m. b.l.
Outlines for Embryology course

M 33 88
# EMBRYOLOGY COURSE
## Tentative Schedule for 1935
### Subject to change without notice

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<td>Fish</td>
<td>Goodrich</td>
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<td>Coelenterata</td>
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<td>New moon</td>
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<td>July 1</td>
<td>Mon.</td>
<td>Echinoderms</td>
<td>Schotte</td>
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<td>8</td>
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<td>Crustacea</td>
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<td>Cell lineage, Annelids and Mollusca</td>
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<td>Annelids and Mollusca, later stages</td>
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<td>22</td>
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<td>Annelids and Mollusca</td>
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<td>27</td>
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<td>Places in laboratory vacated by noon.</td>
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| Thurs. June 20 | Introductory instructions  
Historical outline  
Phases of embryonic development  
Spawning habits  
Handling of material       | Dr. Goodrich  |
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Fertilization, cleavage and early stages of Teleost development | Dr. Goodrich  |
| Sat. June 22   | Development of Teleosts (cont.)  
Problems of gastrulation and early embryo formation | Dr. Goodrich  |
| Mon. June 24   | Development of genetic characters in fish                                                  | Dr. Goodrich  |
| Tues. June 25  | Ovulation in Anura                                                                      | Dr. Rugh      |
| Wed. June 26   | General embryology of the Squid                                                          | Dr. Grave     |
| Thurs. June 27 | Orienting factors in nerve development                                                    | Dr. Weiss     |
| Fri. June 28   | Comparative embryology of Coelenterates                                                   | Dr. Barth     |
| Sat. June 29   | Experimental Studies on Coelenterate Development                                           | Dr. Barth     |
| Mon. July 1    | The Echinoderm egg and fertilization in Echinoderms                                      | Dr. Schotte   |
| Tues. July 2   | The normal development of Echinoderms  
2:00  
Metamorphosis in the Echinoderms                                              | Dr. Schotte   |
<p>| Wed. July 3    | Genetics and Embryology                                                                  | Dr. Sturtevant|
| Thurs. July 4  | The mitotic figure of dividing cells                                                     | Dr. Fry       |
| Fri. July 5    | Parthenogenesis                                                                          | Dr. Schotte   |
| Sat. July 6    | Problems of experimental embryology and morphology in Amphibia                           | Dr. Schotte   |
| Mon. July 8    | Transplantation experiments in Echinoderms                                               | Dr. Schotte   |</p>
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<td>Characteristics of organ forming areas in the chick</td>
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<td>Topics from the History of Embryology</td>
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<td>Tues. July 16</td>
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<td>Thurs. July 18</td>
<td>Embryological processes in the adult mammal</td>
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<td>Fri. July 19</td>
<td>General embryology of the Mollusea (continued)</td>
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<td>Sat. July 20</td>
<td>Problems of determination regeneration</td>
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<td>Embryology of Polyzoa</td>
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<td>Regeneration in Tubularia and the problem of organization in the Coelenterates</td>
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<td>Localization and development in the Ascidian egg</td>
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<td>Disorganization and reorganization in the adult Ascidian</td>
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<td>Dr. Conklin</td>
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Two types of eggs will usually be studied; the pelagic egg and the non-pelagic or demersal egg.

The demersal egg
Examples: Fundulus heteroclitus
Fundulus majalis
Cpsanus tau

1. 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 and roll it about gently beneath a second piece of filter paper until the jelly and outer filtres 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.

2. THE OVUM. To study unfertilized eggs they should be obtained from females that have been kept in fresh water for about 20 minutes. Studies must be made soon after stripping. 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.

3. FERTILIZATION. Note exact time of fertilization and be prepared to study immediate changes. Note time of change of yolk plates; of formation of porovitelline space. What are your conclusions in regard to the rapidity of the activation of the egg? If practicable find micropyle on unfertilized egg. Incubate and note spreading of fertilization reaction from locus of micropyle. Separation of membrane and possibly entrance of sperm may be observed if microscope is placed in horizontal position with micropyle on horizon of the egg.
4. **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 sometimes be seen but these may be more advantageously studied in the pelagic eggs. What is the relation of the pole of the ovum to gravity? How does this compare with the condition in the frog egg; with the chick egg? Do any processes take place in the unfertilized egg similar to those in the fertilized egg?

