink. For this purpose the eggs should be taken immediately after shedding since the micropyle (funnel) may disappear as the jelly swells. Examine and sketch some unfertilized eggs under high power noting features described above.

Centrifuge a sample of unfertilized eggs at about 10,000 g for ½ hour and sketch one in "side view" noting the following five layers of stratified material:— oil cap (centripetal end), hyaline zone, mitochondria, yolk zone and pigment layer (centrifugal end). Where is the nucleus located? Have the granules in the cortex of the egg been displaced?

Fertilization:— Inseminate a sample of eggs, using one drop of freshly diluted 1% sperm (one drop of "dry" sperm in 5 ml of sea water) for each 10 ml of freshly washed dilute egg suspension containing about 5,000 eggs per ml. Stir the dish immediately after adding the sperm and observe the process of membrane elevation. How soon does it begin? when is it completely separated from the surface of the egg? When does the perivitelline space attain its maximum width? Measure the diameters of an unfertilized egg and an egg at 10 to 15 minutes after fertilization. Is there any appreciable difference in volume apart from that of the perivitelline space?

The spermatozoa enters the egg within a few second after attachment. To observe the process place a drop of eggs in the center of a vaseline-ringed slide and add a drop of sperm of just sufficient concentration to fertilize all of the eggs. Add a coverslip and locate as quickly as possible an egg that shows only one spermatozoa on its surface. Note the changes that occur upon penetration of the sperm. Where does membrane elevation first begin? In the cortex of the unfertilized egg there is a single layer of granules which disappear (Moser, 1939) upon fertilization and contribute (according to Runnstrom, 1944) to the formation of the fertilization membrane. These are best seen in the hyaline zone of the centrifuged eggs. Inseminate a sample of centrifuged eggs on a slide, as described above and observe the behaviour of the cortical granules.
Dark-field illumination shows a bright reddish "luminous" layer on the surface of the unfertilized egg. The luminosity diminishes and becomes paler upon fertilization (Runnström, 1928; Ohman, 1945) Using the dark-field stop disc for the condenser of your microscope examine a sample of unfertilized and fertilized eggs.

Preliminaries to Cleavage:- At 10 minutes after fertilization a hyaline layer (about 1 micron wide) forms on the surface of the egg. This layer later follows the cleavage furrows and is the material by which the blastomeres are held together. In calcium-free sea water the hyaline layer disappears.

The sperm cannot be distinguished in the living egg. At about 15 minutes after fertilization (at 20°C) a sperm aster is visible as a spherical region containing clear rays extending from a clear center. This attains its maximum development at 20 to 30 minutes. Then a clear streak appears in the egg slightly above the equator and at 45-50 minutes this replaced by two clear areas, the asters of the first cleavage spindle.

Cleavage:- The following figures give the average time for the first three cleavages (after Fry, 1936).

<table>
<thead>
<tr>
<th>Cleavage</th>
<th>Minutes after fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>42 (25°C) 113 (15°C) 107 (20°C)</td>
</tr>
<tr>
<td>Second</td>
<td>145 (20°C)</td>
</tr>
</tbody>
</table>

Different batches of eggs vary slightly (1-2%) in average cleavage time and, while within a batch of eggs most will develop at the average rate, some may vary by about 10%. For any temperature between 15°C and 25°C the average time of development can be calculated from that at 20°C by means of the following formula:-

\[
\log (\text{time at temp.}) = \log (\text{time at } 20^\circ C) - \frac{(t - 20)}{10} \log 2.6
\]

Temperatures above 30-32°C are lethal for Arbacia eggs.

The first three cleavages divide the egg into eight equal sized blastomeres. The planes of the first two cleavages are meridional (in the polar axis), that of the third is equatorial or horizontal (at right angles to the polar axis). Follow the progress of the cleavage furrow in dividing eggs. Note that the hyaline layer forms the surface of the furrow, and later, when the cells flatten against one another, that it forms the boundary between them.

At the fourth cleavage the upper four cells divide meridionally forming eight equal cells called mesomeres, while the lower four cells divide unequally and horizontally forming four large cells called macromeres and below them, at the vegetal pole, four small clear cells called micromeres. At the fifth division the eight mesomeres divide equally and horizontally forming two tiers of cells termed an1 (at the animal pole) and an2 (see Hörstadius, 1939) while the macromeres and micromeres divide meridionally. At the sixth cleavage the an1 and an2 cells divide in more or less radial direction while the macromeres divide horizontally forming two tiers termed vog1 and vog2. Vog2 is next to the micromeres which have also divided at this time but which do not form distinct layers. Layers of cells are not readily distinguished in later cleavage stages and no special designation is applied to the cells after the 64-cell stage. It has been shown (see Hörstadius 1939) than the an1 and an2 and vog1 cells form the ectoderm (gut) and part of the mesoderm (coelom) and the micromeres form mesoderm(skeleton). Sketch the various stages up to the sixth cleavage.
Arbacia p. 4.

Blastula:— At the eight cell stage there is a very small central cavity which enlarges, as cleavage continues, to form the blastocoel. At about 6 hours after fertilization a smooth-surfaced spherical young blastula is formed, the wall of which is one-cell thick. Cilia soon develop on the surface and the blastula is rotated by their action within the fertilization membrane. At about 10 hours the blastula hatches out of the fertilization membrane. It has been shown (Kopae, 1941) that the blastula releases a "hatching enzyme" at this time that weakens and dissolves the membrane sufficiently for the blastula to break thru. A small tuft of long cilia develops at the animal pole of the blastula which is the forward end when it is swimming. At the base of this apical tuft the blastula wall is thickened, forming the apical plate. At the vegetal pole the blastula wall becomes flattened and the micromeres migrate into the blastocoele, forming the primary mesenchyme which gives rise to the skeleton. Sketch early and late blastulae.

Gastrula:— At about 20 hours after fertilization the cells at the vegetal pole invaginate to form a blind tube, the archenteron. This reaches the opposite end of the blastocoel in about five hours. The gastrula contains about 1000 cells and its outer wall as well as the wall of the archenteron has a single layer of cells. The primary mesenchyme cells form a ring around the blastoporal end of the archenteron. Secondary mesenchyme and, later, coelom are budded off from the tip of the archenteron. Draw beginning and completed gastrulae.

Prism:— At the completion of gastrulation the tip of the archenteron turns to one side of the gastrula which becomes flattened over an area extending from the animal pole nearly to the blastopore. This is the first sign of bilateral symmetry, the flattened area representing the ventral side of the embryo. The primary mesenchyme cells aggregate in two groups, one on each postero-ventral side, and each group secretes a triradiate spicule, the beginnings of the skeleton. Where the tip of the archenteron touches the ectoderm there is formed a depression which later acquires an opening into the archenteron to become the stomadeum. The archenteron becomes divided by two constrictions into oesophagus, stomach and intestine. The apical tuft disappears, a ciliated band surrounds the oral field, the embryo begins to elongate in the dorso-ventral axis and the direction of swimming changes so that the ventral side is forward. Draw a prism larva.

Fluteus:— After about 48 hours the embryo enters the pluteus stage which is fully developed at the end of the third day. The original apical plate grows out in a ventral direction to form the oral lobe which includes the stomadeum and anterior part of the oesophagus. Two short outgrowths, the oral (antero-lateral) arms are formed on the oral lobe, and, at the anal side, two longer anal (aboral or postoral) arms grow out in the same general direction. The original triradiate spicules form skeletal rods which extend into the oral arms (oral rods) the anal arms (anal rods), dorsally through the body (body rods) and laterally (ventral transverse rods). The rods are each made up of three or four parallel parts joined by cross bars. Different species of sea-urchings differ in this regard, so the structure of the skeletal rods is a useful characteristic in hybridization studies. The embryo continues to elongate in the dorso-ventral direction and becomes pointed at the postero-dorsal end where the body rods meet.
The axis running thru oesophagus, stomach and intestines becomes J-shaped. The stomach expands to form a spherical structure that fills a large part of the body of the pluteus and sphincter muscles connect it with oesophagus and intestine. The two coelomic sacs extend posterolaterally from the oesophagus. That on the left side becomes larger and later acquires a dorsal opening called the pore canal. The right coelom buds off cells to form the madreporic vesicle but otherwise remains rudimentary. The left coelom undergoes extensive later development in the formation of the structures of the adult sea urchin. These changes do not occur until the second week when metamorphosis begins in properly fed larvae and will not be studied here. It should be mentioned, however, that the adult organs are built up in and around a structure termed the Echinus rudiment which is formed by the fusion of an invagination (amniotic invagination) of the ectoderm on the left side with the mid-portion (hydrocoel) of the left coelum. The left side of the pluteus becomes, then, the future oral face of the adult. Draw a 3-day old pluteus in postero-ventral and side view.

References:
Hartmann, M., Schartau, O., Kuhn, R. and Wallenfels, K. 1939. Uber die Sexualstoffe der Seeigel Naturwiss. 27:433

Asterias forbosii (or A. vulgaris)

A) Obtaining gametes, maturation and fertilization:

The sexes are separate in Asterias, but it is not possible to distinguish them on the basis of external characteristics. Only animals with soft, bulging arms are fully ripe, and it is a waste of material to open small, hard-skinned starfish in an attempt to obtain gametes. Fill two 1750 cc. finger bowls with clean sea water from a 2-liter flask in which the sediment has been permitted to settle by about fifteen minutes of standing after withdrawal of the sea water from the tap. With large scissors, make a small puncture in one arm close to the disc, and pipette a few drops of cells from the gonad to ascertain the sex of the animal. If the animal is a female, remove this arm completely, and slit it along the mid-dorsal line to expose the bulging pair of ovaries, of a typical pale salmon color. Then with a pair of forceps carefully detach each plume-like ovary by grasping it near its point of attachment at the disc end, closing the gonaduct, and rinse it with as little injury as possible in the first bowl of sea water, then transfer it to the second bowl. The animal from which the arm has been separated may be returned to a separate aquarium of running sea water, and other arms may be used for gametes later in the day. Such an injured female will not keep indefinitely, however, and gametes are rarely usable at the time the animal begins to show autotomy. Do not cut up to ovaries in the bowl of sea water, merely allow the eggs to exude from the blunt end of the ovaries for a period of five minutes. At the end of this time, remove
the ovaries to another container, or discard them. The best eggs are those first shed. Gently stir the water in the large finger bowl and allow the eggs to settle. Settling occurs very slowly. Then pour off the supernatant sea water and carefully replace with an equal volume from a 2-liter flask. Then leave the eggs undisturbed, without shaking or stirring, for about 20 to 30 minutes. During this time small samples may be removed with a pipette for examination under the microscope, and the steps in germinal vesicle breakdown observed. Note the jelly-mill about the eggs. This may be demonstrated more readily in dim illumination or by adding a trace of Janus Green to one slide preparation. Eggs from a ripe female which was kept under proper conditions of coolness and adequate oxygen supply from the moment of collection, and properly manipulated in obtaining gametes, should show 85 to 90% germinal vesicle breakdown at approximately the same time. Retain a good sample of eggs in a small finger bowl to follow the maturation stages through the second polar division in the un inseminated eggs.

