EMBRYOLOGY COURSE

The Staff

1940

Hubert B. Goodrich
Professor of Biology
Wesleyan University

William W. Ballard
Assistant Professor
of Biology and Anatomy
Dartmouth College

Donald P. Costello
Assistant Professor of Zoology
University of North Carolina

Viktor Hamburger
Associate Professor of Zoology
Washington University

Oscar Schotte
Associate Professor of Biology
Amherst College

Assistants

D. E. Copeland
Harvard University

J. J. Milford
New York University
EMBRYOLOGY CLASS
1940

Alper, Carl, Brothers College, Drew University
Atkinson, William E., University of Virginia
Belanger, Leonard F., Université de Montréal (Harvard University Medical School)
Cass, R. Elizabeth, Russell Sage College (Ohio Wesleyan University) (Syracuse University)
DuBois, Rebeckah, Vassar College
Fetter, Dorothy, Brooklyn College (University of Michigan) (University of Chicago)
Fincke, Robert T., Indiana University (New Mexico State College) (University of New Mexico)
Foulks, James G., University of Rochester (Rice Institute)
Friedman, Robert Simon, Harvard University (Boston University)
Goldman, Philip W., Harvard University (Cornell University)
Halsted, George Oliver, Princeton University
Hartmann, J. Francis, Cornell University (Holy Cross College)
Hartung, Ernest W., Jr., Harvard University (Dartmouth College)
Heath, James P., Stanford University
Henderson, John M., McGill University
Hopper, Arthur F., Jr., Yale University (Princeton University)
Johnson, Virgil C., University of Oklahoma (University of South Dakota)
Jolly, Margie, DePauw University
Jones, Sarah R., Connecticut College (Goucher College)
Karelsen, June V., Oberlin College
Krantz, Marion, Bennington College (Städt Luisen Gymnasium München)
Lee, Richard E., Harvard University (Massachusetts State College)
Ludwig, Francis W., o.s.a., University of Pennsylvania (Villanova College)
McFarland, William, Washington and Jefferson College
Miller, Gerald, Oberlin College
Nichols, Myron M., DePauw University
Pond, Sidney M., Wesleyan University
Robinson, Edwin James, Jr., New York University, Washington Square College (Dartmouth College)
Samorodin, Albert J., University of Minnesota (Cornell University)
Sawyer, Charles H., Yale University (Middlebury College)
Sherman, Frederick G., Northwestern University (University of Tulsa)
Steele, Kenneth C., Dartmouth College
Sweeney, Frank P., Amherst College
Yancey, Maude J., North Carolina College for Negroes (Knoxville College)
## EMBRYOLOGY COURSE

### Schedule for 1940

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<th>Topic Description</th>
<th>Instructor</th>
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<td>June 18</td>
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<td>Fish</td>
<td>Goodrich</td>
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<td>24</td>
<td>Mon</td>
<td>Fish</td>
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<td>25</td>
<td>Tue</td>
<td>Fertilization &amp; Cell Lineage</td>
<td>Costello</td>
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<td>29</td>
<td>Sat</td>
<td>Coelenterata or Tunicata</td>
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<td>July 1</td>
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<td>Coelenterata</td>
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<td>2</td>
<td>Tue</td>
<td>Squid</td>
<td>Hamburger</td>
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<td>4</td>
<td>Thu</td>
<td>Echinoderms</td>
<td>Schotte</td>
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<td>8</td>
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<td>Echinoderms</td>
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<td>12</td>
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<td>Annelida &amp; Mollusca</td>
<td>Hamburger</td>
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<td>Annelida &amp; Mollusca</td>
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<td>18</td>
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<td>Crustacea</td>
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<td>Tunicata or Coelenterata</td>
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<td>23</td>
<td>Tue</td>
<td>Tunicata</td>
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LECTURES IN EMBRYOLOGY COURSE
1940

June 18 Tue Introductory Announcements. Fish Spawning. Fertilization in Fundulus. Dr. H. B. Goodrich

June 19 Wed Teleost Embryology, cleavage and gastrulation. Dr. H. B. Goodrich

June 20 Thu Gastrulation in Amphibia and in birds. Dr. O. Schotte.

June 21 Fri Recent studies on gastrulation Dr. H. B. Goodrich in fish. Topics in organogeny. Dr. H. B. Goodrich

June 22 Sat Topics in Organogeny (cont.). Dr. H. B. Goodrich Teleost hybridization. Dr. H. B. Goodrich

June 24 Mon Development of color patterns and of Mendelian characters in fish. (Illustrated) Dr. H. B. Goodrich

June 25 Tue Fertilization. Dr. D. P. Costello

June 26 Wed Cell Lineage and Spiral Cleavage. Dr. D. P. Costello

June 27 Thu Sizecontrolling factors in Amphibian Embryology. Dr. Twitty

June 28 Fri Experiments in Fertilization and localization in the Nereis egg. Dr. D. P. Costello

June 28 Fri Physicochemical structure of the egg nuclues. Illustrated with motion pictures. Dr. W. Duryee

June 29 Sat The development of Tunicate Eggs. Dr. W. W. Ballard

July 1 Mon General Embryology of Hydrozoa. Dr. W. W. Ballard

July 2 Tue Embryology of Chephalopoda. Dr. V. Hamburger

July 2 Tue Early Differentiation of the Fucus Egg. Dr. D. Whitaker

July 3 Wed The Spermatophore of the Squid Dr. V. Hamburger
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<td>The Echinoderm egg, its structure and its relation to the orientation of the embryo.</td>
<td>Dr. O. Schotte</td>
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<td>July 5</td>
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<td>The Development of the Echinoderm egg.</td>
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<td>July 8</td>
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<td>Some aspects of experimental neuro embryology.</td>
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<td>July 8</td>
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<td>Metamorphosis of Echinoderms.</td>
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<td>Parthenogenesis.</td>
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<td>Experimental embryology of Echinoderms.</td>
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<td>Experimental embryology of Echinoderms II.</td>
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<td>July 11</td>
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<td>Embryonic Induction.</td>
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<td>July 11</td>
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<td>Induction in Regenerates.</td>
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<td>Development of Annelida.</td>
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<td>Morphogenetic action of plant hormones.</td>
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<td>Experimental embryology of Annelida and Mollusca.</td>
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<td>July 15</td>
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<td>Development of Mollusca</td>
<td>Dr. V. Hamburger</td>
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<td>Control of feather color pattern by grafting of melanophores.</td>
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<td>July 17</td>
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<td>Genetics and development.</td>
<td>Dr. Curt Stern</td>
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<td>July 17</td>
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<td>Microscopical observations of certain embryological processes in the living adult mammal.</td>
<td>Dr. E. R. Clark</td>
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<td>The embryology of Crustacea. Drupal. P. Costello</td>
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<td>July 18</td>
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<td>Evolution of nutritive and respiratory devices in embryos of viviparous fishes.</td>
<td>Dr. C. L. Turner</td>
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<tr>
<td>July 19</td>
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<td>Tissue culture and morphogenesis.</td>
<td>Dr. P. A. Weiss</td>
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July 22 Mon  Asexual reproduction in Coelenterates and in Tunicates.  Dr. W. W. Ballard

July 23 Tue  Ascidian Metamorphosis.  Dr. Caswell Grave
OUTLINES AND INSTRUCTIONS FOR LABORATORY WORK
IN EMBRYOLOGY COURSE

1940
Development of the Teleost

Two types of eggs will usually be studied; the pelagic egg and the non-pelagic or dermersal egg.

The dermersal, (non-pelagic) egg.
Examples: Fundulus heteroclitus
           Fundulus majalis
           Opsanus tau

1. CARE OF FISH. Fish are usually brought to the laboratory in mixed lots of male and females. After stripping of eggs and sperm 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 five minutes before fertilizing. After ten minutes add and change sea water and leave in shallow water (not more than 1/4 inch 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 condition in which they are spawned but for many purposes it is desirable to prepare the egg as follows for microscopic study: remove the egg to 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 female that has been kept in fresh water for about 20 minutes. Strip and keep the egg in dilute sea water (70% fresh water 30% sea water) to retain the morphological characteristics observable at time of extrusion. Note details of structures 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 plate; of formation of perivitelline space. What are your conclusions in regard to the rapidity of the activation of the egg? If practical, find micropyle on unfertilized egg. Insaninate and note spreading of fertilization reaction from locus to micropyle.

5. FORMATION OF THE BLASTODISC. Note the gradual accumulation of the protoplasmic cap. This is the blastodisc or germ disc. Compare polar and lateral views. Polar bodies may be more advantageously studied in pedagic eggs. What is the relation of the pole of the ovum to gravity? How does this compare with the condition in the frog 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 blastodisc — the indication of the first cleavage plane. This usually occurs from two to three hours after fertilization. The rate of development varies with the temperature. Note the geometric and time relations of the subsequent cleavages. Do the cleavage planes divide the entire ovum? The entire blastodisc? During interkinesis the nuclei are sometimes visible. Distinguish between central and marginal cells. Are the latter completely bounded by cell walls? 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. As developmental rate varies with the temperature no time chronology can be given for stages of fundulus as observed under varying temperature conditions. For list or stages see paper by Osenheimer 37 and for chronology at 25°C see paper by Solberg '38).