5. **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 and a half 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?

6. **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 blastodis (not visible except in sections); the central periblast. In the late blastodisc (18-20 hrs.) observe particularly the behavior of the marginal cells distinguish between circular and radial cleavages. The large pinhead 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.

7. **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 (blasta). Soon the margin of the disc appears relatively thicker (germ ring) due both to an actual thickening and to the thinning of the central part of the disc. The germ ring can best be observed in F. majalis. During the next few hours the germ ring grows completely over the surface of the yolk mass. The uncovered portion of the egg is the blastopore. The final covering of the yolk or the closure of the blastopore occurs after the first stages of the formation of the embryo. Under favorable conditions the beginning of gastrulation may be observed in the appearance of a slight indentation at edge of germ ring at a time when the yolk is about 1/3 covered. Staining with neutral red may help in identification of germ ring.

8. **THE FORMATION OF THE EMBRYO.** (Beginning 24 to 36 hrs.)

   a. While the germ ring is extending around the yolk, the formation of the true embryo takes place. Its first indication is a cellular thickening known as the embryonic shield, caused by a more active proliferation and 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 blastodisc 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 blastodisc to near the center of the blastoderm. 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? Study the shield to determine what parts of the future embryonic axis it represents. How does this compare with the development of the chick blastoderm?

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 $3/4$ 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 Kupffer'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?

9. 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, medulla, 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 formation of pericardium; first blood vessels; (and especially their mode of formation from wandering mesenchyme cells); first action of the heart; form and position of heart. Compare course of circulation on 4th and 6th days. Illustrated by diagrams.

d. Mesenchymal cells. Note wandering mesenchymal cells especially abundant beneath posterior end of embryo on 2d 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.

f. The young fish may be studied just after hatching by anaesthetizing with chloretone.

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

When possible comparisons will be made with pelagic eggs. Those of the cunner, (Tautogolebrous adspersus Cuv.) the scup (Stenotomus chrysocuc, Linn.) and the mackerel (Scomber scombrus, Linn.) are most likely to be obtained. 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.

10. 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. 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 sertmatozoa. Crosses between F. heteroclitus and the cunner (Tautogolebrous) 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. 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 to those suggested under (b) should be observed in obtaining unfertilized eggs.

d. 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 work of course

This bibliography contains only a few of the older papers (for
more complete references see general works and papers here men-
tioned with special reference to bibliography there listed.)

I. GENERAL REFERENCE WORKS.

Hertwig, O. (editor) Handbuch der vergleichenden und experimentelle

1913

Co. 1919

Morgan, T. H. Experimental Embryology, New York, 1927

Schleip, W. Die Determination der Primitiventwicklung, Leipzig 1929

Ziegler, H. E. Lehrbuch der vergl. Entwickelungsgeschichte der
niederen Wirbeltiere. Jena 1902.

II. SPECIAL REFERENCES ON MORPHOLOGY OF DEVELOPMENT

Agassiz, A. & Whitman, C. O. On the development of some Pelagic

Agassiz, A. & Whitman, C. O. The Development of Osseous Fishes.

Balfour, F. M., A Monograph on the Development of the Elasmobranch
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Clapp, C.M. Some points in the development of the toad-fish.
Jour. Morph. 5, 1891

15, 1899.

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Teleosts and in other Vertebrate Types. Q. J. M. S.
28, 1886.

His. W. Untersuchungen uber die Bildung des Knochenfischembryo.