If the animal opened is a male, the testes will be white or ivory. Since it is important to use a fresh sperm suspension, this animal may be placed in a dry fingerbowl until the eggs are ready for fertilization. Then a single testis is removed, rinsed in clean sea water, and a small piece from the blunt end cut off and placed in 200 cc sea water. Two or three pipettes of this suspension should be added to a 1750 cc finger bowl of eggs, with an immediate but not violent rotational movement to ensure complete mixing. The optimum period for fertilization is after the breakdown of the germinal vesicle and before the 1st polar body has been extruded. It is, therefore, convenient to inseminate when the distal end of the first maturation spindle begins to protrude above the previously smooth surface of the oocyte, in a fair percentage of the eggs showing germinal vesicle breakdown. Eggs inseminated in the stage of the intact germinal vesicle are non-fertilizable, even tho they may elevate a fertilization membrane they do not develop further. The details of sperm penetration may be readily studied, if the observer examines the eggs without delay. It was in the egg of the starfish that Fol (1879) first observed the actual penetration of an egg by a sperm. Chambers (1930) has confirmed these early observations. Microscopic examination of clean slides and covers, and good illumination are prerequisites for observing the finer details of this process in the laboratory.

It must be remembered that the egg of Asterias is very delicate as compared with most eggs used for routine laboratory work. Satisfactory results are not obtained without taking adequate precautions. Important precautions are: (1) to avoid contaminating either type of gamete with perivisceral fluid; it is because of this that the gonads are rinsed; (2) do not overinseminate; (3) do not crowd the eggs; there should be no more than one layer of well-spaced eggs on the bottom of the dish; (4) use only fresh, motile sperm.

Cleavage:
The blastomeres of Asterias are rather loosely connected, because the perivitelline space is wider and the hyaline plasma membrane thinner and weaker than in the Arbacia egg. Note the relation between the first and second polar bodies and the fertilization membrane. Chambers has pointed out that in the absence of fertilization membrane, the blastomeres tend to separate completely. Because of the relative transparency of the yolk of this egg, details of living astors may be seen.
A detailed study of the cleavage of this form is usually not undertaken by students of the course unless they have a special interest in this material, but the later stages are of considerable significance:

Later Stages:

To raise Astorias to late embryonic stages, it is necessary to change the sea water in the culture dishes at about half-hour intervals during early cleavage to eliminate the excess sperm which would otherwise foul the culture. Then, when the first swimming stages (blastulae) appear, the upper half of the culture, containing the more normal top-swimming blastulae, is poured off into a series of tall battery jars which are subsequently filled to the top with fresh sea water. Care must be taken to avoid carrying over dead embryos of unfertilized eggs. Tall jars are superior to shallow dishes, since evaporation in considerably reduced. It is essential that relatively few larvae be placed in a jar. Early bipinnaria may be obtained without special feeding but the cultures of Astorias larvae must be fed dictyons (prepared by Just's methods) to obtain brachiolaria or later stages.

References:

Costello, D. P. 1935 Fertilization membranes of centrifuged Asterias Eggs. Physiol. Zool. 8 65-72
Dolago, Y 1904 Elevage des larves parthenogenetiques d'Asterias glacialis. Arch. de Zool. Exper. 43 Ser.2
Just, E. E. 1939. Basic methods for experiments on eggs of marine animals. Blakiston, Phil.

Asterias Forbesii

B. Development

Prepare your own cultures for early stages and use those prepared by the assistant for stages from blastula on. Development up to the early Bipinnaria (Dipleurula) can be followed on living material. Older Bipinnaria, Brachiolaria larvae and metamorphosis stages will be studied on stained whole mounts. Whole mounts of early Bipinnaria are also available for comparison. It is advisable to begin with early gastrula stages and follow the development through to the Dipleurula.
Study fertilization, cleavage and blastula later on, using your own cultures. Consult the illustrations in MacBride, Agassiz (1877) and Gemmill (1914) See time table in appendix.

1. Fertilization (see lab directions for "Fertilization")

2. Cleavage. The first two cleavages are meridional, that is, they go through animal and vegetal poles and are perpendicular to each other. The third cleavage is horizontal, the eight cells of this stage are approximately equal in size. In the 16-cell stage, no definite arrangement of cells in rows takes place, and cleavage from now on is irregular. Throughout these early cleavage stages the blastomeres have a tendency to assume spherical shape, resulting in a rather loose arrangement of cells.

3. Blastula. Eventually the cells arrange themselves in an epithelial wall enclosing the blastocoel. The surface cells acquire cilia, and the blastula rotates within the vitelline membrane. The two polar bodies are still visible, either attached to the animal pole, or detached from the embryo. The embryo hatches in the late blastula stage.

4. Early gastrula. The vegetal pole area thickens and flattens and invagination begins. The blastopore is destined to become the anus. The larva elongates along the animal-vegetal axis.

5. Middle and late gastrula. The gastrula becomes pear-shaped. The blind inner end of the archenteron becomes thin-walled and expands. From this end mesenchyme cells wander out into the blastocoel. In a slightly later stage, two outpocketings of the distal end become distinct, the primordia of the coelomic sacs. At the same time, the archenteron bends towards one side which is the future ventral side. This is the first sign of the change of radial into bilateral symmetry. Note the ciliation in the archenteron.

6. Transition to Dipleurula-larva. The blind end of the archenteron bends sharply towards the ventral body wall, makes contact with an endodermal depression, the stomodaenum, and the mouth breaks through. The two coelomic vesicles have been constricted off from the archenteron. The left one is larger from early stages on. It forms a tubular outgrowth to the dorsal body wall which opens to the outside. This is the *pore-canals*.

7. Fully formed Dipleurula (early Bipinnaria) larva. This larva represents an early larval type common to Asteroidea, Echinoidea, Ophiuroidea and Holothurina (see Korschelt, vol. 1 p. 499). Study carefully a ventral, dorsal and lateral (preferably left) view

Observe the following:

Shape of larva. Notice convexity of ventral side and mouth opening underneath the overhanging oral lobe.
Locomotion

Ciliation. Small cilia cover the entire surface. The ciliary band is at first continuous, a longitudinal band with two cross bars. The longitudinal band above the upper cross bar loops towards the midline. Eventually the loops meet in the midline, and a frontal field, the pre-oral ciliary band is separated in the upper ventral part of the larva, overhanging the oral field. This separate frontal field is characteristic of Asteroid larvae. Observe carefully the course of the entire longitudinal band. Observe the beat of the cilia in dark field illumination, if available.

Alimentary tract. The three parts, characteristic of Echinoderm larvae: oesophagus (with constriction near entrance into stomach), stomach and intestine. Observe in lateral view the bend of the intestine. Study ciliation in oral field and different parts of the tract.

Coelom. Study the two coelomic vesicles from all sides. They are clearly visible at the lower end of the oesophagus near its entrance into the stomach. A subdivision of the vesicles is not yet clearly demarcated, but the narrow tube connecting the larger left coelomic vesicle with the dorsal body wall, the pore canal, and its opening, the madreporic pore, can be readily seen. Loose mesenchyme cells are scattered in the body cavity which is the persisting blastocoele.

Vital staining with neutral red is helpful; but study unstained specimens first. Study also stained whole mounts of these stages.

The following stages are rarely found in dredges of plankton and difficult to raise from eggs in the laboratory. Prepared and stained whole mounts will be provided.

8. Fully formed Bipinnaria. Consult figures in Gemmill, MacBride, and Agassiz. This larva is characterized by a number of pairs of lobes or arms which grow out from the margin of the ectoderm and which carry the ciliary band along. They are not supported by skeleton. Pairs of arms follow each other in succession. Young stages may not have all arms developed.

Arms: Identify unpaired median dorsal, paired anterior dorsal, posterior dorsal, posterior lateral, postoral and preoral arms. See Gemmill, plate 18, fig. 7, and MacBride, p. 465.

Intestinal Tract

Coelom. The coelomic vesicles have grown out into long tubes and have fused in the anterior part of the larva. No further subdivisions have yet occurred.


The Bipinnaria arms are long, hollow tubes. The three Brachiolaria arms (brachia) are short. They contain diverticula of the coelom. They are not ciliated but their end discs differentiate small papillae and can adhere to the substrate.
A sucker, the gland cells of which secrete a sticky substance, is formed between the brachia. Brachia and sucker serve for attachment of the larva to the substrate in later stages of metamorphosis. (Gemmill, p. 250).

**Intestine**

*Coelom* in different stages of subdivision

*Disk* or developing starfish, on left side.

In late stages of metamorphosis, the anterior part of the larva in front of disk shrinks to form the stalk which is attached to the substrate by sucker and branchia and which carries the Asterias anlage at its distal end. For details of metamorphosis consult Gemmill, MacBride, and Korschelt.

**Echinarchnus parma**

**Obtaining gametes**

The sexes are separate in *Echinarchnus* (the sand dollar) but it is impossible to distinguish the male from the female by superficial examination. A cut is made about one-quarter inch from the margin around the entire animal through both oral and aboral calcareous skeletal parts. Then a scalpel is carefully inserted, just beneath the oral skeleton, separating the oral and aboral portions. The oral portion is lifted away and discarded, taking care not to destroy the gonads, which adhere to the aboral portion. The aboral portion is then placed (outside surface down) on a clean, dry Syracuse watchglass. If the animal is ripe, gametes will ooze from the gonads. Allow the opened male to remain undisturbed until the eggs are to be inseminated. The ovaries of the female are a reddish purple color, and the eggs are usually mixed with an opalescent or milky perivisceral fluid. From the female carefully pipette the eggs to a small finger bowl of sea water. After allowing the eggs to settle, carefully pour off the supernatant fluid and replace with fresh sea water. If the females are not in good condition (if the eggs do not readily stream from the ovaries), the gonads may be removed with a forceps to a finger bowl of sea water, and the egg suspension strained through clean, washed cheesecloth previously soaked in sea water.

**Fertilization**

The eggs of *Echinarchnus* are larger than those of *Arbacia* (135 microns as compared with 75) and surrounded by a much thicker jelly-hull in which beautiful red pigment granules are suspended. The egg itself, free of the jelly, is pale yellow. Examine the unfertilized eggs under low and high magnification. Then inseminate the eggs as was done in the case of *Arbacia*, and examine the eggs immediately after adding the diluted sperm suspension. Because of the relatively large size of the egg, the fertilization reaction may be readily followed. Membrane elevation proceeds from the entrance point of the sperm around the egg cortex in a wave. The membrane begins to elevate in from seven to twenty-two seconds after sperm penetration, and is completed in from nine to thirty seconds after it begins. Since sperm
penetration occurs from fourteen to forty-five seconds after insemination, both processes (i.e., sperm penetration and membrane elevation) may be completed within about 40 seconds after insemination (Just, 1919).

Cleavage of the egg of Echinarachnius is not markedly different from that of Arbacia, and unless this form is of special interest to the student, detailed drawings of the cleavage need not be made. One culture should be prepared and kept to provide plutei for comparison with those of Arbacia.

References:

Bibliography on Echinoderm Development

1. Normal development and metamorphosis


II. Experimental: general reviews:


Child, C. M. 1941. Patterns and problems of development. Univ. of Chicago Press.

Dalcq, A. 1938. Form and Causality in Early Development. Cambridge Univ. Press.


Curt Herbst in 1892 discovered that treatment of developing sea-urchin eggs with sea water containing lithium salts results in the formation of exogastrulae and other related types of abnormal embryos. This has been the subject of numerous investigations since that time (see Child, 1940, 1941; Lindahl, 1940 and your experimental embryology texts for complete references). The exogastrulating action of the lithium is interpreted as a result of a general vegetalization of the egg in which the vegetal, endodermal and mesenchymal, material increases at the expense of the animal, ectodermal, material. Other agents may bring about this effect but none are, as yet, known to give as consistent results as lithium.