7. THE PERIBLAST. (First appearance is from 16 to 24 hours after fertilization). The uncleaved 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 late blastodisc (18-20 hrs) 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. Subsequently to the nucleation of the periblast note the change in form and size of the blastodisc (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 form tion of the embryo. Under favorable conditions the beginning of gastrulation may be observed in the appearance of a slight indentation at edge of 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 hrs.)

a. While the germ ring is extending around the yolk, the forma-
tion of the true 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
blasto derm. The embryonic shield can most readily be identified when
seen in profile. As the blastoderm spreads over the surface of the
yolk the embryo grows rapidly in length. Does this growth occur at
the posterior or anterior end of the embryo?

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 7/8 covered, 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
Rupffer's vesicle. How is it formed?

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 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 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, optic lens, neuromeres, mid, fore and hind brain regions
and trace the development into cerebrum, optic lobes, cerebellum,
modulla, etc. (Drawings at twenty-four 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; for-
mation 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. Illustrated 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.

Note earliest appearance of chromatophores. Note early and later forms. Do the black and red types vary in form and in relation to blood vessels? When do they first appear on the brain? Finer details of cellular structures may be studied by removing the embryo from the membrane and mounting beneath a supported cover slip.

e. 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.)

f. The young fish may be studied just after hatching by anesthetizing with choretone.

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 5 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 FISHES 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 (Scromber scombrus, 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 aquaria. Female cutters 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 18° C. Artificial aeration 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. Mitotic cleavage spindles can be outlined by staining with neutral red.

12. SUGGESTIONS FOR EXPERIMENTAL WORK ON FUNDULUS.

In most cases the experiments may best be performed by a group of two to four students working on one problem. Plans and preparations should be carefully perfected before the experiment is started.

a. Germinal localization. By use of fine glass needles the blastodisc may be punctured and some of the protoplasm will flow out.
Similarly one of the cells of the two cell stage or one or more of the cells of later stages may be destroyed. In planning such an experiment the operation should be performed upon at least a dozen eggs and an equal number from the same lot of fertilized eggs should be kept as a control experiment under identical conditions. Make comparisons at the same time intervals of different stages of development of the two lots. Is it to be expected that puncture of any one of the cells of the four-cell stage will give identical results? Keep careful records. See bibliography for reference to previous experiments.

b. Experimental hybridization. Fertilize the egg of Fundulus heteroclitus with the sperm of Fundulus majalis. Make also the reciprocal cross (egg of F. Majalis fertilized by sperm of F. heteroclitus.) In planning this experiment the two sexes should be kept in separate aquaria for several hours and a few moments in fresh water gives an additional precaution against the presence of undesired spermatozoa. Crosses between F. heteroclitus and the sunner (Tautogolabrus) may also be attempted. In all cases control experiments should be carried out and a careful comparison should be made of the normal and hybrid development.

Some points that may be noted in these comparisons are rates of development, gross morphological structures, appearance of chromatophores, etc. See bibliography for reference to previous experiments.

c. Cyclopin monsters and other defects may be produced by treatment with alcohol or magnesium chloride using methods outlined by Stockard '10.

d. The activation of the egg. Sea water has a certain activating effect on Fundulus eggs. This may be studied by placing unfertilized eggs in sea water and carefully comparing changes in structures and rate of reaction with normally fertilized eggs. Further work may be done by noting effect of hypo and hyper-tonic solutions of known concentrations. Similar precautions of those suggested under (b) should be observed in obtaining unfertilized eggs.

e. Studies may also be made in regard to the possibility of fertilizing eggs treated as mentioned above. How long and under what conditions can an egg remain in the solutions and yet be fertilized? How long does the sperm retain its fertilizing power? What dilutions of sperm will effect fertilization?
REFERENCES ON DEVELOPMENT OF FISH
With special reference to (lecture and laboratory) work of course.

This bibliography contains only a few of the older papers (for more complete references see general works and bibliographies in papers here listed.

I. GENERAL REFERENCE WORKS.

Brockel, A. Traite d'embryologie des vertebres. 2nd ed. 1935.


Oppenheimer, J. M. Historical introduction to the study of teleostean development. Osiris, 2, 1936.


II. SPECIAL REFERENCES ON MORPHOLOGY OF DEVELOPMENT.


Cunningham, J. T. On the relations of the Yolk to the Gastrula in Teleostean and in other Vertebrate Types. Q. J. M., S. 28, 1886.


Wilson, H. V., The Embryology of the Sea Bass (Serranus atrarius). Bull. U. S. Fish Com. 9, 1891.

III. HABITS.


IV. CIRCULATORY SYSTEM


V. GERM CELLS


Olshoelberg, Peter. The early history of the germ cells in the brown trout. Amphiopus wilderi (Cage) up to and including the period of sex differentiation. Jour. Morph. 35, 1921. (This paper has a complete bibliography of work on germ cells in other groups.)


VI. EXPERIMENTAL WORK


Batallon, Nouveaux essais de Farthenogenere experimentale chez les vertibres infesienes Arch. Ent. Mech. 12, 1904.


Hoadley, L. Viscosity changes during early cleavage stages of Fundulus eggs. Science 68 (pp 40 9) 1928.


Luther, W. Potenzprüfungen an isolierten Teilstücken der Forellenkeimscheibe. Zool. Anz. Suppl. 9, 1936.


Pasteels, J. Études sur la gastrulation des Vertèbres meroblastiques. III. Oiseaux; IV Conclusions générales Archives de Biologic. 48, 1937.


VII. DEVELOPMENT OF GENETIC CHARACTERS


Goodrich, H. B. Mendelian inheritance in Fish, Quart. Review of Biology 41, 1929 (contains bibliography of genetic work on fish.)


Use only the pipettes at the stock dishes to obtain gametes. Carelessness will result in contaminating the stock of unfertilized eggs with spermatozoa. Use the ordinary low power of the compound microscope (approx. 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 the sea water by evaporation is not rapid. The phenomena can be readily seen with this magnification, which affords excellent definition. Higher power will be useful if it is desired to observe spermatozoa in detail under a coverslip. Care must always be taken to avoid inseminating heavily. Too many spermatozoa 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.

It will aid observation and study to sketch the egg before fertilization and at stages during and after fertilization. Otherwise some of the changes may be overlooked because of failure to observe or remember the preceding 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. Observe the large immature nucleus (gerninal vesicle or oocyte nucleus), and the oil droplets and yolk particles in the cytoplasm surrounding the nucleus. Note also the cortex of the egg.

After becoming familiar with the unfertilized egg, inseminate by adding a drop of freshly prepared sperm suspension. Stir the eggs by a circular movement of the dish at once and observe changes. 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. #19). 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. The time schedule in the descriptive text below will 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 supernumerary 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. From 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. #11, 13, 14). 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 38-40 minutes,
and it lies in the space between the egg and the vitelline membranes.
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 the 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. 21).

Place some very recently fertilized eggs of Nereis in a drop of
fresh, thick Chinese ink suspension (made up in 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 par-
ticles of ink will penetrate. This is due to inhibition of jelly
cutflow 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 in a number of eggs the
relation of the first cleavage plane to the polar bodias and the en-
trance point of the sperm as marked by the ink (ref. #11, 14, 17).

Nereis: Exaggerated Entrance Cones:

Place some nereis eggs inseminated about 8 minutes earlier in a
Syracuse watch glass containing alkaline NaCl (pH 10.3-10.5). Ob-
serve immediately. The vitelline membranes will elevate due to a
sudden inhibition of jelly release through the membranes and a sub-
sequent 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 mem-
brane 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 icebox
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 re-
moved 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, 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 (in distilled water) at the bottom of the centrifuge tubes prevents injury to the eggs. This amount of centrifuging separates the various formed components of the egg in several strata (ref. #7). Fertilize the centrifuged eggs after washing off the sucrose with sea water, and observe asymmetrical jelly-extrusion.

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).

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. 18, 22).

When the egg is taken from the female it contains a large immature nucleus (germinall vesicle), as does the Nereis egg, but unlike the egg of Nereis, it spontaneously undergoes partial maturity when placed in sea water, even if not fertilized. A number of species of eggs partly mature when they enter sea water and fasten (ref. #18) has shown that this is dependent upon the presence of Calcium in the sea water.