Ryder, J.A. A Contribution to the Embryology of osseous fishes
with special reference to the cod. Report of Com.of
Fish and Fisheries. 1882. Washington, 1884

Sumner, F.B., Kupffer's Vesicle and its Relation to Gastrulation

Sumner, F. B., A study of Early Fish Development, Experimental and
Morphological. Arch Entw'mech. 17, 92 - 119. 1904

Stockard, Charles R., A Study of wandering mesenchymal cells on the

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

III. HABITS

Craig-Bennett, A. The reproductive cycle of the three-spined stickle
back, Gasterosteus aculeatus, Linn. Phil. Trans. Roy.

Gudger, E. W. The habits and life history of the toadfish. Opsanus

Newman, H. H. Spawning behavior and sexual dimorphism of Fundulus
IV. CIRCULATORY SYSTEM


V. GERM CELLS


Okkelberg, Pater The early history of the germ cells in the brook lamprey, Entosphenus wilderi (Gage) 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.


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

Kopsch, Fr. 1896
Experimentelle Untersuchungen über Keimbautrand
Orteiches Vitalartung, bei Scyllaembryonen zeit
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Experimental production of cyclopia in the fish
McEwen, R. S.
Experiments on localization in the egg of Fundulus
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Morgan, T. H.
The early development of Haemichromis bimaculata
with special reference to factors determining the
embryonic axis. Jour. Morph. and Physiol. 49, 1930
Spek, Josef
Experimental studies in the teleost eggs. Anat.
Anz. 8, 1893
Stockard, C. H.
28, 1891.
Worley, L. G.
Development of the egg of the mackerel at different

VII. Development of Genetic
VII. DEVELOPMENT OF GENETIC CHARACTERS

Bancroft, F. W.
Heredity of pigmentation in Fundulus hybrids
Jour. Exp. Zool. 12, 1912
Goodrich, H. B.
A study of the development of Mendelian Characters
Mendelian inheritance in Fish. Quart. Rev. of
Biology 41, 1929 (contains bibliography of genetic
work on fish.)
One step in the development of hereditary pigmen-
tation in the fish Oryzias latipes. Biol. Bull. 65,
1933.
The development of hereditary color patterns in fish
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and I. B. Hansen. The post embryonic development
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Moenkhaus, W. J.
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oclitus and Menidia notata with especial reference
to the behavior of the maternal and paternal chromat-
Morris, Margaret
The behavior of chromatin in hybrids between
Newman, H. H.
Hybrids between Fundulus and Mackerel. Jour. Exp.
Zool. 26, 1918. (See this for reference to other
paper by same author).
The initial block in normal development in cross
Pinney, M. E.
EMBRYOLOGY OF THE SQUID

If you are not familiar with the anatomy of the adult squid you may find it advantageous to examine now and then, the dissected specimens which are placed on demonstration tables.

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 adhere closely to it. Seen more distinctly some hours after fertilization.

2. In one end of the chorion find a depression and minute canal extending 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 gela tincus 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.

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? (See Hoadley Jour. Exp. Zool. 1932)

See schedule of rate of development below. Draw.

III.

CLEAVAGE

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 Wataasc.
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 13 cells.

Note: It may be of advantage to place the egg in the depression of a small glass bead, or of paraffine so that it will 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.

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 from observations on a mass of eggs spawned June 29, 1926 which hatched on July 11 and 12, temperature 21°C.

<table>
<thead>
<tr>
<th>Time</th>
<th>Event Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>00 minutes</td>
<td>Fertilization</td>
</tr>
<tr>
<td>20 &quot;</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</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. (3½ days)</td>
<td>Appearance of mantle and eye stalks.</td>
</tr>
<tr>
<td>130 hrs. (5½ days)</td>
<td>Eyes pronounced, arms appearing,</td>
</tr>
<tr>
<td></td>
<td>mantle well formed, siphon developing.</td>
</tr>
<tr>
<td>156 hrs. (6½ days)</td>
<td>Mantle grooves, well developed</td>
</tr>
<tr>
<td></td>
<td>eye stalks, arms, etc.</td>
</tr>
<tr>
<td>6 to 8 days</td>
<td>Largest size of yolk sac attained</td>
</tr>
<tr>
<td>11 to 12 days</td>
<td>Yolk sac rapidly absorbed and young squids hatch.</td>
</tr>
</tbody>
</table>

V. SPREADING OF THE BLASTODERM

2. 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. Periblast nuclei and blastocones may be observed as well as a beautiful blastoderm in which cell outlines are distinctly visible. Draw.