Equipment:— Scissors, blunt forceps, 6” square of cheesecloth, 3 finger bowls, 12 syracuse dishes, 4 stender dishes with lids, 3 slides and coverslips, 1 graduate (100 ml), 3 ordinary pipettes, 1 narrow long-tipped pipette, 1 fine pipette.

Solutions:— 1 liter filtered sea-water
100 ml Li-sea-water (20 ml of m/2 LiCl + 80 ml sea water).

Treatment:— Obtain eggs and sperm of Arbacia or of Echinarchimnius in the usual way. Inseminate a fairly large sample of the eggs in a finger bowl. At the time of first cleavage transfer a sample with not more than 1 ml of sea water, to a finger bowl containing about 50 ml of the Li-sea-water and at the same time, another sample to a control dish of sea water. After 2, 4, 8 and 12 hours transfer samples (ca.0.05 ml) through three dishes of 10 ml of sea water and culture in half-filled, covered, stender dishes.

Development:— Observe the eggs at various times during the lithium treatment and compare their rate and form of cleavage with the controls. Examine the cultures twice a day during the next three days, and sketch various types of exogastrulae, noting inhibition of development of arms and octodermal structures, tripaprite structure of archenteron, etc. Determine the approximate proportion of normal to abnormal embryos in the four cultures.

Li-treatment of Isolated Animal Halves. Animal halves of sea-urchin eggs isolated in the 8-or 16-cell stage fail to gastrulate generally forming "Deuterostomulae". Von Ubisch (1929) made the interesting discovery that lithium treatment would enable some of the animal halves to develop into normal plutei. Students who are skillful in micro-dissection may substitute this experiment in place of the above, after discussing details of procedure with the instructor.

References:—
Child, C. M., 1940 Physiol. Zool. 13:4-42
Von Ubisch, L. 1929, Arch. Entw.-mech., 117:80-122
Tamini, E., 1943, Reale Instituto Lombardo (Red. Sci.), 76:363-392
In 1896-1899 Morgan, Mead, Hertwig and Loeb demonstrated that
development could be initiated and normal larvae obtained by
treatment of unfertilized eggs with salt solutions. Since that
time artificial activation of eggs has been the subject of a
great many investigations. (See review by Tyler, 1941, and your
experimental embryology texts for discussion and references).
The present exercise is essentially a repetition of Loeb's
classical double treatment method (modified after Just, 1939).

**Equipment:**- Scissors, blunt forceps, 6" square of cheesecloth,
10 finger bowls, 6 syracuse dishes, 4 standard dishes with lids,
3 slides and coverslips, 1 graduated pipette (5 ml, wide tip),
1 graduate (100 ml), 3 ordinary pipettes, 1 narrow long-tipped
pipette.

**Solutions:**
- 2 liters filtered sea-water
- 20 ml butyric acid sea water (8 ml M/10 butyric
  acid + 100 ml sea water)
- 200 ml hypertonic sea water (32 ml 2 M NaCl + 100 ml
  sea water).

**Treatment:**- Obtain eggs of Arbacia or of Echinarchlhius as pro-
viously described. Guard against accidental introduction of
sperm into the dishes of eggs. To 3 ml of a dense suspension of
the eggs add 3 ml of the butyric acid sea water. After 90, 105,
120 and 135 seconds remove samples with not more than 1 ml of the
solution to finger bowls containing 200 ml of sea water. Determine
in which of the four bowls the eggs show the highest percentage of
membrane elevation. Pour off about half of the water from the
bowl to facilitate concentrating the eggs, then transfer the eggs,
with not more than 3 ml of the solution, to a graduate and make
the total volume up to 50 ml with sea water. At 10 minutes after
the start of the butyric acid treatment pour the eggs gently into
a finger bowl containing 66 ml of the hypertonic solution. After
20, 25, 30 and 35 minutes in this solution transfer samples, with
not more than 1 ml of solution, to 100 ml of sea water, wash
once and culture in covered finger bowls or standard dishes.

**Development:**- Examine, at intervals of about ½ hour for 3 hours,
some of the eggs that have received only the butyric acid treat-
ment and note the repeated formation and disappearance of a
monaster. In the doubly treated eggs look for accessory cytoasts
and for amphisterns. Note the time and pattern of cleavage and
sketch some of the early cleavage stages. At about 3 hours esti-
mate the percentage cleavage and the following day the percentage
of "top swimmers" in the four-dishes. Remove the top swimmers to
another bowl, culture for two or three more days and estimate
the percentage of normal plutei obtained.

**References:**
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Development of Isolated Blastomeres of Arbacia
and Echinarchichinus

Hans Driesch in 1891 demonstrated that whole embryos may be obtained from isolated blastomeres of the sea-urchin egg. The present exercise is essentially a repetition of that classical experiment with some additional features provided by later work.

Equipment: - scissors, blunt forceps, 6” square of cheesecloth, 3 finger bowls, 12 syracuse dishes, 3 standor dishes with lids, 6 embryological watch glasses, 1 test tube (ca. 5/8”x6”), 2 fine glass needles, eyepiece micrometer, 3 vaseline-ringed slides and coverslips, 3 ordinary pipettes, 1 narrow long-tipped pipette, 1 fine pipette.

Solutions: - 1 liter filtered sea-water
100 ml hypertonie sea-water (30 grams NaCl in 1 liter of sea-water)
100 ml of Ca-free sea-water (1000 ml M/2 + 22 ml M/2 KCl + 195 ml M/3 MgCl2.6H2O + 103 ml M/3 Na2SO4 + 6 ml M/2 NaHCO3, adjusted to pH 7.9-8.3 (based on Lyman and Fleming 1940).
50 ml of 5% formalin in sea-water

Removal of Fertilization Membrane. For the purpose of isolating blastomeres the fertilization membrane must first be removed. This is accomplished by shaking a suspension of freshly fertilized eggs. Obtain eggs and sperm of Arbacia or of Echinarchichinus in the usual way. Inseminate (noting time) a sample to test for fertilizability and to determine first cleavage time. Ten minutes later fill a test tube about four-fifths full with a freshly washed sample of eggs and inseminate, mixing by inverting the test tube once. About one-half minute later pour about 1/2 of the eggs from the test tube gently into a syracuse dish and examine for membrane elevation. When the membranes have separated from the surface of practically all of the eggs (about 1 to 2 minutes after insemination), shake the test tube ten times rapidly up and down using a full fore-arm swing and holding long axis of tube in direction of swing with thumb over open end. Pour about 1/4 of the eggs into a syracuse dish, immediately shake tube again ten times, remove another 1/4 of the eggs, repeat a third time and remove the remaining 1/4. Examine the three dishes of shaken eggs and select the one containing the highest percentage of naked eggs. Wash twice with filtered sea-water.

An alternative method of membrane removal uses a pipette with a narrow opening. The opening can be several times the egg diameter. A concentrated suspension of eggs at the time of membrane elevation is rapidly drawn into the pipette, and expelled into an empty dish and examined. If one treatment is inadequate it may be repeated.

Separation of Blastomeres. For this purpose the student may use either the Ca-free sea-water (Herbst, 1900) or the hypertonie sea-water (E.B. Harvey, 1940) listed above. The time of first cleavage is determined on the control sample that was fertilized 10 minutes before the experimental set.
2. Ca-free sea-water. At about five minutes before the time of first division, concentrate the membrane-loss eggs in the center of the dish by gentle revolution of the dish (the center of the dish should follow the circumference of a circle about 1 to 2 cm. in diameter) and transfer, with the narrow long-tipped pipette, a sample of the eggs with less than 0.1 ml of sea-water through three dishes of 10 ml of Ca-free sea-water. Examine, under high power, a sample of the eggs in the Ca-free sea water and compare their ectoplasmic layer with that of the control eggs. After 10 to 20 minutes remove a sample to a dry finger bowl. If the blastomeres have not separated draw the sample rapidly in and out of the pipette several times. Fill the bowl with sea water and transfer once to a stender dish half filled with fresh sea water and cover. This will serve as a mass culture of isolated blastomeres along with some whole eggs. To study pairs of blastomeres from the same egg pick out of the Ca-free sea water dish, under the dissecting microscope, eggs in which the blastomeres are still together or are close enough together to be recognized as sister. Transfer each pair along with a whole egg to a separate embryological watch glass containing sea water. If the blastomeres of the pair were not completely separated at the time of selection bounce the egg in the dish a few times or separate the blastomeres by means of a glass needle before transferring to the sea water. After one or two cleavages mount a pair of isolated blastomeres along with a whole egg on each of two or three of the vaseline-ringed slides. To do this place a small drop containing the eggs in the center of the ring, add a coverslip and press it down so that it touches the drop and a continuous seal is made with the vaseline, but avoid having the drop touch the vaseline.

b. Hypertonic sea-water. With this method, the eggs (one drop) are placed in the solution (about 10 ml) when most of them have just completed the first cleavage but before maximal separation of the blastomeres has occurred. Examine, under high power, a sample of the eggs in the hypertonic solution and note the effect on the ectoplasmic layer. Ten minutes later, transfer the eggs with a minimum amount of solution through three dishes of normal sea-water. The treatment, if successful, causes the ectoplasmic layer, by which the two blastomeres are joined, to become thin and gelatinous, and the two blastomeres are often widely separated with only a thin film between them. Such pairs may be cultured along with control whole eggs, in embryological watch glasses and one vaseline-ringed slides as described above. They may be picked out at a later stage of development since the pairs generally remain attached by a thin hyaline strand until they are swimming blastulae. Only a few twin blastulae are ordinarily obtained by this method since the great majority fuse together during early development.

Development. Observe and sketch the isolated blastomeres in their 4-, 8-, and 16-cell stages. How many micromeres are formed at what stage? Does the isolated blastomere cleave as though it were still part of a whole egg? At the beginning of gastrulation fix some "half"-embryos and whole embryos in 5% formalin and measure their respective diameters. What approximate ratios are obtained for their respective volumes, surface areas and wall-thickness?
Examine and sketch the embryos in the completed gastrula, prism and pluteus stages. Do the "half"-embryos develop at the same rate as the controls? Are the "half"-plutei complete in regard to all structures seen in the whole plutei? Determine whether or not both members of the pairs of isolated blastomeres form normal plutei. (See Hörstadius, 1940; Tyler, 1942 and experimental embryology texts for further analysis).

References:

F. R. Lillie (1912) demonstrated that ripe eggs of Arbacia and of Nereis give off a substance, called fertilizin, which activates and agglutinates the species sperm. Similar fertilizins have since been reported in many species of invertebrates and they, together with the antifertilizins from sperm with which they react, have been the subject of many investigations, of which most of the more recent are listed below along with some of the older ones. The present exercise includes more tests with this material than can be completed in the time allotted. Only the simpler tests in the first part of each of the following sections should be undertaken during the class period assigned to the work. The additional material is presented as a guide to further work for those who may elect such investigations at the end of the course.

**Equipment:**
- 4 finger bowls, 50 syracuse dishes, 1 graduate (100 ml), 1 graduated pipette (5 ml), 4 ordinary pipettes (droppers), 1 large pipette (25-50 ml cap.), 25-50 ml rubber bulb), 2 centrifuge tubes (15 ml).