Chaetopterus 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. 20). When eggs are fertilized after having been in sea water for 15 minutes or longer, the following schedule applies to 50% of the eggs in a population reared at 210 C. (ref. #20) (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 fertilized. The spindle cannot be distinguished as such in the living egg, but it will be observed that the relatively clear region of the nucleolus 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 fertilization membrane may be seen. It is not conspicuous and does not elevate much above the egg surface. By 10 or 12 minutes, the eggs, 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 (16-18 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 antipolar end of the egg (40-43 minutes).

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 at 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 diffuse 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.
Fertilization

General References:


Further references relating to laboratory work:


**Lunar Periodicity**

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 low power eyepiece and high power objective are useful. Refer to Conklin, 1897, Embryology of Crepidula.

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 and fusion with the latter.

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 micromeres 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.

On slides C and D find stages in the development of the gastrula. Note the blastopore, and the increase in number of endoderm cells.

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 Kleinenberg's micro-sulphuric 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. After removing the xylol used in clearing, 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. Milford in 1939. An older method sometimes yielding good results is as follows:

1. Fix for 30-120 minutes in Mayer's Micro-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 distilled water, to which 1 drop of micro-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 coverglass.
Cell-lineage References

1. General:

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


2. Special:

Child, C. M. 1900 Arenicola, Sternapsis Arch. f. Entwickl. 9.
Heath, H. 1899 Development of Ischnochiton G. Fischer, Jena.
Wilson, E. B. 1892 Nereis J. Morph. 6.
Woltereeck, 1903 Polygordius Arch. f. Entwickl. 9.
Wilson, E. B. 1898 Cell-lineage W. H. Lectures, 1898.
         Ann. N. Y. Acad. Sci. 11.
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. When removed to fingerbowl, the individuals are apt to die promptly unless kept below room temperature. 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 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 variability in structure of the medusa-state. The best example of a complete medusa with a degenerate polyp stage is the idealized Trachylinid 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 sporosacs. 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 gonosome (Obelia) or separately (Bougainvillia); shedding of medusae, which mature slowly as separate individuals before forming eggs or sperm.
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.

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 study 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 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.

See if you can tell which of the gonophores on a blastostyle are the older. Determine the general structure and position of the tentacles on one of the older gonophores.

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.

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 found. 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 eversion 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-umbrella, though the term has lost its appropriateness.

The medusa of Obelia geniculata has 24 tentacles when liberated, while that of Obelia commissuralis has 16. Both forms may be available in the laboratory. Neither has the gonads developed when liberated.

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

PODOCORYNE

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. Pococoryne is included here because of its startling metagenetic contrast to Hydractinia. Medusae of P. are nearly perfect, and may produce several generations of new medusae by asexual budding before getting around to their main business of gamete production. The sporosacs of H. bear very little resemblance to medusae. (Goette '16).

B. Study of Forms with Imperfect Medusae

Examples: Pennaria, Tubularia.
Life Histories: Zygote shed from short-lived imperfect medusan (Pennaria) or retained in reduced sessile medusa (Tubularia); Development to planula larva and metamorphosis to polyp (r.) or development to actinula larva and growth to polyp (T.); 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 (T) or within the limits of the colony during their detachment (P).

PENNARIA. (July, August, September).

Gonophores bud off singly around the lower portion of the hydranth. They form slightly reduced medusae with rudimentary tuft-like tentacles. Before opening out as transparent bell-shaped forms they suggest coccomats. 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).

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 in demonstration of the shedding will be made when possible. It starts early in the evening and continues through midnight.

The ripe medusae gradually start to twitch, and those which are males emit puffs of whitish sperm, and those which are females eject with greater trunal 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 fertiliza-
The medusae finally drop off, swim very feebly about, 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 for free medusae as evidence of shedding. If they are found, look for developing eggs.

The eggs are very simple and 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.

Illustrations of medusa development in Goette '07; of cleavage in Hargitt '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 two circles of tentacles on a hydranth. The gonophores are quite severely reduced medusae which never become free-swimming, usually have no evident radial or circular canals and develop nothing but buds for tentacles. With rare exceptions male and female gonophores occur in separate colonies, "male" colonies being a very small minority. 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.

Examine a ripe male gonophore, considering it as a very degenerate medusa. Notice 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 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.

Open some female gonophores and make a study collection of 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 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 McBrirde '14, Korschelt '36 and Hyman '40. For cleavage cf. Allen '00, Hargitt '09. For gonophore development, cf. Goette '07.

c. Study of Forms with Degenerate Medusae.

Examples with blastostyle inside gonotheca: Campanularia, Gonothyrea.
Examples with naked gonophores: Hydractinia, Eudendrium.

Life Histories:

(a) Campanularia, Gonothyrea: 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 by transformation of hydranths; 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).

CAMPAANULARIA. (June, July)

It is not safe to try to distinguish this genus from Obelia or the anatomy of the feeding individuals. Even the gonosomes 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 in Campanularia the medusae are not set free, nor do they even attain any characteristic medusa form, so far as can be seen in life. Each gonophore on the blastostyle of a female colony contains a very large, irregularly shaped egg which is fertilized, cleaves, forms a morula, gastrulates by delamination and reaches the free-swimming planula stage in situ. Then the planula is released from the gonangium.

Because the gonophores are so inconspicuous and the embryos so obvious, the colonies which produce female gonophores and later contain embryos can be 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 and study with various magnifications under the microscope.

Select and study 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 and were showing maggot-like movements within the gonotheca.

Put a few mature planulae aside in sea water (not more than 2 or 3 to a watch glass) and cover them. Examine them later for stages in metamorphosis. 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 fully 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.

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 are shed and become active upon coming in contact with the sea water. Crush a male gonophore under a cover slide while watching it under the microscope.

For illustrations of Campanularia gonophore development cf. Goette '07.

GONOTHYREA. (July, August).

As in Obelia, 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.

Observe 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).

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 fully 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 an encrusting hydroid-kiza network covered with a rust-red shell and often studded with sponges.

The gonosomes 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.
"Male" and "female" colonies can be told apart if ripe, since the eggs within the sporosacs are dull green against the red hydrorhiza, and the sperm when mature are a white mass. Very rarely shells may be found bearing both a "male" and a "female" colony, with a sharp no-man's-land ditch between them.

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.

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. 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 early in the morning and re-illuminated an hour or two before shedding is desired.

Materials for a study of the entire development of Hydractinia from egg to polyp will be made available.

Eggs are heavily yolky and usually green. The cleavage is quite irregular. (Bunting '94, Beckwith '14.) Thick bridges often remain between the cleaving blastomeres. Cells may be nearly equal or very unequal, and ameboid changes in shape are often seen. The blastomeres may be so loosely tied together as to suggest yeast colonies, even after the fourth cleavage. Gastrulation is said to start at the 16-cell stage by mixed delamination and multipolar proliferation.

By the acquisition of cilia and by elongation, the embryo becomes a planula in about 24 hours. It is a heavy swimmer, but rolls and crawls actively on the bottom like a planarian. The big end is to the fore during this locomotion, and the big end produces the adhesive disc by which it attaches for metamorphosis. Following attachment there is a variable period of delay, then the little free end shrinks down and a succession of tentacles and a mouth are produced. The attached end then sends out actively a number of anastomosing and encrusting hydrorhiza processes, from which sprout new polyps.

Illustrations of cleavage in Bunting '94, Beckwith '14; of developing gonosome in Goette '07, '16.

**ADDENDUM.** (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 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.

Eggs are fertilized within the female gonophore or sporosac, and develop to the planula stage before being liberated. (Hargitt C. W. 1904). 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.

Eudendrium 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 CYANEA.** (June)

Both these jellyfishes have oral lobes extending downward. In mature specimens granular material will be found entangled on the lobes. Tease off some of this material into a drop of sea water on a slide, and examine under the microscope. Most of the particles are masses of embryos, and must be further teased apart with needles. At different points on the oral lobes embryos of different stages can be found, from spherical cleaving eggs to oval gastrulating forms and fully formed stocky, active planulae. (Hargitt, G. T. 1909) (Hein 1900). Is cleavage regular? Is gastrulation embolic or not?

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. The number of tentacles is usually 4 or 8 at the end of a week, 16 at the end of a month.

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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Tunicates.
1940

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)

Ever since the publication of Conklin's description of the development of Styela eggs (1905), these have been a classic example of the determination of embryonic organs and tissues in the undivided egg following fertilization. With certain concessions to recent experiments (Ti Chou Tung '34, Rose '39), Styela is still one of the best cases of "mosaic development". Refer to Conklin's figures while observing the eggs.

a) Methods.

Though truly hermaphroditic, Styela is ordinarily self-sterile like several other ascidians. 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 migrations 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 inches from the animals is sufficient. Eggs and sperm are discharged in clouds at the desired time.

b. Mature Unfertilized Egg.

The mature unfertilized egg should show the following:

(1) Chorion, a tough membrane with perhaps a few follicle cells adhering to its outer surface.
(2) Small spherical test cells between chorion and the egg itself.
(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.