**Solutions:**
- 1 liter filtered sea water; 10 ml 1N HCl; 10 ml 1N NaOH

**Fertilizin:**
- Obtain eggs and sperm of Arbacia by one of the usual methods. Wash the eggs once and concentrate the suspension to about 25% by volume. After about 15 minutes mix 2 drops of the supernatant egg water with 2 drops of a 1% sperm suspension (one drop of "dry" sperm in 5 ml of sea water) and examine with the microscope. Note the agglutination of the sperm and, a few minutes later, the reversal of the clumping. Are the sperm still motile after reversal?

To 2 drops of a strong egg-water (in which eggs have stood several hours, or obtained by acidification—see below) and to 2 drops of sea water in a control dish add 2 drops of 1% sperm. After reversal of the agglutination add 2 drops of egg-water to each dish. Do the reversed sperm re-agglutinate?

To 5 ml of a strong egg-water and to 5 ml of a control dish of sea water add 1 drop of 'dry' sperm. Shake the dishes. What difference in behaviour of the drops of sperm do you observe and how do you account for it?

To 1 ml of a strong egg-water and to 1 ml of a control dish of sea water add 2 drops of 1% sperm. After agglutination has reversed add 1, 2, 4 and 8 drops from each to dishes containing 5 ml of a dilute suspension of eggs (about 100 eggs per ml). Determine the percentage fertilization in each of the 8 dishes. Has the egg-water treatment had any effect on the fertilizing power of the sperm?

Titration of fertilizin solutions may be done by testing serial dilutions of the solution with a standard sperm suspension. The dilutions may be prepared with an ordinary pipette (dropper) as follows:

- Place 2 drops of sea water in each of a set of dry dishes. Add 2 drops of egg-water to the first dish, rinse the pipette with sea water, mix the drops, draw up most of the mixture, expel 2 drops into the second dish and return remainder to the first dish. Repeat this procedure with the succeeding dishes. Then add 2 drops of 1% sperm to each dish and examine at once. The first dish contains a four-fold dilution of the egg water, the second eight fold, etc. The fertilizin titer can be expressed as the greatest dilution of egg-water that gives a microscopically perceptible agglutination reaction. Titrate your egg water using eight 2-fold dilutions.
Evidence concerning the source of the fertilizin may be obtained from the following tests. Divide about 200 ml of a freshly prepared 10% egg suspension in two equal parts and acidify one part to about pH 3 to 3.5 (requires about 1 ml of 1N HCl per 100 ml). A few minutes later draw off 50 ml of supernatant from each dish, neutralize the acid egg-water with 0.5 ml of 1N NaOH and determine the fertilizin titer. Examine the acid-treated eggs with the microscope and note the absence of the gelatinous coat. Neutralize and wash the acid-treated eggs. After several hours determine fertilizin-titer along with that of the similarly aged control. Acidify the control to pH 3 to 3.5, draw off the supernatant, neutralize and compare its fertilizin titer with that of the first acid-egg-water. Is there evidence of secretion of fertilizin by the eggs? What is the apparent source of the fertilizin?

To test for activating action of fertilizin allow a dilute (1%) sperm suspension to stand for 1 hour or until motility has decreased considerably; then add 2 drops to 2 drops of a strong egg water and to 2 drops of sea water. Examine the two dishes microscopically and note roughly the activity exhibited by the spermatozoa. Absorption tests may be made by adding a concentrated sperm suspension (10%) or greater) to an equal volume of moderately strong egg-water, centrifuging after 1 hour and testing the supernatant as well as a similarly diluted sample of the egg-water for agglutinating action on dilute (1%) sperm. Does absence of agglutinating action necessarily mean binding of fertilizin by the sperm? What other tests would be necessary?

Specificity may be examined by testing Arbacia fertilizin on sperm of closely related and distantly related animals, that are available in the laboratory. Whose reactions are obtained absorb the Arbacia egg-water with the foreign sperm, as described above, and test the supernatant on both species and foreign sperm. Antifertilizin:- This material may be prepared from a concentrated (10% or greater) sperm suspension by (a) freezing and thawing the suspension, (b) heating to 100°C. for one minute or (c) acidifying to pH 3. The treated suspension is then centrifuged or filtered and the supernatant or filtrate will be found to contain the active material.

To demonstrate the agglutinating action of antifertilizin add 2 drops of the solution prepared by methods a, b or c to 2 drops of a 1% suspension of freshly washed eggs. Shake the dish several times and examine macroscopically and microscopically after 1 to 5 minutes. Note the formation of a precipitation membrane on the surface of the gelatinous coat of the egg.

Titration of the antifertilizin is performed in the manner described above for fertilizin. Prepare a set of 8 two-fold serial dilutions of 2 drops of the antifertilizin solution and add to each 2 drops of a dilute (ca. 100 eggs per drop) freshly washed, egg suspension. Examine at once and again after ½ hour. Note differences in width of the gelatinous coat and in the precipitation membrane in the different dilutions and at different times. The end point may be taken as that dilution beyond which a precipitation membrane is no longer visible. Determine the titer of your preparation. Does the egg-agglutination reaction reverse spontaneously?

To demonstrate neutralization of the fertilizin add 2 drops of strong antifertilizin solution to 2 drops of a moderately strong egg-water. Prepare a control of 2 drops of egg water plus 2 drops of sea water. After ½ hour add 2 drops of a 1% sperm suspension.
to both dishes. Note the degree and duration of the agglutination reaction. Titrations may be performed with duplicate serial dilutions of the egg water to one set of which is added a constant amount of the antifertilizin solution while the other gets an equal volume of sea water then sperm added after \( \frac{1}{2} \) hour; or with duplicate serial dilutions of the antifertilizin plus constant egg-water to one set and sea water to the other, then eggs added after \( \frac{1}{2} \) hour.

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Introduction to Hydrozoa

a) Sensitiveness of the material

While working with coelenterates in the laboratory it is essential to remember that the hydroids are very sensitive to environmental conditions. They do not survive well in the laboratory even in aquaria of running water. Do not crowd either the adult stems or the eggs and embryos. In general, your procedure will be to look over a good-sized colony of hydroids under the lowest power of magnification, and to clip off a few pieces containing the best embryological material. These can be rinsed in running sea water, and segregated in plenty of sea water in a dish for more detailed study.

b) Types of Life History Illustrated

There are two phases of embryology in the Hydrozoa. Characteristically, a long series of asexual reproductions (by budding and other methods) is interrupted at irregular intervals by isolated examples of sexual reproduction. This alternation of sexual and asexual generations is closely paralleled by an alternation of structure, since zygote production is usually accomplished by medusae and the production of buds by polyps.

In some hydrozoa no medusa-form is known, in others no polyp-form is known, and there are all stages between. The hydrozoa available at Woods Hole illustrate well the structural variability of the medusa-state. The best-known example of a complete medusa with a degenerate polyp stage is the idealized jelly-fish Gonionemus, but it is unfortunately now nearly extinct at Woods Hole. The genera picked for study are arranged in the order of diminishing completeness of the medusa-form, the first having free-swimming medusae and the last mere spongæa. The utter degeneration of the medusa-form is illustrated by the familiar Hydra.

c) Order of Study

Because of seasonal variations and unpredictable fluctuations in the Coelenterate fauna, some of the material described may not be available, and the order of study will have to be announced.

Laboratory Procedure

A. Study of Forms with Perfect Medusa

Examples: Bougainvillia, Obelia, Podocoryne.

Characteristic life history: Zygote shed from medusa; Development to planula larva; Metamorphosis to polyp; Asexual Multiplication of polyps by budding, which produces a colony; Medusae formed by special buds, in a gonosoma (Obelia) or separately (Bougainvillia); Shedding of medusae, which mature slowly as separate individuals before forming eggs or sperm.

BOUGAINVILLIA. (June, July, August; not always available in June)
The gonophores are borne singly or in clusters on the main stem and branches, and in this genus develop into complete medusae. The medusa-buds are scattered irregularly throughout the colony, there being no orderly arrangement according to age. Select buds that show various stages of medusa development and mount them under cover slips, and study their unfolding structure. Draw off water from under the cover slip with absorbent paper to produce a slight pressure on the buds. Sketch three stages in medusa development.

When all of its parts except the gonads are fully formed, the medusa breaks loose and swims away. It lives independently one or two months, the gonads gradually maturing. Find a well-developed specimen that is swimming actively and sketch its diagrammatic structure. Identify manubrium, radial and circular canals, velum, oral tentacles, 4 groups of marginal tentacles.

Illustrations of medusa in Hargitt, C. W. '01, '04; Nutting '01, of medusa development in Goette '07. Also Hyman '40.

**Obelia**  (June, July, August)

The *Obelisines* gonosomes are several times as large as the hydranths. Examine specimens and see if they are located at random along the stalks, or in regular places.

An Obelia gonosome has an enlarged transparent covering, the gonotheca, with a blastostyle extending thru it from base to tip. The outer end, or tip, of the blastostyle expands to make a loose plug for the gonotheca when mature.

A cluster of gonophores is borne on the blastostyle inside the gonotheca. The gonophores mature as medusae, and break loose, escaping to the outside past the blastostyle plug. They are commonly caught in tow nets. Their free-swimming life lasts two months or so, the gonads maturing slowly.

The older gonophores should show developing tentacles, when pressed slightly under a cover slip. Which gonophores on a blastostyle are oldest?

With needles, press on the gonotheca of a well-matured gonosome and examine under high magnification the gonophores that are released. Those that are oldest may show swimming movements. Younger stages of development may be teased out from the gonosome and studied. Sketch several stages.

If ripe Obelia colonies are kept for an hour or two in a dish of sea water on the desk (remember not to crowd them), swimming medusae can usually be detected in a good light with the naked eye. Examine some of them when available, and note that their structure is slightly imperfect compared with that of Bougainvillia.

The velum is reduced to a narrow and somewhat lobed membrane near the bases of the tentacles. This makes possible an aversion of the bell when the medusa comes to rest, so that the manubrium sticks out from the center of the convex side, like the handle of a post-hurricane umbrella. Watch the swimming movements, and see how this
happens. In the everted condition, the manubrium is still morphologically sub-umbrellar, though this term has lost its appropriateness.

The newly shed medusa of Obelia geniculata has 24 tentacles, while that of Obelia commissuralis has 16. Both forms may be available in the laboratory. Neither has gonads developed at this stage.

Illustrations of Obelia embryology in Hyman '40, Goette '07

PODOCORYNE (June, July. 1 or 2 colonies will be collected with each 100 Hydractinia colonies from Sheep-Pen Harbor. None from Pasque.)

The highly specialized colony grows in an encrusting mat on snail shells, etc., and is almost exactly like hydractinia. Both have three types of individuals: feeders, gonosomes, stingers (Cf. descriptions of Hydractinia below, p. 7) Podocoryne is included here because of its startling metagenetic contrast to Hydractinia.

Medusae of Podocoryne are nearly perfect, and may produce several generations of new medusae by asexual budding before getting around to their main business of gamete production. Cf. Goette, '16 (The sporosacs of Hydractinia bear very little resemblance to medusae, being highly degenerate).

Sketch the three types of polyps and show several stages in medusa development. Gonads can be made out along the radial canals of the swimming medusae, very immature but sexually distinguishable. The asexual colony gives off either male or female medusae, hot both!

B. Study of Forms with Imperfect Medusae.

Examples: Pennaria, Tubularia.
Life Histories: Zygote shed from short-lived imperfect medusae (Pennaria) or retained in reduced sessile medusa form. (Tubularia): Development to planula larva and metamorphosis to polyp (Pennaria) or development to Actinula larva and growth to polyp (Tubularia): Asexual multiplication of polyps by budding to produce colony; Gonophores formed by special buds on hydranths; Maturation of gonophores (imperfect medusae) and fertilization either in situ (Tubularia) or within the limits of the colony during their detachment (Pennaria).

Pennaria (July, August, September; begins to ripen middle of July) Gonophores bud off singly around the lower portion of the hydranth. They form slightly reduced medusae with rudimentary tuftlike tentacles. Before opening out as transparent bell-shaped forms they suggest coconuts. A single colony bears gonophores of one sex only, but in the living individuals, sex can be diagnosed only with difficulty until they mature, when the pinkness of eggs and the whiteness of sperm appear. (Smallwood '89). "Male" and "female" colonies are actually asexual, bearing male and female gonophores respectively.

Mature Pennaria colonies festooned with shedding medusae provide
an astonishing and beautiful spectacle that every student should certainly see. The material ripens in the season of warmest water, and a demonstration of the shedding will be made when possible. It starts early in the evening and continues through midnight. It is usually best seen in material brought into the laboratory the preceding day (i.e. the second night).

The ripe medusae gradually start a rhythmic twitching. Those which are males emit puffs of whitish sperm, and those which are females eject with greater travail the three to six opaque pink eggs. In the south, Pennaria medusae generally break loose from the colony and swim about during this discharge, but at Woods Hole they generally remain attached, and the eggs may not be ejected until long after fertilization. The medusae finally drop off, swim very feebly if at all, shrivel rapidly and die in a few hours.

Put small selected stems from ripe "male" and "female" colonies together in a fingerbowl after careful rinsing, at 3-4 p.m. and leave them overnight. They are extremely sensitive to overcrowding! Next day, remove the stems and look with naked eye for free medusae as evidence of shedding. If they are found, look for developing eggs.

The eggs are very simple and slightly ameboid, with no apparent membranes. Follow their development as far as possible. If the water is changed several times, the planula stage should be reached in 24 hours, and stages in the very simple metamorphosis to the polyp form may be observed. (Hargitt, G. T., 1900, 1909).

tease apart male and female gonophores and examine the eggs and sperm. Sketch several stages in development of the medusa; sketch the mature male and female medusae; sketch several cleavage stages if found.

Illustrations of medusa development in Goette '07; of cleavage in Hargitt, C. W., '00. Also Hyman '40

TUBULARIA (June, July)

The Gonosomes of a well-matured specimen form long racemes or clusters of gonophores crowding and drooping from the region between the circles of tentacles on a hydranth. The gonophores' quite severely reduced medusae which never become free-swimming, usually have no evident radial or circular canals and develop nothing but buds for tentacles. Male and female gonophores occur in separate colonies. Diagnosis of sex by sight is impossible in the immature, but gonophores that contain embryos are easy to tell from those that are filled with a cloudy mass of sperm. Early stages of developing embryos are found by teasing with needles, those near the hatching stage are visible in situ.

Examine a ripe male gonophore, considering it as a very degenerate medusa. Notice and sketch its mode of attachment, its shape, the structure of its free end and the position of the sperm surrounding the dark red manubrium. Crush it on a slide and inspect the motile sperm under high power.
Examine and sketch a ripe female gonophore. Usually the tentacles at its distal end appear only as four short blunt knobs, but one or more of them are sometimes slightly elongated. The eggs come from favored oocytes that progressively swallow up their neighbors lying in the space around the spadix (manubrium).

When ripe the egg is very large and somewhat irregular in shape (Allen '00, Lowe '26). After fertilization, cleavage is often chaotic, apparently either a coeloblastula or a morula may be formed (Lowe '26), and gastrulation of the former has been described (Benoit '25), as a mixture of delamination and multipolar proliferation. The embryos are developed up to the "Actinula" stage within the gonophore. The Actinula larva is to be considered as a precociously metamorphosing form, part planula and part polyp.

By teasing some female gonophores open with needles, collect and sketch eggs and larvae in various stages. The larvae just taking shape are flattened with blunt marginal processes. At first these are not symmetrical in outline but radial symmetry is attained later. When the larva has reached the Actinula stage it has a mouth and aboral tentacles, and a rounded aboral body that later becomes attached at its tip.

Study and sketch the structure of a fully formed Actinula larva. Look over some ripe "female" colonies for Actinulae beginning to escape from the gonophores. Actinulae will not develop further without feeding.

Tubularia anatomy and development is illustrated in the texts of MacBride '14, Korschelt '36 and Hyman '40. For cleavage cf. Allen '00, Hargitt, G. T. '09. For gonophore development, cf. Goette '07.

C. Study of Forms with Degenerate Medusae.

Examples with blastostyle inside gonotheca: Campanularia, Gonothryea.

Life Histories:

a) Campanularia, Gonothryea; Zygote develops into planula larva inside sessile degenerate medusa; Planula escapes, lives free awhile, metamorphoses into a polyp; Asexual multiplication by buds; Colony formation; Degenerate medusae (gonophores) formed on a blastostyle; Gonophores mature in situ; Sperm are shed, eggs fertilized in situ.

b) Hydractinia, Eudendrium: Zygote develops into planula larva either inside gonosome (Eudendrium) or after being shed from gonosome (Hydractinia); Planula metamorphoses into polyp; Asexual multiplication by buds; Colony formation; Gonosomes formed from Hydrocoriza (Hydactinia) or by transformation of hydranths (Eudendrium); Gonophores (highly reduced medusae or sporosacs) borne on gonosomes; Eggs and sperm formed in the sporosacs; Eggs fertilized in situ (Eudendrium) or during shedding (Hydractinia).
CAMPANULARIA. (June, July)

It is not safe to try to distinguish this genus from Obelia by the anatomy of the feeding individuals. Even the gonosomae are similar in appearance in the two genera, each consisting of a transparent gonotheca with the blastostyle extending from base to tip and gonophores budding from it.

The striking difference is that Obelia produces nearly perfect free-swimming medusae, whereas Campanularia produces gonophores so utterly degenerate that their medusa-like structure can only be made out in sections. Each gonophore on the blastostyle of a "Female" colony contains a very large irregularly shaped egg which is fertilized in situ, cleaves, forms a morula, gastrulates by delamination and reaches the free-swimming planula stage, still in situ. Campanularia, therefore, releases from its gonotheca not medusae but planulae.

Because the gonophores are so inconspicuous and the embryos so obvious, the colonies which produce female gonophores and later contain embryos are loosely spoken of as "female" colonies, although they are asexual.

Select from a "Female" colony a gonosome showing eggs in the basal gonophores. Mount it on a slide, study with various magnifications under the microscope, and sketch it.

Select and sketch another gonosome with planulae showing near the tip. Squeeze or open the gonotheca with needles and liberate the planulae. Notice their ciliated ectoderm and watch their movements. If the planulae are well matured, they are two or three times longer than broad. They show maggot-like movements even while within the gonotheca.

Campanularia is very favorable for the study of planula metamorphosis. Put a few mature planulae aside in sea water (not more than 2 or 3 to a watch glass) and cover them. They should attach to the glass in 4 to 10 hours. Each should then open a mouth, bud out tentacles, secrete hydrotheca and perisarc, and become a full formed individual polyp in two or three days. When the planulae have attached, the water should be changed in the dish at least twice a day. Sketch several stages of attachment and metamorphosis.

The gonosomes of "male" colonies are similar in form to the female. The gonophores, when mature, are rounded and have a thin milky-gray color. The sperm become active when they are discharged into contact with sea water, as may be seen by crushing a male gonophore under a cover slide while watching it under the microscope.

For illustrations of Campanularia gonophore development c.f. Goette '07.

GONOTHYREA (July, August).

As in Abelia, the medusae develop within the gonotheca. When mature, instead of swimming away they remain attached to the end of the blastostyle, projecting outside the gonotheca in groups of
three or four, like toy balloons. Within the balls of the medusae the eggs of "female" colonies are fertilized by the shed sperm from "male" colonies. The zygotes develop to the planula stage before being set free, after which the medusae drop off.

Sketch planulae in various stages of development in the projecting attached medusae of a ripe "female" colony. (Wulfert '02).

Gonothyrea cleavage illustrated in Wulfert '02; Medusa development in Goette '07. Cf. also texts of Hyman '40 and Korschelt '36.

HYDRACTINIA. (June, July, August)

Colonies of this form are fairly common on Littorina snail shells inhabited by the small hermit crab, Pagurus. There are three types of individuals in the full developed colony: ordinary polyps (feeders), threadlike coiling forms with no mouth and an apical knob of nematocysts (stingers, commonest around the lip of the shell), and gonosomes. The three types all arise from a hydrorhiza network covering a rust-red spine-studded crust.

a) Gonosomes and Gonophores:

The gonosomes or reproductive individuals are usually without tentacles and have a large knob of nematocysts on the proboscis; each bears a number of gonophores, which are medusa-buds reduced to the status of sporosacs. Ripe "male" and "female" colonies can be told apart with the naked eye since the eggs within the sporosacs are dull green against the red hydrorhiza, and the sperm when mature are a white mass.

Remove several "male" reproductive individuals showing ripe sporosacs, and crush them slightly under a coverslip on a glass slide. This may be done by drawing off some of the water with blotting paper. The sporosacs fall far short of being perfect medusae. Note stages in the production of sperm within them. Burst a mature sporosac and study the sperm under high power.

Similarly, crush several "female" reproductive individuals, and observe the eggs with their large germinal vesicles, in various stages of development. Sketch the "female" gonosome with its female gonophores and contained eggs.

b) Cleavage and Development.

If a number of "male" and "female" colonies of Hydractinia are put together in a large dish of sea water (or a pair of prime colonies in a fingerbowl) and left overnight, eggs should be shed and fertilized between 7 and 9 a. m. WST. The shedding can be controlled by light. If fertilization and cleavage stages are needed later in the day or in the evening, the colonies may be kept illuminated during the preceding night, put in the dark for a couple of hours and reilluminated one hour before the time when shedding is desired. Eggs are shed in 55 minutes, sperm in 50 minutes. Cf. Ballard, '42.
Materials for the study of the entire development of *Hydractinia* from egg to polyp will be made available. If possible, observe the shedding of eggs and sperm. Sketch eggs undergoing first three cleavages, elongated gastrula, swimming and attached planulae, metamorphosing form and young polyps.

Eggs are heavily yolky and usually green, but occasionally gray, orange or pink. Maturation takes place during the half hour preceding shedding and polar bodies are lost soon after, a loose jelly being the only covering of the egg.

Cleavage may be irregular or even chaotic, but usually the slightly ameboid egg undergoes three equal total cleavages, each at right angles to the proceeding. The separating pairs of blastomeres tend to retain broad protoplasmic connections with each other on the side opposite to the cleavage furrow, until just before the succeeding cleavages begin. It is soon apparent that there is much variation in the time and degree of shifting of positions of the blastomeres, but the extraordinary and chaotic cleavage patterns commonly seen in the classroom are often the result of drying up and concentration of sea water, or other unfavorable circumstances.

Mitotic synchronism quickly disappears. Gastrulation is said to start even as early as the 16 cell stage, by mixed delamination and multipolar proliferation. The gastrula loses its spherical form and spends a few hours as an irregularly bumpy oblong mass, then returns to the spherical form and gradually lengthens into the planula form.

At the end of 24 hours the embryo is a "preplanula" (Teissier '27) with an elongated ovoid form, recognizable polarity and ciliation which enables it to swim heavily. During several days it lengthens, one end becoming slimmer and slimmer, while it rolls and crawls along the bottom like a planarian. The big end which goes first in this movement is the end which later produces the adhesive disc by which it attaches for metamorphosis. It becomes the aboral end of the polyp.