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 gray yolk rises to occupy the upper pole, all except the space that surrounds the maturation-spiral 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 horns 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 spermatozoon 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.

First cleavage to 2 cells after 40 minutes.
2nd " 4 " 30 "
3rd " 8 " 30 "
4th " 16 " 20 "
5th " 32 " 20 "
6th " 64 " 20 "
7th " 112 " 20 "
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 B5.2).

(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 end, becomes T-shaped, the stem of the T bordered by the yellow mesoderm-mesenchyme 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.

Ross'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 '26).

B. COLONIAL ASCIDIANS.

1. AMAROUCIUM: TADPOLES, METAMORPHOSIS, EPICARDIAL BUDDING.

a) Methods.

The larvae of Styela and Molgula are so small that study of their internal organization is difficult. The compound ascidian Amarouciuim 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 Amarouciuim 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; 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 study a series of embryos and developing larvae that show the gradual emergence of tadpole form. None of these squeezed tadpoles will swim immediately, and few will attach normally or develop farther. Twitching movements like those of vertebrate embryos or fetuses will be observed.

c) Tadpole structure and Behavior.

Watch *Amaroucium* 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 *Amaroucium* tadpoles have been fixed in Bouin's 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 '21).

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 test swells and metamorphosis has started. Within a couple of hours the flesh of the tail is withered and drawn in, and 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.

e) Later Stages.

Observe metamorphosed *Amaroucium* 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.

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 an agent in asexual reproduction and colony formation.

Below the siphon is the pharynx with its three rows of numerous stigmata (visceral cilia). It opens into a short esophagus 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 occurs in laboratory cultures about 17 days after attachment of the tadpole. It is accomplished by strobilization, i.e. segmentation of the post-abdomen. The old heart is isolated and degenerates; the parent individual regenerates a new heart; the organs of the new individual all differentiate from the epicardium (endoendrerm). This method of asexual reproduction is distinguished from others in tunicates by being called Pharyngeal or Epicardial Budding. (Kowalewsky '74). The epicardial buds while differentiating into new zooids move up and take their place around the parent. Examine a specimen in which this has occurred. Use low power magnification.

g) Colony Structure

The foregoing stages should be compared with the structure of a large Amaroucium colony. With a razor, remove a thin slide from the side of a colony which has been marcoitized with chloroform. Draw several zooids to show the arrangement of the colony, internal arrangement of the zooid and position of the developing embryos.

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 may also be obtained by squeezing the colonies and hunting in the debris. When placed in a little sea water in a watch glass they soon attach to the dish and commence metamorphosis. (Herdman 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 (Grave 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 dense 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.
Tadpoles attach to a substrate within an hour or two after hatching. The metamorphosis is extremely rapid.

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 to 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 blastozooid) appears from the sexually developed animal (the oozooid) 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 ectoderm. This is the Atrial type of budding.

c). Week-Old form.

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

The first blastozooid 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 oozooid does not develop gonads. Thru rearrangement of 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, 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.

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 a week.
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.
1940

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<th>Author</th>
<th>Year</th>
<th>Title</th>
<th>Journal/Book</th>
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</table>
TUNICATE ASEXUAL REPRODUCTION


TUNICATE REGENERATION, ETC.


TUNICATE TADPOLES AND THEIR METAMORPHOSIS.


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 midamental 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.

I. MORPHOLOGY OF THE EGG

Study mature eggs taken from the ovary of the squid.

1. The egg is surrounded by a transparent chorion which does not s lure closely to it. Seen more distinctly some hours after fertilization.

2. In one end of the chorion find a depression and minute canal extruding entirely through it. This is the micropyle. What is its function? Is there also a vitelline membrane?

3. Is the egg spherical? Is it homogeneous? Is it symmetrical from all points of view?

Make an accurate drawing of the egg and its chorion including the micropyle.

4. Study egg masses which have been laid by the squid. What is the origin of the gelatinous matrix in which the eggs are imbedded? What is the exact form of the egg masses? Examine a bit of it carefully to see if each egg has its own gelatinous sheath. The jelly is secreted by the midamental glands and is deposited upon the eggs as they pass through the oviducts.

II. FERTILIZATION AND MATURATION

If it is possible to obtain ripe eggs from the ovary these may be artificially fertilized. Such eggs are more favorable for study than those laid in the normal manner, as they are without the jelly envelopes. Place artificially fertilized eggs in a watch glass of sea water and watch the formation of the polar bodies. How many are formed? (Sea Hooldeley 1930).

See schedule of rate of development on next page.
Trace the first cleavages and note their relation to the axes and symmetry of the egg. The first cleavage furrow lies in the plane separating the right and left halves of the future embryo. See figures by Watase.

Note that the segmentation is meroblastic and also bilateral thus differing from the typical molluscan eggs which are holoblastic. Draw stages up to 8 or 16 cells.

Note: It may be of advantage to cover a depression slide with a thin film of vaseline, or paraffine so that the eggs can be made to stand up endwise for better observation. The same result may be accomplished by crowding a larger number of cleaving eggs together in a depression slide so that some of them stand on end. Try placing the microscope in a horizontal position and jar the slide until some eggs fall into a favorable position for observation.

IV. SCHEDULE OF RATE OF DEVELOPMENT

The following schedule indicates the rate of development but there may be considerable variation due to temperature differences. This schedule was taken by Dr. B. Grave from observations on a mass of eggs spawned June 29, 1936 which hatched on July 11 and 12. temperature 21°C.

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>00 minutes</td>
<td>Fertilization</td>
</tr>
<tr>
<td>20 minutes</td>
<td>1st Polar body</td>
</tr>
<tr>
<td>1 hr. 20 min.</td>
<td>2nd Polar body</td>
</tr>
<tr>
<td>2 to 3 hrs.</td>
<td>1st cleavage, 2nd cleavage 1/2 hr. after 2nd cleavage.</td>
</tr>
<tr>
<td>12 hrs.</td>
<td>Blastoderm over top of egg as cap</td>
</tr>
<tr>
<td>48 hrs.</td>
<td>Blastoderm half way over egg</td>
</tr>
<tr>
<td>72 hrs. (3 days)</td>
<td>Blastoderm nearly covering whole egg.</td>
</tr>
<tr>
<td>84 hrs. (3 1/2 &quot; )</td>
<td>Appearance of mantle and eye stalks.</td>
</tr>
<tr>
<td>130 hrs. (5 1/2 &quot; )</td>
<td>Eyes pronounced, arms appearing, mantle well formed, siphon folds developing.</td>
</tr>
<tr>
<td>156 hrs. (6 1/2 &quot; )</td>
<td>Mantle groove well developed, eye stalks and arms prominent, siphon folds fused into a tube.</td>
</tr>
<tr>
<td>6 to 8 days</td>
<td>Largest size of yolk sac attained.</td>
</tr>
<tr>
<td>11 to 12 &quot;</td>
<td>Yolk sac rapidly absorbed and young squids hatch.</td>
</tr>
</tbody>
</table>

V. SPREADING OF THE BLASTODERM

1. Study eggs which have been segmenting for longer periods, several hours to two days. Note the extension of the blastoderm about the yolk and its relation to the axes of the egg. Paribsblast massed and blastocoones may be observed as well as a beautiful blastoderm in which cell outlines are distinctly visible. Draw.

2. Compare with the figures of Watase, Brooks and Nafe. Does the blastoderm finally completely enclose the yolk? See embryos three or four days old.
VI. THE DIFFERENTIATION OF THE ORGANS

Study an embryo in which the blastoderm has spread over the entire yolk. Certain prominences or irregularities on the surface of the egg are appearing (3 to 5 days) as follows: (figures in MacBride).

1. On the animal pole the skull gland with a depression in its center.

2. Lateral eye prominences.

3. Between the eye and the mantle on each side there is an inconspicuous ridge which will develop into part of the siphon. They are called the lateral siphon folds. Turn the embryo over to see them clearly.

4. The arms are appearing as paired protrusions. How many at first? All these will become more prominent as development proceeds. Draw.

VII. COMPARATIVE STUDY OF OLDER EMBRYOS FIVE TO TEN DAYS OLD

1. Place embryos of various stages in a watch glass and compare them noting the further development of the organs mentioned above and also the appearance of new organs. A careful comparison of older and younger embryos will enable you to recognize most of the organs and in this way you can trace them back to their beginnings. It will be desirable to tear off the jelly envelopes with needles or puncture them. Use figs. in Brocks, Korschelt ('36) and Neaf.

For purposes of comparison call the mantle dorsal and the yolk end ventral. The siphon side is posterior. Study mostly posterior aspects although profile views should be consulted frequently. A frequent turning of the embryo will be advantageous for a better orientation of parts:

Recognize the most obvious things first.