Following attachment of the attenuated planula, there is a delay of a few hours to several days, and then the tapering free end shrinks down almost to the substrate, where it shortly produces a mouth and a succession of tentacles. The new polyp elongates, its attached and meanwhile actively sending out a number of anastomosing and encrusting hydrorhiza processes from which sprout new polyps.

Illustrations of cleavage in Bunting '94, Beckwith '14; of later planula development in Teissier '37; of developing gonosome in Goette '07 and '16.

**EUDENDRIUM** (July, August)

The gonosomes of this genus are degenerate sessile medusa-forms or gonophores, strikingly different in the two sexes, borne at the bases of special hydranths which lose their tentacles and degenerate while the gonophores are ripening.
"Female" colonies bear loose irregular tufts of sporosacs attached to the stems, each ripe sporosac being bright orange in color. "Male" colonies bear light pink sporosacs arranged in groups of two to four or more in a line, the lines radiating from a common point on the base of the degenerated hydranth. The ripest male sporosacs occur at the periphery of the cluster and are white with sperm. Sketch both male and female sporosacs.

Eggs are fertilized within the female gonophore or sporosac, and develop to the planula stage before being liberated. (Hargitt, C. W., '04). Tease out embryos from different colonies and study all stages found. The eggs are so rich in yolk that they cleave like insect eggs. The gastrulation is by an extraordinary syncytial delamination. Metamorphosis is simple.

Endocerium cleavage illustrated in Hargitt '04 (zool. Jahrb.); Gonosome development in Goette '07. Cf. also texts by Korschelt '36 and Hyman '40.

Development of Scyphozoa

AURELIA OR CYNELA (April - June)

Both these jellyfishes have oral lobes extending downward. In mature specimens granular material will be found entangled on the lobes or contained in small brood sacs in the lobes. Tease off some of this material into a drop of sea water on a slide, and examine under the microscope. Embryos of different stages can be found, from spherical cleaving eggs to oval gastrulating forms and fully formed stocky, active planulae. (Hargitt, G. T. '09) (Hein '00). Is cleavage regular? Sketch the embryonic stages that are available.

Select a number of active planulae and place them in clean watch glasses of sea water for further study on later days. Their gradual change in form, attachment to the bottom, acquisition of tentacles and elongation into the sessile scyphula stage can be easily followed. The resemblance of the scyphula to a simple polyp is obvious. Attachment plus the formation of 2 to 4 tentacles occurs on the second day, as does the development of an open mouth. There are 8 tentacles at 4 days, 16 at 2 weeks, 24 at 1 month. Sketch the scyphula in side view and in top view.

The scyphula or scyphistoma stage lasts throughout the winter. The animals increase in size and undergo asexual reproduction by transverse fission into ephyrae ("Strobilization"), and by other methods (Percival '23). The tiny ephyrae (larval jellyfishes) are liberated and gradually transform into the adult form over a period of many months.

Aurelia life history illustrated in the texts of Macbride '14, Korschelt '36 and Hyman '40.
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In Tubularia we have a system in which each part is able to reform or reconstitute into a differentiated hydranth such as develops normally from the egg. When the stem is cut thru, the opening closes and the stem cells are converted directly into a hydranth without further cell division. There are, therefore, no special reserve cells which supply the cells for the hydranth nor any outgrowth from the cut surface. Thus, a hydranth can form in two entirely different ways: 1) by cell division of the egg with the orderly formation of a larval stage (the actinula) and 2) by a direct transformation of adult stem cells into a hydranth. By studying the second process we can separate the various embryonic processes and deal with differentiation alone.

The process of differentiation by definition being the process of becoming different we must either start with a system in which there are differences or create differences in a homogenous system. Tubularia stem shows some differences but those differences are such that the chemical environment can override them establishing a new polarity. If the stem be cut into a number of small pieces each piece will form a hydranth at the distal end but none at the proximal end, thus exhibiting a polarity. However, if each distal end be placed in a glass tube this polarity can be reversed and a hydranth placed in a glass tube this polarity can be reversed and a hydranth will develop at the opposite end, the proximal. Obviously then both cut ends tend to form a hydranth, but the tendency is greater at the distal end and this in some way represses the formation of the proximal hydranth (Dominance).

Dominance is important embryologically as it allows for the formation of a single structure from a mass of tissue which is capable of forming more than one structure. The region which has the greater tendency to form a structure represses adjacent tissue from forming the structure. This might be explained on a nutritional basis with the region of greatest activity drawing materials away from adjacent regions. In a crowded mass of cells as obtained in the gastrula stage there must be a keen competition of the various cells for nutriments and a mutual inhibition by cells caused by their excretory products. In both of these phenomena the more rapidly metabolizing cell has the advantage and is most likely to differentiate.

The above situation can be brought about experimentally by taking a mass of cells and placing a barrier to free diffusion on one side of the mass. This has the dual effect of an increase in excretory products and a decrease in oxygen tension and the result is that differentiation is inhibited on this side while the hydranth forms from the cells of the opposite side.

In the natural environment with the perisarc around the cells it is highly probable that this perisarc acts as a natural barrier to free diffusion and that the hydranth forms at the cut end because these cells are released from an inhibition caused by both excretory products and low oxygen tension.

COLLECTION AND CARE OF TUBULARIA

Theoretically Tubularia is a solitary form but practically so many
individuals grow together that a dense tangled mass usually results in the older forms. Young short stems are the best for experimental work and can be obtained from floats and rocks in swift current. In general it is best to collect your own stems. Since the stems need running water and a low temperature they do not keep well in the laboratory. In nature the hydranths drop off about the end of July and the stems remain dormant until the water cools down in the fall. At Woods Hole the stems appear in mid June and can be used until August. However, since the waters of Cape Cod bay on the North Shore are much colder, Tubularia may be obtained from the North end of the canal throughout August.

The stems as collected vary in length, thickness and in general physiological condition (some are crushed, some starved, others very old with large gonophores. Therefore, we cut the stems off and sort them out in a large finger bowl being careful not to crush them. For most work the short stems are about 10 mm in length and are suitable. These are selected for uniform diameter and appearance and the hydranth is cut off a few mm from its base. It is necessary to cut off some of the stem with the hydranth as this part of the stem does not regenerate consistently, especially in older stems.

Of course, even after selection the stems show some variability in regeneration and so it is best to pool all the stems for one experiment and select at random for the various parts of the experiment. Thus, if you are treating the stems in 4 different ways you should separate the stems into 5 lots at random using one lot as a control. The number in each lot depends on the nature of the experiment. Many experiments are of the all or none nature and 10 stems in each lot are sufficient. In experiments where rate of regeneration is compared under different conditions it is best to use about 25 stems in each lot. This gives a very high accuracy in averaging rates.

The stems are kept in running water and cool by placing them in Syracuse watch glasses which are first submerged in large fingerbowls through which sea water is running. Some care must be taken that the stems are not washed away.

The instruments used for cutting and handling are a pair of sharp scissors, a pair of forceps and a medicine dropper. In using the forceps care must be taken that only the parts which are finally cut off and discarded are handled. After the stems have been cut to size they are transferred with a pipette, and must not be handled with forceps.

When the temperature of running sea water gets above 25 c it is necessary to keep the stems in a refrigerated bath or regeneration will not be consistent.

The Warburgs appear to be an excellent place for Tubularia as the constant shaking keeps them well aerated and bathed with solution with the result that they regenerate rapidly and form large hydranths.

DOMINANCE IN TUBULARIA REGENERATION

Four lots of stems with 10 stems in each lot will be used. 1) long stems from 10 to 15 mm in length after cutting; 2) short stems about 6 mm long. 3) short stems with a ligature tied in the middle of the stem; 4) very short stems about 1 or 2 mm long. Select stems about 20 to 25 mm long for #1 and #2, 3 and 4 stems up to 10 mm are suitable. These can then be trimmed to size being very careful to
remove some of the stem when cutting off the hydranth.

This experiment is of the all or none nature; that is, the proximal hydranth will usually either develop in all stems or be absent in all stems because of dominance of the distal hydranth.

For this experiment it will be necessary only to record the number of hydranths developing in each of the four lots. It may be safely assumed that the distal hydranth always develops so that if one end fails to form a hydranth it is almost certainly the proximal end.

After the stems have been cut to size they are placed in the watch glasses in the circulating sea water. Care must be taken that the incoming sea water does not wash the stems away. Have the sea water enter at the bottom of the large finger bowl and do not have the water run too fast.

At 48 hrs. the hydranths should be fully formed within the perisarc and some should be emerging. Record both the numbers. Discard stems which do not show regeneration at either end.

**EXPERIMENTAL CONTROL OF POLARITY**

This again is an all or none type of experiment and only 10 stems are needed in each lot. Draw out glass tubes about 1 mm in inside diameter so that the stems will fit into these tubes loosely. Take 10 stems about 6 mm long and insert the distal end of the stem into the tube leaving the proximal end free. Use 10 controls to determine the behavior of the proximal and distal ends under normal conditions.

**GRADIENTS IN TUBULARIA**

Gradients are quantitative differences which can be detected in a variety of ways but perhaps the most significant difference is a difference in the rate at which the stem forms a hydranth at different levels of the stem. (Accompanied with this is a difference in size of the regenerating hydranth.) We will attempt to measure the time from cutting of the stem to the fully formed hydranth. Since the first hydranths develop at about 24 hours and others continue to develop at later times it is best to start the experiment as early in the morning as possible so as to have all the next day in which to make observations. The stems should be examined every 2 hrs. after the first hydranths have formed for further regeneration. The criterion used for a fully formed hydranth is the presence of the tentacles and especially a sharp constriction at the base of the hydranth while still within the perisarc. Simply look at the ends of the stems under low power and record the time when this constriction is first noted.

Select stems about 25 mm of the stem as before. Then cut the remainder into pieces about 5 or 6 mm. long discarding which is left. As these pieces are cut place them in separate dishes labelled distal, middle and proximal thirds. Continue 2 hr. observations as long as possible and take a final observation the second day at 48 hours and average the times, by totalling the total number of hours for each lot and dividing by the total number of stems. If some stems have not yet regenerated include these in the total number as they would regenerate if given time.
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A comprehensive survey of developmental processes in the tunicates would include examination not only of eggs and embryos but also of metamorphosing larvae, various types of vegetative reproduction, and regenerating forms. Because these developmental forms must be constantly related to the structure of larvae and adults, it is necessary that the student should first of all review in a standard text the anatomy and life history of the common types of tunicates.

A. SIMPLE ASCIDIANS

DEVELOPMENT TO THE TADPOLE STAGE.

Styela (Old name Cynthia)

Conklin's classic description of the development of Styela eggs (1905) provides the morphological background for the experiments (Conklin '31, Tung '34, Dalcq '38, Rose '39, Tung, Ku-Tung '31, etc.) which are actively shaping our conception of the organization of Tunicate eggs and embryos. Conklin's figures should certainly be referred to during the following studies.

a. Methods

Though truly hermaphroditic, Styela is ordinarily self-sterile like several other Ascidians (Morgan '38). It sheds the eggs and sperm between 4 and 7 p. m., and fertilization takes place when ripe gametes from two different individuals get mixed. It is easy to have eggs shed and fertilized on normal schedule in the laboratory, but this entails the disadvantage of having to study the migration of yellow pigment within them by artificial light.

The classic method of obtaining eggs and embryos from Styela has been to mince the gonads from a number of large individuals together in a dish of sea water. This liberates all stages in the maturation of eggs and sperm, and usually a few eggs will be fertilized, whatever the time of day or night, and will commence normal development.

S. M. Rose '39 has developed a method of controlling the natural spawning in the laboratory by illumination, and this is the best way to get fertilization for experimental material. It works well except for a few weeks in mid-summer, when the animals are spent. By such control, the same batch of animals can be induced to shed a number of times on successive days. They are kept darkened until eleven or twelve hours before fertilization is desired, and then an artificial day is started by turning on an electric light. A 40 watt bulb 18" from the animals is sufficient. Eggs and sperm are discharged in clouds at the desired time.

b. Mature Unfertilized Egg.

Sketch the mature unfertilized egg, which should show the following

(1) Chorion, a tough membrane with perhaps a few follicle cells adhering to its outer surface.
2. Tunicates

(2) Small spherical inner follicle cells ("nurse cells") between chorion and egg itself. They contain yellow granules.
(3) Peripheral layer of egg, a clear layer containing minute yellow granules.
(4) Central part, consisting of gray yolk platelets.
(5) Germinal vesicle, a very large eccentrically placed mass.

C. Post-Fertilization Rearrangements

This is a difficult process to observe as most eggs have too little pigment, and few are fertile in "minced" cultures. It is better to omit this section and to concentrate on cleavage and gastrulation. Then, if time is available and if the eggs this year contain sufficient pigment, these rearrangements may be studied.

Carefully watch for the rearrangement of egg substances starting within 2-8 minutes after fertilization. The clear yellowish peripheral matter streams to the lower pole over the yolk, followed by the clear protoplasm from the animal pole. If the yellow pigment is not visible, try a more brightly colored egg. Use only daylight for illumination, and have the diaphragm on the microscope wide open. Roll the egg around by moving the cover slip with a needle. The yellow inner follicle cells may also be migrating; watch the egg cortex.

The grey yolk rises to occupy the upper pole, all except the space that surrounds the maturation-spindle complex. Soon the yellow substance accumulates on one part of the lower hemisphere, where it assumes crescentic form. Immediately above the broad part of the yellow crescent is a layer formed by the clear cytoplasm.

The most remarkable characteristic of Styela is that now at the conclusion of these movements the position of the future larva is visibly marked on the surface of the uncleaved egg. The broadest part of the yellow crescent is at the posterior pole, and the borns wrap around the right and left sides. The animal pole, where the germinal vesicle lay, becomes the ventral-anterior side of the larva. The vegetal pole, where the spermatocyst entered, is the future dorsal side. To avoid confusion, fix firmly in your mind the relationship between egg orientation and definitive axis of embryo.

D. Cleavage

The following approximate time schedule for the embryology of Styela is from the great monograph of Conklin ('05a). Follow the events of cleavage in as much detail as possible. Observe gastrulation and watch the tadpole take shape. Sketch a succession of cleavage stages, showing bilateral symmetry and location of yellow crescent material. Sketch at least 2 stages of gastrulation and 2 of elongating pro-tad poles. (This schedule is for normally shed eggs. If eggs are obtained from "minced" cultures, cleavage is delayed, the eggs apparently maturing at variable intervals after striking the sea water.)

First cleavage to 2 cells after 40 minutes
2nd " 4 " " 30 "
3rd " 8 " " 30 "
4th " 16 " " 20 "
5th " 32 " " 20 "
6th " 64 " " 20 "

3-

7th cleavage to 112 cells after 20 minutes
8th " 218 " 20 "
To neural plate stage, 2 more hours.
Fully formed tadpole 12 hours after fertilization

(1) First cleavage. Equal, separating the two horns of the yellow crescent from each other, likewise bisecting the clear protoplasm anterior to the yellow.

(2) Second cleavage. Nearly equal, vertical, at right angles to the first. The two posterior cells (B3 on the left and B3 on the right) contain little yolk and practically all the yellow crescent substance. The two anterior cells (A3 on the left and A3 on the right) contain much yolk, and practically none of the yellow crescent substance. The clear protoplasm goes equally to the four cells.

(3) Third cleavage. Horizontal, the upper quadrant (cells a4, a4, b4 and b4) somewhat smaller than the lower quadrant cells A4, A4, B4 and B4.) The yellow crescent substance is almost entirely confined to the two posterior dorsal cells (B4, B4).

(4) Fourth cleavage. The planes of cleavage vary in different quadrants, but the new cells do not overlap the sagittal plane of the embryo. Two of the antero-dorsal cells and two of the postero-ventral cells of the 16-cell embryo are crowded away from this sagittal plane, but all other cells touch it. The dorsal and ventral hemispheres at this stage are mirror images. The yellow pigment lies in four posterior cells (B5.1, B5.2, B5.3 and B5.4).

(5) Fifth cleavage. Cleavage in the dorsal (vegetal) hemisphere precedes that in the ventral (animal) hemisphere, and cleavage in the anterior part of each hemisphere precedes that in the posterior part.

At the 32 cell stage, the yellow substance is almost entirely confined to six dorso-posterior cells, three on each side of the midline (B6.2, B6.3, B6.4, B6.2, B6.3, B6.4). They give rise to mesoderm and mesenchyme.

Six yolk-filled cells at the vegetal (definitive dorso-posterior) pole anterior to the yellow mesoderm cells will give rise to endoderm; A6.1, A6.3, B6.1, A6.1, A6.3, B6.1.

Four cells at the anterior border of the embryo just below the equator (A6.2, A6.4, A6.2 and A6.4), and two just above the equator (A6.5 and A6.5) will give rise to the notocord and neural plate.

All the rest of the cells are ectodermal.

(6) Later cleavage, gastrulation, neurulation. The gastrula passes through disc-shaped, saucer-shaped and cup-shaped stages, starting with the 7th cleavage. As it finally becomes egg-shaped, the gastrula's blastopore, found at the small hind and,
becomes T-shaped, the stem of the T bordered by the yellow mesoderm-mesenchym cells. The cells overhanging the crossbar of the T-shaped blastopore constitute its dorsal lip. They overgrow it, finally engulfing the yellow cells which from then on are only seen dimly through the translucent ectoderm.

Watch the gradual elongation of the embryo and the appearance of definite tadpole form.

**Molgula.**

The eggs of Molgula follow almost exactly the pattern of development seen in Styela, but they do not have pigment. The animals are self-fertile. A few fertilized eggs may be obtained at any time by mincing the gonads in sea water, although spawning normally occurs at daybreak.

Rose's method for controlling the spawning of Molgula or Ciona is to put a few individuals in a large dish of sea water and store them in the dark, for instance, in a desk drawer, until eggs are needed. Molgula will shed 15 minutes or so after being brought out into the light, Ciona immediately.

Start some Molgula eggs developing, and follow the cleavage through gastrulation. If developing eggs are isolated in a watch glass, tadpoles and young stages of metamorphosis are obtainable. Tadpoles are fully formed in 8 hrs. (Conklin '05a) (Grave '2).

**B. COLONIAL ASCIDIANS**

1. **AMAROUCIUM: TADPOLES, METAMORPHOSIS, EPICARDIAL BUDDING.**

(Usually not available until July)

a) Methods.

The larvae of Styela and Molgula are so small that study of their internal organization is difficult. The compound ascidian Amarouciium is viviparous, and the large tadpoles are easily obtained from parent colonies, provided the material is ripe and has been quite recently collected.

Squeeze a mass of fresh Amarouciium over a little sea water in a fingerbowl. Many highly colored fragments of the adult individuals will be ejected, together with eggs and embryos. Fill the bowl with water and pour off the coarser particles whirling near the top. Tadpoles and eggs in all stages of development may presently be found at the bottom.

Tadpoles usually leave the parent colony shortly after sunrise. These undamaged and fully developed individuals can be collected in the laboratory, and they are the best material for the study of swimming and of metamorphosis. They collect at the top of the water on the side of the tank nearest the light.

If colonies are kept in a shrouded aquarium the shedding can be postponed until a more seasonable hour. Swarms of active tadpoles usually appear within fifteen minutes of bringing ripe colonies out of the dark. About a third of the tadpoles will emerge within half an hour, if illuminated first at nine o'clock in the morning;
5- Tunicates

if the colony is kept dark until mid-afternoon about three quarters of them will emerge within half an hour. Nearly all of them will commence metamorphosis within an hour of being shed.

b. Early stages
From the debris in the fingerbowl, select and sketch a series of embryos and developing larvae that show the gradual emergence of tadpole form. None of those squeezed tadpoles will swim immediately, and few will attach normally or develop further. Twitching movements like those of vertebrate embryos or fetuses will be observed.

c. Tadpole structure and behavior
Watch Amorucium tadpoles swarming in a dish of sea water. What are the reactions to light and gravity? How do they propel themselves? Place a few tadpoles on a slide in a small drop of water and study their anatomy under the microscope. (Grave'20, 21.) Not all structures can be seen in a living tadpole. Note the thick test with contained test-cells, the adhesive papillae, the atrial siphon (near the tail), the oral siphon (usually larger); and the sensory vesicle between the two siphons. The small pigment cup with lens is a light-perceiving organ, and there is a small round black static organ also within the sensory vesicle. In the tail, note the muscle cells and notocord.

Some Amorucium tadpoles have been fixed in Bouins and stained with borax carmine. In one of these stained tadpoles, identify the pharynx with its visceral clefts, the central yolk mass, the intestines and the nervous system (Grave'20). Sketch a tadpole, showing structural details seen.

d. Metamorphosis
Isolate a dozen or so tadpoles in separate drops of water in watch glasses, for study of behavior during metamorphosis. When in the course of the next hour the tadpoles have firmly attached to the glass, add more sea water to the dish. After attachment (sometimes before there is time to attach, the flesh of the tail is withered and drawn in, the test swells and metamorphosis has started. Within a couple of hours, movements of the body may be observed. The results of the extensive and rapid internal reorganization that is going on can be observed best two days or a week later. (Grave'35). Make several timed sketches of the external aspects of metamorphosis.

e. Later Stages
Observe and sketch metamorphosed Amorucium individuals which have been growing for four days or so after attachment. They are fastened to watch glasses which have been stored in frames under water. Gently flush debris from them at the tap, and avoid tipping off their cover of water. After making your records you will return the specimens, still living in their watch glasses, to the frames they came from.
Examine the specimens under low power first, for orientation. If they are growing upright they may be flattened out by gently lowering a cover glass on them. (If grown on cover slips, turn it over).
At one end of this animal are the atrial and oral siphons, at the other is the post-abdomen, with the heart at its tip. Watch the heart for periodic reversal of beat. Identify the epicardium, a usually pigmented strand of tissue running throughout the post-abdomen from pharynx to heart region. It is the agent in asexual reproduction and colony formation.

Below the siphons is the pharynx with its three rows of numerous stigmata (visceral clefts). It opens into a short oesophagus which connects with a round yellow stomach marked by muscular bands. The intestine turns sharply after leaving the stomach, and ends near the atrial siphon. The endostyle is sharply indicated on the wall of the pharynx, delimiting the two atrial pouches. These pouches surround the pharynx and open to the exterior at the atrial siphon.

f. Epicardial Budding

Asexual reproduction of new individuals may be seen in laboratory cultures about 17 days after attachment of the tadpole. Or, swarms of buds in all stages of growth and migration can usually be found at the bases of the tiniest transparent fingerlike lobes of a large healthy colony. Demonstrations will be made of them, which should be sketched at low magnification.