1. Note that the yolk mass gradually constricts in the neighborhood of the embryo and remains as a very prominent oval mass for a long time. It may be seen also as a dark plug within the embryo, extending as a point into the mantle area and also similarly into the eye stalks. What finally becomes of the yolk mass? Observe the contractions of the yolk.

2. Note the development of the mantle until it fits like a hat over the dorsal end of the embryo. Is there a mantle chamber beneath the mantle in any of the embryos?

3. Note that the eyes become prominent. Do they resemble the eyes of vertebrates? Lenses present? Optic cup?

4. The statocysts appear early as a pair of spherical sacs. Do any of them contain statoliths? They move closer together as development proceeds.

5. Dorsal to the statocysts and beneath the mantle there is a pair of gills. They are not very prominent at first but become so later,
appearing feather-like. Between the gills the rectum appears as an unpaired ridge.

6. The lateral siphon folds retain their position dorsal to the eyes for a time. Two inner siphon folds also appear just ventral to the statocysts. These all finally coalesce to form the unpaired siphon. See the older embryos. What is the composition of the fully formed siphon?

7. The arms develop in two lateral groups which appear first as folds ventral to the eyes. In older embryos they take on the adult forms and seem to enclose the yolk. Where is the mouth? Do the arms have suckers?

8. In older embryos find the rectum and ink bag beneath the mantle and directly between the gills. Note the anal valves as in the adult.

9. In the oldest embryos see also fins, branchial hearts, systemic heart, chromatophores.

VIII THE NEWLY HATCHED SQUID

Study the young squid which has just hatched from the jelly envelope. If it swims too vigorously slow it down by adding a little chloroform but do not kill it. A few shreds of lens paper added to the mount will impede swimming.

Recognize the organs that belong to the adult squid as follows:

1. Mantle. Is it contractile? Its relation to the head?
2. Fin present?
3. Eyes. Similar in appearance to the vertebrate eye?
4. Arms and suckers. How many?
5. Siphon with central tube and lateral valves. Is it or any part of it contractile? Compare with the siphon of the adult.
6. Within the mantle chamber recognize two feathery gills and the rectum with rectal valves at its tip.
7. Within the rectum is the spherical ink sac.
8. At the base of the gills near the apex of the mantle are pulsating branchial hearts.
9. Between the branchial hearts is the systemic heart, also pulsating.
10. Two types of chromatophores in the mantle and on the arms. Note color and activity.
11. Several other internal structures may be seen indistinctly: central yolk mass, blood vessels, liver, ganglia, muscles of siphon and head.
Directions for the study of the Spermaphore
(See Drew '11 and '19)

I

Open a living squid in the usual way by cutting through the mantle from the siphon to the tip, with forceps transfer a large number of spermaphores to a shallow dish of sea water.

Watch to see the ejaculation of the spermaphores. Are spermatozoa set free? Observe them under the microscope (low and high power objectives) noting size, shape and activity.

II

Place some spermaphores in strong formalin and after fixing for five minutes pour off the fixing fluid and stain for one minute in Ehrlich's Tri-acid stain by flooding.

Pour off the stain and wash off the excess by several changes of sea water. Study under the low and high power of the microscope. Make out the following structures.

I. The large sperm mass at the larger end.
II. The cement body, the flask shaped middle portion.
III. The ejaculatory apparatus consisting of numerous membranes, the innermost of which contains a spiral filament.
IV. The cap and cap thread at the smaller tip end.
V. There is a liquid space between the sperm mass and the outer membranes, the whole being turgid while living.
VI. Covering the cement body and the ejaculatory apparatus recognize three tunics and three membranes. How many of these cover the entire spermaphore if any? How many extend only from the cement body forward over the ejaculatory apparatus? See Drew's figures.

Draw and label, being careful to observe the relation of certain membranes to the cap and cap thread.

VII. By what means does the spermaphore overt or ejaculate and what is the result? How are the spermatozoa expelled from it?

Note: - The only living portion of the spermaphore is the sperm mass which consists of great numbers of spermatozoa. The membranes are all secretions of the glands located in various parts of the sperm ducts.
References on Development of Cephalopods.

1939

W. I. Brooks

G. A. Drew

1919. II. The spermatophore; its structure, ejaculation and formation. J. of Morphology, vol. 22.

(Both papers are recommended for collateral reading; consult figures in #II for details of the structure of the spermatophore).

L. Bradly

F. Korschelt
1928 Entwicklung des Darmkanals und Nervensystems der Cephalopoden. Festschrift für Leuckart (good figs. of development of intestine)

F. W. Macbide
1914 Textbook of Embryology. I. Invertebrate.

A. Naef

(Complete series of figures of development of Loligo on plates I-VII; serialisation of stages. Consult particularly plate 7, figs. 4 and 4a for newly hatched squid. Note that Naef describes the Mediterranean species, L. vulgaris, which develops more slowly than L. Pe).

A. Fortmann

A. Fortmann and Bidder

S. Ranzi

1931. Duplicitas cruciata in embrioni di Cefalopodi, ibid. vol. II.

S. Watase 1891 Studies on Cephalopods. I. Cleavage. J. of Morphology vol. 4. (See figures)

L. W. Williams The Anatomy of the Common Squid. (Excellent Figures of the structures of the adult squid)
The study of the typical development of echinoderms will include observations on the following forms: A) the starfish (Asterias), B) the sand dollar (Echinarchnus), and C) the sea urchin (Arbacia). The following points will be considered:

1. Morphology of the gametes.
2. Maturation of the ovum.
3. Fertilization
4. Cleavage of the zygote.
5. Gastrulation.
6. Organogeny and metamorphosis.

This will be followed by several experimental studies. A partial list of experiments suggested will be found below.

In order that material of different stages be available at all times, the instructor in charge will place stock cultures of different ages on the tables. Please be careful to avoid contaminating these. This may be avoided by using only the pipette found in each dish for securing material from that culture.

Echinoderms are dioecious, i.e. the sexes are separate. It is impossible, however, to distinguish the male from the female by superficial examination. It is necessary to open animals until ripe individuals of each sex are found. This should be done in the following ways. A) starfish: Wash the animal and all instruments and hands in fresh tap-water to kill any adhering spermatozoa. Now rinse well in running sea-water from the tap. (Why?) One of the arms should now be removed. With forceps remove the gonad which lies free in the cavity of the ray and place in a dish without sea-water. The gonads of the males are white; those of the female are orange-yellow. The gametes will flow from the isolated gonad. Female gonads should be placed immediately in a finger bowl containing sea-water. The male gonads should be left in a watch crystal in order that the sperm may be obtained "dry". When eggs cease to flow from the female gonad, the fleshy part should be removed and the ova washed several times in the following way. Add a considerable amount of sea-water to the eggs and allow them to settle. Now pour off the water and add a fresh supply. This does not remove very small fragments of the ovary but it does serve to eliminate a great deal of the body fluid which may chance to be present. (B) Echinarchnus: Wash the animals as described above. Cut across the animal with a heavy pair of scissors. The gonads will be exposed. Place the males in a clean dry dish in order that "dry" sperm will be available. Wash the eggs extruded from the ovaries (which are purple in color) as described above for the starfish. C) sea-urchin: After washing the animals, puncture the soft tissue at the peristome with a sharp pair of scissors and cut around the equator exposing the
... ands. The sex glands of the male are grayish white; those of the female are scarlet red. After rinsing out the contents of the cavity with fresh sea-water, place the males upside down in a watch crystal to allow the sperm to run out through the genital pore. This is 'dry' sperm. Pick the ovaries out of the shell and place in a finger bowl full of sea-water. After approximately five minutes strain through cheesecloth and then wash the gametes as described above for the starfish. The gametes are now ready to use in your observations. All should be prepared fresh unless otherwise indicated.

1. **Morphology of the gametes. A. E. C.**

The Ovum: Mount freshly shed eggs in a little sea-water and observe the appearance of the egg. Are all the eggs of the same size? Are they all in the same stage of development? Can you see the nucleus? Does the protoplasm of which the egg is composed appear homogeneous in all regions? Are there any cytoplasmic inclusions? Can you see any pigment within the eggs? Is there more than one kind? Is there any evidence of differentiated structure within the cytoplasm? Do you see any polar bodies? How many? Do you see any evidence of jelly around the egg? Now mount some eggs in a small amount of sea-water containing a heavy suspension of India ink. What structures are now visible that you did not see before? Look at a number of eggs and see if you can find the micropyre. This may be seen as a conical depression in the jelly surrounding some of the eggs. Is the jelly coat always the same thickness? Make drawings to illustrate the points observed and compare the different eggs studied. Do mature eggs of the different forms differ in size?

The sperm: Place a very small amount of the dry sperm in a drop of sea-water on a slide. A sufficient number will be carried on the end of a pair of forceps dipped in the 'dry' sperm preparation. Try to observe the form of the sperm and their motility. For the latter determination use sea-water with India ink suspension. A demonstration of sperm under dark field illumination will be made.

2. **Maturation of the ovum. A. E. C.**

When shed under normal conditions, the eggs of all three of the animals studied are ready for fertilization. When removed from the ovaries in the laboratory some of the ova are not mature. Examine eggs from each of the forms. Those of the starfish are best for the study of the maturation process. Place some eggs from a freshly prepared culture on a slide and note the condition of the nucleus in several. Select oocytes with germinal vesicle intact and observe them over a period of from one and one-half hours to two hours. Record the changes observed by means of sketches. Be sure the illumination employed is correct in order that you may trace the formation of the polar bodies. Do you find any immature eggs in cultures from Echinarchus and Arbacia? Sketch. When the eggs are mature they may be fertilized.

3. **Fertilization. A. E. C.**

In order to avoid pathological poly sperm in the eggs studied a dilute sperm suspension should be used in making the inseminations. This may be assured by the following method. Add one drop of 'dry' sperm to 25 cc. of sea-water. To a mass of eggs in approximately 50 cc. of sea-water, add 6 to 7 drops of this suspension. This cor-
centration is sufficient to ensure fertilization of all of the mature ova in a good batch of eggs. As soon as the sperm are added the water containing the eggs should be agitated in order that the sperm may be as nearly evenly distributed as possible. Place some of the eggs on a slide immediately after removal and observe the changes which take place in the egg. Note the gross changes here. The penetration of the sperm and the elevation of the membrane may be better observed by another method of insemination. Mount a few of the eggs in india ink and observe. Make sketches.

The activity of the spermatozoa and the rapidity with which the sperm enters the egg and the accompanying changes take place varies directly with the temperature of the medium and hence the geneses. For this reason it is desirable to study these changes in preparations which have been chilled to such an extent that it takes place slowly. Make preparations as follows. Chill eggs and sperm in sea-water by the use of an ice bath. Mount a few of the eggs on a slide under a cover glass and add a little of the sperm suspension at one side. Observe constantly until the sperm appear in the field and then watch carefully for the union of the sperm and the egg. How does this take place? Examine carefully the way in which the perivitelline space is formed and the manner in which the membrane is elevated. How many sperm enter each egg?

Eggs should be fertilized each morning and each afternoon for several days in order that you may have cultures at different stages of development for use during the following periods.

When you have prepared cultures as described above, place some eggs in about 25 c.c. of sea-water and add a drop of dry sperm, stir vigorously and allow to stand for three minutes to settle. Pour off the supernatant sea-water and wash to remove excess sperm. Set aside for examination at the time of the first cleavage. Such eggs will show a high percentage of polyspermy.

4. Cleavage of the zygote. A. B. C.

One result of complete fertilization is cleavage of the zygote. Examine eggs of each of the above forms in your cultures and study the morphology of cell division, the time at which each cleavage takes place in each form, and the pattern formed by the blastomeres. Are you able to determine the relation of the cleavage planes to the polar orientation of the egg? Be sure to record the temperature of the sea-water in the cultures (vide seq.). Make sketches of each of the cleavage stages in each form and any other events which are of interest to you. How does the cleavage in the cultures inseminated with dilute sperm suspension compare with that in the cultures inseminated with dry sperm? Now compare the rate of cleavage in the three types of eggs. Are they the same? Can you think of any explanations for discrepancies which appear in your data? The blastula is formed within the first twelve hours. Compare the blastulae of the three forms. At what stage is the vitelline membrane lost? When do embryos first become motil? Your sketches should be an accurate record of the events of cleavage showing the relation between successive divisions, the relationship between the sizes of the blastomeres, and the relative rate of the cleavage in different blastomeres of one individual.
5. Gastrulation. A. B. C.

Gastrulation is foreshadowed in the blastula by a flattening of one of the poles (Which?). The cavity of the blastula or segmentation cavity may now be called a blastocoel. Do you find any loose cells within this cavity after invagination is initiated? What are they? Where do they come from? What cells are concerned in the invagination? The secondary cavity formed by the invagination is the gastrocoel or archenteron (cultures of from 24 hrs. to 120 hrs.). Note the two lateral diverticulae at the apical end, the precursors of the coelom (coelomic sacs). These subsequently sever their connection with the archenteron. What is the relation between the size of the blastulae of the three forms just prior to gastrulation?

6. Organogenesis and metamorphosis. A. B. C.

a. Alimentary tract.

The early development of the gut has been considered in the previous section. In addition to the observations made there you should examine the larvae for the origin and later history of the stomodeum. When is it formed; where does it form; and at what stage does it make its connection with the main portion of the gut? Identify the parts of the alimentary tract. Make sketches and compare the development in the three forms studied.

b. Coelom.

Trace the changes which take place in the two coelomic sacs mentioned above. Does the right or the left sac establish a connection with the ectoderm (stone canal)?

c. Skeleton.

Trace the development of the skeletal portions in the embryo of Arbacia between the 48th and the 72nd hours. These appear first as spicules within the mesoderm. Note the relation between the skeleton and the arms. Make sketches of the stages here and in the embryo of Echinarchaeus. Compare with embryos of the starfish at similar ages. Make sketches to illustrate the development of the skeleton.

d. Gross form of the larva.

In the above exercises you have observed the development of the larval form. This should be studied carefully to determine the location of ciliary bands, and the gradual appearance of a more complex organization which results in the formation of the typical larva of the sea-urchin and the sand dollar (pluteus), and the starfish (bipinnaria). If time permits and material is available a study will be made of the metamorphosis of the bipinnaria larva of the starfish including a study of the brachyolaria larva and the subsequent development of the adult form.
EXPERIMENTAL WORK WITH ECHINODERMS.

A few suggestions as to experimental work with echinoderm eggs are given in the following paragraphs. For the most part, these deal with the analysis of early development. Inasmuch as the study of fertilization occupies a separate section of the subjects investigated, but few experiments are outlined here. Any student wishing to test methods of artificial parthenogenesis should consult the instructor.

Too much emphasis cannot be placed on the necessity for running adequate controls in each of the experiments. In working with living systems it is essential that the experimenter be certain that his tests are in reality concerned with the factors he is investigating and hence that all other possibilities are, insofar as is possible, excluded. This can only be done by keeping an adequate series of controls. There is no more efficient way of obtaining "parthenogenesis than by transferring sperm to a mass of unfertilized eggs on the objective of the microscope.

FERTILIZATION: (Arbacia recommended.)

Concentration of spermatozoa.

Add 1 drop of dry sperm to 33 cc of sea-water. This is a 0.1% (cc.) dilution of sperm. Prepare eggs and place 2 drops in 5 dishes containing 25 cc., 50 cc., 500 cc., and 1000 cc. of sea-water respectively. To each of these dishes add one drop of the 0.1% sperm suspension and calculate after fifteen minutes the number (percentage) of the eggs fertilized. Observe again after 5 hours. At the same time make a 1% sperm suspension and add one drop to eggs in 10 cc., 25 cc., 50 cc., 100 cc., and 150 cc. sea-water. In the last series record not only the percentage of the eggs fertilized but also the degree of polyspermy in each instance. (Note first cleavage).

Effect of hydrogen ion concentration.

Set up a series of finger bowls containing 100 cc. of solution as follows: sea-water plus 0.1 cc., 0.2 cc., 0.4 cc., and 0.6 cc. n/10 NaOH, and 0.1 cc., 0.2 cc., 0.4 cc., 0.6 cc., 0.8 cc., and 1.0 cc. n/10 HCl. This makes ten dishes. Add another dish containing sea-water from the tap. Add 2 cc. of eggs to each dish and examine to determine the effect of each solution on the eggs. Repeat to determine the effect of the solution on the sperm. Repeat once more to determine the effect of each solution on fertilization and cleavage. In testing the effect of each of the solutions upon the gametes of one of the sexes alone, observe the gametes at intervals up to one hour. The hydrogen ion concentration of each of the solutions should be determined by the indicator method. What concentration of sperm did you use in making the insominautions? It should be the same in every case.

Cross-fertilization.

Prepare eggs and sperm of the starfish and sea-urchin. Inseminate the eggs of each with the sperm of the other. Observe the fertilization and development of the eggs. Analyze your results.
What effect does the pH of the sea-water have upon the readiness with which the gametes unite? What effect has the concentration of the sperm? What percentage of the eggs develop as compared with eggs fertilized by sperm of the same species? Is there any evidence of patroclinic influence during the period over which your observations are made? Significance.

Effect of agents in solution on fertilization.

To sea-water in measured amounts add definite quantities of coelomic fluid. What effect do these solutions have on the eggs? On the sperms? Inseminate eggs in these solutions as described above. Now inseminate eggs in sea-water and, after ½, 1, 2½, 5, 10 and 15 minutes, add to the solutions of coelomic fluid. Does the presence of coelomic fluid have any effect on the fertilized eggs in the concentrations employed? Analyze.