Asexual reproduction is accomplished by strobilization, i.e., segmentation of the post-abdomen which contains the epicardial strand. The buds consist at first of inner vesicle (from epicardium) and outer covering (from parent epidermis). All internal organs of the new individuals form from the epicardium tissue, which was a pharyngeal derivative, i.e., endoderm.

This method of asexual reproduction is distinguished from others in Tunicates by being called Pharyngeal or Epicardial Budding. (Kowalevsky '74, Berrill '35). The epicardial buds while differentiating into new zooids move up and take their place around the parent. During the strobilization of the parent's postabdomen, the old heart is isolated and degenerates, and a new heart is regenerated in the parent.

2. BOTRYLLUS: TADPOLES, METAMORPHOSIS, ATRIAL BUDDING

Botryllus is another compound Ascidian, which is found encrusting on rocks, wharves and floats. Certain colonies, brought into the Laboratory, and placed in dishes of sea water, will liberate many tadpoles. Tadpoles and all stages of cleavage and development may also be obtained by mincing the colonies and hunting in the debris. If the normally shed tadpoles are placed in a little sea water in a watch glass, they soon attach to the dish and commence their rapid metamorphosis. (Herman F. C. '24).

a) Structure of Tadpole
The tadpole is not as large as that of Amaroucium, but shows an interesting new feature. Just under the adhesive papillae is a ring of 8 ampullae which are diverticula of the body wall, destined to be parts of the as yet non-functional and incomplete circulatory system (Graves and Woodbridge '24). In metamorphosis they unfold
like petals of a flower, and spread around the edge of the developing tunicate. As the colony grows these increase in number.

In addition, identify the statolith, a densely black cup suspended in the sensory vesicle by a slender stalk and closely associated with light-sensitive elements (Grave and Riley '35); the two siphons; the pharynx with several stigmata; the stomach and intestine. Sketch the tadpole.

Tadpoles attach to a substrate within an hour or two after hatching. The metamorphosis is extremely rapid. (Grave '35 Grave and Nichol '39). They often metamorphose without attaching and stick down a day or so later.

b. Two-Day-Old Form _Atrial Budding_
Tadpoles have been allowed to attach to watch glasses and grow for two days. They are now larger and clearer, and usually so oriented that the observer looks directly down into the Atrial and oral siphons. The large pharynx is in the shape of a truncated cone and bears up the three rows of stigmata (visceral clefts) which let water pass out into the atrial cavities of the two sides. The endostyle lies on the under side of the pharynx and thus appears as a rod under the mouth.

The stomach ordinarily appears as a yellow body under the atrial opening. The intestine, after turning to one side from the stomach, returns to empty near the atrium. The pulsating heart and its vessels are of particular interest, especially the flow of blood around the mouth and pharynx.

A new bud (First blastozoooid) appears from the sexually developed animal (the oozoid) as an evagination of the atrium at one side. It is furnished with a blood supply, and presently the differentiating parts can be distinguished. Coming from the atrium, the whole bud, and all parts of the new individual, are derived from octoderm. This is the Atrial type of budding. (Berrill '41) Sketch an oozoooid showing buds.

c. Week-old form.

In these animals, general structure already studied may be easily seen under low power. The pharynx of the oozoooid has developed 4 rows of stigmata, and the first blastozoooid may also have three or four rows. Blastozoooid buds of the second and third order may be present.

The first blastozoooid bud in Botryllus is single, all the later ones are in symmetrical pairs. The same organ structures may be seen in all the individuals, notwithstanding their diverse embryology, with the minor exception that the oozoooid does not develop gonads. Thru rearrangement of the individuals, the completed colony shows a common atrial pit in the center, with separate pharyngeal openings at the periphery.
For the anatomy of the zooids and structure of various stages in bud development compare the living preparation in the watch glasses with fixed–stained–mounted preparations that are available. These were made from a colony that was nearly transparent when living. Most colonies develop so much pigment in the tunic that whole mounts are useless. Each bud consists at first of a disc, then a sphere. The sphere extrudes sex cells at one or both sides and becomes partitioned into three vesicles, the lateral ones forming at real chambers, the middle one the pharynx. Later stages show differentiation of the rest of the organs from the pharynx-vesicle. (Berrill, '41).

3. **PEROPHORA: SEPTAL BUDDING**

Perophora is a little green–colored ascidian, which by means of stolons forms loose colonies on wharf pilings, etc. Pieces of the colony may be gently stuck to watch glasses with vaseline, and stored in running sea water. After a day or so, stolons will be sent out over the surface of the glass and new blastozooids will be formed at intervals. Examine a watch–glass culture of Perophora which has been growing for two weeks.

Notice the branching pattern of the stolons. The tips show exploratory tendencies like small pseudopodia. The outgoing and incoming blood streams in the stolons are separated by a mesenchyme septum. All stages in the formation of new individuals will be found, arranged like pumpkins on a vine, with the youngest nearest the tips. The youngest buds consist of an outer vesicle derived from the epiderm of the colony and an inner vesicle formed by the splitting of the mesenchymatous stolon septum.

Organogenesis takes place in the inner vesicle, which is derived from mesenchyme (Berrill '35). This method of budding is distinguished as the septal type.
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NORMAL DEVELOPMENT OF STYELA PARTITA- A SIMPLE ASCIDIAN.

The ovarian egg contains a large germinal vesicle. Surrounding this is a layer of yolk. The peripheral portion consists of a layer of protoplasm containing yellow pigment granules. The germinal vesicle ruptures and maturation begins about the time the eggs are released. The contents of the germinal vesicle spread out over the animal hemisphere. The first maturation spindle forms and the chromosomes remain in metaphase until the sperm enters. The entrance point of the sperm is at or near the vegetal pole. Maturation now continues and the two polar bodies are formed. An extensive rearrangement of the cytoplasm now occurs. The peripheral layer of clear protoplasm, together with the yellow pigment, flows toward the vegetal pole. The yellow pigment forms a disk with the sperm nucleus in its center. The clear protoplasm becomes localized in the same region but beneath (towards the egg center) the yellow pigment area.

The sperm nucleus now moves into the posterior vegetal region where it unites with the egg nucleus. The clear protoplasm and yellow pigment follow the sperm nucleus. The yellow pigment remains in the posterior part of the vegetal hemisphere forming a superficial colored crescent. Most of the clear protoplasm, together with the male and female pronuclei, migrates deeper into the egg. During first cleavage the clear protoplasm becomes localized in the animal hemisphere. The different pigmented regions make this a naturally occurring vitally stained egg. These regions correspond rather closely to the different presumptive areas of the egg. These regions are:

1. The yellow pigment in the posterior vegetal region forms the "yellow crescent". This area is presumptive mesoderm.

2. The ventral and anterior portion of the vegetal hemisphere which exhibit the slate gray color of yolk forms endoderm and small amounts of mesoderm and a portion of the neural plate.

3. The animal hemisphere is light gray due to the presence of clear protoplasm beneath the pericheral yolk. This region forms the body epidermis and a portion of the neural plate.
First cleavage is equal and in the median plane of the future embryo. The yellow crescent is bisected. Throughout development the right and left sides are identical.

The second cleavage is slightly unequal. It divides the egg into two larger anterior cells and two smaller posterior cells.

Third cleavage, which is slightly unequal, results in four smaller cells in the animal hemisphere and four larger cells in the vegetal hemisphere.

Throughout early development the pigmented regions maintain their relative positions. By the 64 cell stage each cell contains the material for a single germ layer:

1. The neural plate is composed of 10 cells which are found along the equator at the anterior end. Six of these cells are in the animal hemisphere - they give rise to the brain or cerebral vesicle. The other 4 neural plate cells are found in the vegetal hemisphere. These give rise to the neural tube of the tail region.

2. The remaining 26 cells of the animal hemisphere form epidermis.

3. The presumptive notochord region is contained in 4 cells which are in the anterior portion of the vegetal hemisphere immediately below the presumptive neural plate region.

4. The presumptive mesoderm consists of an expanding ring of 14 cells which lie immediately below the equator in the lateral and posterior regions of the vegetal hemisphere. The chordee and mesoderm form a continuous band around the egg.

5. The presumptive endoderm is contained in 10 cells occupying the ventral portion of the vegetal hemisphere.

AN. POLE

VEG. POLE
Gastrulation begins at the end of the 64 cell stage and results in the mesoderm and endoderm moving to the interior of the embryo. The epidermis and neural plate material remain on the outside. The endodermal cells decrease their exposed area by becoming tall columnar. The ectoderm cells on the other hand become flatter and so increase their area. In this way they spread over the other germ layers. The presumptive notochordal cells turn in at the dorsal lip (anterior) of the blastopore. The presumptive mesoderm turns in along the lateral and posterior lips. The dorsal lip moves posteriorly carrying the notochordal material with it. The portion of the neural plate anterior to the notochord forms the brain or cerebral vesicle. The part over the notochord produces spinal cord. Both the brain and spinal cord are produced by the closure of lateral folds.
EXPERIMENTAL ANALYSIS OF DEVELOPMENT IN STYELA.

Conklin (1905b) studied the development of individual blastomeres and groups of blastomeres. By shaking the eggs or by squeezing squirting them from pipettes some blastomeres could be injured in such a manner that they would remain alive but were incapable of further development.

1. DEVELOPMENT OF HALF EMBRYOS (1/2 or 2/4 blastomeres contain portions of all prospective regions). Cell division in the uninjured half continues in its own normal manner- as though it was part of a whole egg. Notochord usually normal but muscles usually on one side only. Single atrial invagination.


3. DEVELOPMENT OF POSTERIOR HALF EMBRYOS (1/2 or 2/4 contain presumptive epidermis, muscle, mesenchyme, endoderm). Mosaic cleavage. Muscles form- usually fuse at midline. No notochord, neural plate, tail, or eye spot.

Conklin concludes that the developing cells never form more than they would in normal development. The egg is therefore strictly mosaic. The basis for this lies in the orderly distribution of the organ forming substances during cleavage. These organ forming substances are found in definite regions which can be identified by their pigmentation. Each is capable of forming only a definite type of tissue in the embryo. Thus the organ forming substance for muscle is the "myoplasm" of the posterior vegetal cells which can be identified by the yellow pigment. If a partial embryo contains this myoplasm it will produce muscles- otherwise it is incapable of so doing.

The development of Styela has been reinvestigated by Rosc (1939). In these experiments the blastomeres were separated by means of glass needles.

1. DEVELOPMENT OF ANTERIOR HALF EMBRYOS (2/4 blastomeres contain presumptive epidermis, brain, pigmented sensory spots of eye and otolith, spinal cord, notochord, endoderm, mesenchyme). Good differentiation. Form notochord, neural structures including pigmented spots, and gut.


3. DEVELOPMENT OF ANIMAL HEMISPHERE (4/8 blastomeres contain presumptive epidermis and brain with eye spot and otolith). Forms brain epidermis but no brain, eye spot or otolith.

Transplantation experiments revealed the existence of the phenomenon of induction which is of such great importance in the chordates.

5. In the 8 cell stage the two anterior vegetal cells (presumptive notochord, spinal chord, mesenchyme, and endoderm) were combined with the two posterior animal hemisphere cells (presumptive epidermis). The vegetal cells induced pigmented spots in the animal hemisphere cells which would not have produced them in normal development.

6. In the 16 cell stage each of the 4 anterior vegetal cells can induce pigmented sensory spots in presumptive epidermis.

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