Repeat this experiment using in place of the coelomic fluid plus sea-water, solutions of CuCl₂ from n/8,000 to n/80,000 made in sea-water. Is there any similarity in the results?

CLEAVAGE:

Physical changes within cytoplasm. (Arbacia)

Examine the relative viscosity of the cytoplasm of the egg at intervals between the insemination and the first cleavage. Test also the uninsminated egg. This can be done by measuring the relative amount or relative rate of pigment movement through the cytoplasm by means of the centrifuge. When is the egg the most viscous? When the least viscous? Plot your results.

Physical changes in membrane. (Arbacia)

Place fertilized eggs in a solution of 60 parts of sea-water plus 40 parts of distilled water at intervals between the insemination and the first cleavage. How rapidly do they swell? Is the rate of swelling a measure of the relative permeability of the membrane?

Effect of HgCl₂ on pigment.

Certain salts have more or less specific actions on the behavior of certain elements within the egg. Make solutions of HgCl₂ (in dishes allotted for this work.) of the following concentrations: m/50,000, m/40,000, and 50,000. Fertilize eggs of Arbacia and after five minutes place some of the eggs in each of the solutions. Remove a few of the eggs to sea-water from the tap at 3 minute intervals and observe for three hours. Describe the results.

Effect of temperature on development.

Fertilize eggs of Arbacia and place in sea-water in beakers which should be kept at an even temperature. The temperature should not vary a degree during the experiment. Attempt to obtain records of the time at which cleavages (1st, 2nd, 3rd, and 4th) take place in several batches of eggs. Make records of as many temperatures as possible and compute the temperature coefficient for the process.
Effect of osmotic pressure on development.

Boil 100 cc. of sea-water to 50 cc. The salts in the sea-water will be twice as concentrated (ca.) in such a solution as in sea-water. Now make up different solutions using this medium to represent 2/1 sea-water, 7/4 sea-water, 6/4 sea-water, and 5/4 sea-water. In the same series make concentrations of 7/8 sea-water, 6/8 sea-water, 5/8 sea-water and 4/8 sea-water by diluting the sea-water as it comes from the tap with distilled water. Into each of these solutions place fertilized eggs of Arbacia (5 minutes after insemination). Record the behavior of the eggs during the first three divisions. Time of cleavage? Percentage of cleavage? Extent of development obtained? Type of cytolysis?

Modification of cleavage pattern.

The cleavage pattern of the egg of Arbacia may be altered by pressure. Place some of the fertilized eggs on a slide and withdraw the water until the cover presses them slightly. Seal the edge of the cover with vaseline to prevent evaporation. The eggs should be placed on the slide approximately 20 minutes before the first cleavage. Observe the cleavages of the egg through the 8-cell stage. Return to sea-water in a watch glass and allow to develop. What effect does such treatment have on the formation of the embryo? Repeat allowing the egg to remain under pressure for longer periods of time.

Effect of dislocation of cytoplasmic inclusions on development.

Centrifuge fertilized eggs of Arbacia at different times between fertilization and the first cleavage. Examine and place in containers for further observation. What effect does the centrifuging have on the eggs? Does the egg recover? What is the relation between the first cleavage plane and the dislocated substance? Select eggs which cleave in such a way that all of the pigment is left in one of the first blastomeres. Do they proceed to cleave at the same rate? Observe the formation of the embryo. Does the dislocation of the cytoplasmic substances appear to have any influence on the development of the embryo?

Development of isolated blastomeres (Arbacia).

Fertilize eggs of Arbacia. About 15 minutes after fertilization remove the jelly and membranes by drawing them into a very fine capillary pipette with a bore 2/3 the diameter of the egg membrane. This may also be done after the eggs have cleaved to form the 1st two blastomeres. With a fine glass needle separate the two blastomeres while still spheres and remove to separate dishes. Keep accurate records of the behavior of both halves of each egg during its subsequent development. Analyze.

Development of fused eggs.

Fertilized ova of Arbacia may be made to fuse by the following method. The results obtained enable the experimenter to draw certain conclusions concerning the organization of the zygote.
Place fertilized eggs of Arbacia in the following solutions:

- 45 cc. sea H2O 55 cc. m/2 NaCl
- 30 cc. " " 70 cc. " "
- 20 cc. " " 80 cc. " "

In these solutions eggs fuse or agglutinate. Leave in the solution 2 to 10 hours and then transfer to pure sea-water and observe subsequent development. The fusions should be treated as individuals and not collectively from early stages. Analyze your results.

Experiments on Parthenogenesis

General Remarks.

It is advisable to perform most of the experiments on Arbacia eggs as they seem to lend themselves better to this kind of experimentation than Astarias eggs. Before opening every sea-urchin it should be rinsed in fresh water and scissors and hands of the experimenter should be washed also in fresh water. If a male sea-urchin is opened particular care should be taken in washing hands and instruments with fresh water before opening a new sea-urchin.

a) Parthenogenesis in one treatment (Dr. Ethel Harvey).

The washed eggs (cheese-cloth!) are put into the hypertonic sea-water solution (1 liter sea-water to which are added 30 g. of NaCl) for 15, 30 and 25 minutes. Agitate dish during treatment and observe under microscope the formation of fertilization membranes.

Transport the so treated eggs into sea-water and observe results. Compare the number of successful developments in the different batches with variable times of treatment. Compute the approximate percentages of successful parthenogenesis and compare the times of development with the times of normal development. (Control fertilizations should be made after the different treatments are started.)

b) Parthenogenesis in two treatments (Jaques Lobo's treatment corrected).

I. To an egg mass of 50 cc. add 8 cc. of 2½ M Mol NaCl solution. Leave for 40 minutes, wash well in sea-water.

II. Bring the egg mass back to 50 cc. and add to this suspension 2.8 cc. N/10 butyric acid. Let act for 30-40 seconds and wash well in sea-water.—Same remarks about observations to be made as above.—Reverse the sequence of treatments, i.e. butyric acid first, hypertonic salt solution as second treatment. Compare results.
I. NORMAL DEVELOPMENT


Korschelt, E. Vergleichende Entwicklungs geschichte der Tiere. Fischer Jena, 1936.


II. METAMORPHOSIS


III. EXPERIMENTAL

A very good general bibliography on experiments performed on Echinoderm eggs up to 1929 can be found in


Some other fundamental papers:
A. FERTILIZATION


A general study on the cytological aspects of fertilization is found in

Wilson, E. B. The cell in development and heredity. New York, 1925.

B. POLARITY OF EGG AND ORIENTATION OF EMBRYO


von Uebisch, L. Entwicklungspysiologische Studien an Seeigelkeimen
II. " " " " 129, 1927.


C. Parthenogenesis

Fauré-Premiet. La cinétique du développement. Paris 1925.
(Consult for further bibliography.)


D. Hybrid Development


Tennent, D. H. Several important papers on the behavior of chromosomes in cross-fertilized eggs (1907-1922). (Journ. Exp. Zool. Vol. 9 and 12.)


E. HEROGENY


F. Physiology of development


Hörstadius, Sven The mechanics of sea urchin development, studied by operative methods. Biological Reviews Vol. 14, No. 2, 1939. (This is a comprehensive review in English of Hörstadius' work).


Embryology of Annelida

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

The two forms to be studied are Hydroides (Eupomatus) hexagonus and Nereis.

Cultures of advanced stages will be prepared. If you wish to prepare your own cultures proceed as follows: Hydroides will spawn immediately after being removed from its calcareous tube. Remove several and place them in finger bowls (one worm per dish), keep 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 hours, gastrulation after 8-12 hrs., trochophore after 20 hrs. to two weeks. The trochophores are best for study when 2-5 days old.

Fertilization of Nereis has been studied in a previous lab. period.

1) The typical trochophore of Hydroides. Consult the excellent figs. in Shearer (11).

Mount trochophores two, three, and five days old in a light suspension of Chinese ink. Put a few shreds of lens paper under the cover slip to entangle the larvae and hold them quiet. Stain lightly with neutral Red or Methylene Blue if desirable.

Observe: 1. Shape of the trochophore larva.

2. Apical tuft of cilia. What is its function?

3. Equatorial band of cilia called the prototroch. Its function?

Determine its exact distribution as seen in side and polar views.

4. Are there other cilia? (Mesotroch or telotroch or fine cilia?)

5. Digestive tract (mouth, oesophagus, stomach, intestine and anus. Are these ciliated also? Feed them a suspension of Chinese ink or carmine powder. By what means do they take food?

6. Eye spots or pigment spots. How many?

7. Apical plate, an ectodermal thickening beneath the apical tuft. The cerebral ganglia are formed from it.

8. A larval kidney (Protonephridium) may be seen similar in position to that shown in Hatschek's figures. It appears as a slender cord extending diagonally from oesophagus to intestine.

9. Body cavity or blastocoele surrounding the digestive tract. Is this the same as the cleavage cavity or is it a coelom?

10. A large vacuolated cell called the anal vesicle at the posterior end. Draw the trochophore as seen in side and polar views.
11. **Larval muscles.** Study their insertions and their functions.

2) The metamorphosis of the trophophore into the segmented worm to be studied in *Nereis.* Consult figs. in E. B. Wilson, 192, plate XX.

Use larvae two, three, five and seven days old; mount them in shreds of lens paper. Observe changes from the trophophore to the three-segmented worm.

**Note:**

1. Three pairs of seti sacs with bundles of seti protruding.

2. The digestive tract consisting of stomodaeum, mid intestine and protodaeum.

3. Mandibles may be seen in the stomodaeum in the oldest larvae.

4. Two to four oil globules in the mid intestine wall. (Of the younger larvae.) What finally becomes of the oil drops?

5. Several bands of cilia?

6. Eye spots in head vesicle. How many pairs? Do older and younger larvae have the same number?

7. Frontal antennae (= tentacles) on head vesicle (= pro stomium) tentacular cirrhi on peristomium.

8. Anal cirrhi and anal flagellum at posterior end.

9. The young *Nereis* trophophore very soon goes into the three segmented condition but no further segments are added until about two weeks have elapsed (see Fwanoff '28). What part of the adult worm does the trophophore represent?

10. Note the differences to *Hydroides:* Lack of blastococcle; incomplete intestine; early loss of apical tuft; position of larval kidney, etc.

3) Gastrulation in *Hydroides* and transformation of the gastrula into the trophophore. Prepare your own cultures. Gastrulation occurs approximately 7-10 hours after fertilization. Consult figs. in Shearer ('11) and Hatschek ('86).

4) Gastrulation in *Nereis* is epibolic. Study embryos 16 and 30 hrs. after fertilization. Consult figs. in Wilson ('92).
References on Annelida

Child, C. M. 1900 Early development of Arenicola. Roux' Archiv. Bd. 9


Just, E. E. 1922 On the rearing of sexually mature Nereis from eggs. Am. Nat. vol. 56


________ 1906 Observations and experiments concerning the elementary phenom. of embryonic development in Chaetopterus (Formative stuffs) Jour. Exp. Zool. III.


Shearer, C. 1911 Development and structure of the trochophore of Hydroides, Quart. J. Micr. Sci. vol. 56


Wilson, E. B. 1892 Cell lineage of Nereis. Journ. of Morph. v. 6


________ 1904 Beitraege zur praktischen Analyse der Polygordius-Entwicklung, etc. Roux Archiv. Bd. 18.

Nos. 1, 3 and 4 are required, nos. 2, 5 and 6 should be done if time permits.

Gastropoda.

1) Study the typical Veliger larva of Crepidula fornicata. 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. I (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.

2) 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)

3) 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 in 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. Are they contractile?

The velum is constantly being retracted between the shell valves.

4) Study the Trochophore stage of Cumingia (12-17 hrs. after fertilization) and its transformation into the Veliger larva. (16-24 hrs. after fertilization).

The trophophore is of short duration, (less than ten hours.) It is best studied 12 to 17 hrs. after insemination. Mount some trophophores in a li ht suspension of india ink, and entangle them in shreds of lens paper.

1. What is the exact shape in side and polar views?
2. Position of the band of cilia. (prototroch)?
3. Apical tuft present?
4. None of the internal organs can be seen because they are obscured by yolk.
5. Is the yolk equally distributed or is one end lighter? Draw side and polar views in outline, showing ciliation.


5) Study the development of the ship-worm Teredo navalis. Different stages will be available. The gastrula shows the invaginating shell gland on the dorsal side, and the blastopore on the ventral sides. The trochophore is very typical, similar to the trochophore of Hydrodides, but has formed the shell. The Veliger larva is also typical.

Literature (consult figs.)


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Libinia, The Spider Crab

Examine female crabs which are carrying eggs. The color of the eggs gives some indication of their stage of development, the youngest eggs being bright vermilion while older stages are bright red. The oldest chocolate colored eggs are not suitable for study.

With forceps remove a few hundred eggs (each student should take them from his own specimen) and place them in a finger bowl from which the rest of the class may take samples.

The earliest stages of division cannot be seen in the living eggs. Troplastic division occurs after the fourth nuclear division at which time the nuclei have migrated to the periphery. Find several stages of cleavage. Are the nuclei visible?

Later stages of development can best be seen after the eggs have been placed in strong HgCl_2 which whitens the embryonic area. Find embryos which show:

a) the 5 embryonic rudiments (examine by reflected light)
b) the nauplius stage with the first 3 pairs of appendages. Are any biramous at this time?
c) later stages having 5 or more pairs of appendages. In these the stomodeum, ventral fold, dorsal shield, telson, and ganglia may be found.
d) the Zoea and Megalops stages (from cultures prepared by the instructor). Warm the larvae with MgSO_4 or other anaesthetic. Under the high power, details of muscles, the compound eyes, the contractile heart and intestine can be observed.

The Barnacle, Balanus conchiferus.

With a strong knife break open the shell and find the egg Lamella at the base. If the eggs are ripe and in the process of development, the lamella is firm and can be taken out entirely. Place it in a watchglass of sea water and tease out a few eggs with a needle.

Fertilization is internal. In the youngest stages available you can observe the polar bodies, and the approach of the germ nuclei. This is best seen in preparations that have dried a little so that the eggs are slightly pressed by the cover glass.

Study the cleaving egg up to the 16-cell stage. These eggs are delicate; usually the same lot cannot be watched through all of these cleavages. If they die, find other eggs in the stage that you need.

Later stages of interest are: a) gastrula; b) the 3 segment stage; c) the 5 segment stage; d) the nauplius just hatched; 4) the nauplius after the first and second moult.
In the nauplius notice the appendages, the labrum, the median eye, paired ganglia or brain, the digestive tract.

Literature List on Crustacea


Brooks, Embryology and Metamorphosis of Macrura.

Davydoff, Traite d'Embryologie comparee des Invertabres (with good literature list).


Korschelt and Heider, Textbook of Embryology.

MacBride, Textbook of Embryology.

Müller, Mr Darwin.


Artemia (crustacean):

The "brine shrimp" Artemia is a primitive (Phyllopod) crustacean which is found throughout those parts of the world which are sufficiently arid to have lakes and other bodies of water which are more saline than the sea. The adults are about half an inch long and are very abundant in such saline bodies as the Great Salt Lake (Utah) (7 to 11 times as salty as the sea), Mono Lake (Calif.), etc. They also occur in abundance in salters in which sea water is evaporated in the sun to manufacture salt, as on the margins of San Francisco Bay, where the cysts used in the Laboratory were obtained.

Artemia develop and live well in sea water, but they are rarely found in the sea in nature, probably because they are defenseless and are eaten. Their tolerance for salt is so great that they can thrive in strong brines in which they have no enemies.

The cysts, or "eggs", as they are sometimes called, are produced in great numbers at certain seasons and float on the brine so that they may be blown in windrows on the shore by wind. These cysts are actually nauplii in a dried and resting condition within the original egg shell. They keep for years. When placed in a suitable medium, such as sea water, they absorb water and hatch in 20-45 hours, depending on temperature, etc.

Examine some of the dried cysts under the microscope. They are dark brown or sepia colored and do not transmit light. When dry, they are the shape of a sphere which has been caved in on one side. After soaking in sea water for an hour or two they absorb enough water to round out. Examine some embryos in the process of hatching. The hard, outer shell cracks, and the nauplius emerges in a quiescent state within a delicate membrane or sac which remains attached to the shell for some hours. The nauplius begins to move and finally breaks out of the sac to swim about. Examine some recently hatched nauplii. They are so active that it is
necessary to anesthetize them, and they are so hardy (the adults live and swim for some time in Bouin's fluid) that it is difficult to anesthetize them, but they will finally come to rest in strong alcohol. Observe the 3 pairs of cephalic appendages, the single median eye, and on the ventral side the enormous labrum or upper lip. The digestive tract may also be seen.

Observe some larvae which have been hatched for 2 or 3 days. Even if the larvae are not fed, the first moult takes place to form a metanauplius. This differs from the recently hatched nauplius principally in being longer and larger and in having the buds of 3 more pairs of segment appendages (on the thorax). Note the strong positive phototropism of the stock culture.

If older stages are available, observe them also. Adulthood is reached after 14 or 15 moults (Heath) during which the number of appendages gradually increases greatly.

**Bibliography for Artemia**


July 23, 1940

To Whom it Concerns:

This certifies that, in the opinion of the staff of instructors..................has satisfactorily completed the course in Embryology given during the summer of 1940 at the Marine Biological Laboratory, in Woods Hole, Massachusetts.

The course has comprised:

35 lectures
126 hours of supervised laboratory work
2 field trips

[Signature]

Instructor in Charge.