3. Compare with the figures of Watase and Brooks. Does the blastoderm finally completely enclose the yolk?

VI. THE DIFFERENTIATION OF THE ORGANS

Study an embryo in which the blastoderm has spread over the entire yolk. Certain prominences or irregularities in the surface of the egg are appearing (3 to 4 days) as follows:-
1. On the animal pole the shell gland with a depression in its centre.

2. 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 and over to see them clearly.

All these will become more prominent as development proceeds. Draw.

VII. COMPARATIVE STUDY OF OLDER EMBRYOS

1. Place embryos of various ages 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.

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?

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? Lens present? Optic cup?

4. The otocysts appear early as a pair of spherical sacs. Do any of them contain otoliths? Do they move closer together as development proceeds?

5. Dorsal to the otocysts and beneath the mantle there is a pair of gills. They are not very prominent at first but become so later, appearing feather like.

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 otocysts. These all finally coalesce to form the unpaired siphon. See the older embryos.
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? (Only three pairs of arms arise thus; others develop later.)

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. Do these hearts contract periodically?

Problem:—Secure an egg mass recently spawned and examine a few eggs daily to observe successive changes up to the time of hatching. This study of ten minutes daily will give a clear conception of the sequence of events and rate of development.

Place some spermatothoraces in 4% formalin and after fixing five minutes pour off the fixing fluid and stain for one minute in Ehrlich’s Ehrlich’s stain by flooding.

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

1. The large sperm mass at the larger end.
2. The cement body; the black clubbed middle section.
3. The circulatory apparatus consisting of anemia and part of intestine at which remains a spiral filament.
4. The cap and cap thread at the smaller tip near.
5. There is a liquid space between the sperm in its case and other membranes, the whole being held together by the covering membrane.
6. Recognize the yellow mass above, the yellow membrane covering the yellow mass above, the thin black middle, and inner. Consult figure.
7. The yellow membrane covering the cement body and the ejaculatory apparatus. Recognize the three tunics and three membranes, but many of these cover the entire spermatothorax if any; the only 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.

Set up a scale to determine the size of the spermatothorax as well as the general size.
Squid Embryology continued

Directions for the study of the Spermatophore

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 spermatophores to a shallow dish of sea water.

Watch to see what happens to them one after another. How do you interpret it? Does adding fresh water increase or decrease this activity? Why?

II

For better observation place a group of spermatophores in 1/4 saturated MgCl₂ and try to induce ejaculation or turning inside out by pulling the long cap thread with forceps. This treatment is supposed to slow down the process.

Add sea water if nothing happens.

III

Place some spermatophores in 40% 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 stain 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 portions.
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 white living.
VI Recognize covering the sperm mass three membranes or tunics outer, middle and inner. Consult Drew's figures.
VII Covering the cement body and the ejaculatory apparatus recognize three tunics and three membranes. How many of these cover the entire spermatophore 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.

VIII By what means does the spermatophore avert or ejaculate and what happens? How are the spermatozoa expelled from it?
1935

COELENTERATA

Introduction

In connection with the following laboratory work certain precautions must be observed. In the first place, the hydroids are very sensitive to changes in environment and do not survive well in the laboratory even in running water aquaria. Since the room temperature is considerably above water temperature, the individuals soon die and disintegrate, when removed to finger bowls. Tubularia for example will not regenerate at temperatures above 25°C. It is important therefore in working with the stems of hydroids to use few stems in large volumes of water and to change the water frequently. Do not crowd either the adult stems or the eggs and embryos. Much time is often wasted in experiments lasting 12 - 24 hours by placing too much of a colony in too little sea water.

In general it is advisable to select a good sized colony, examine it under low power to find the gonads and to clip off the branches containing the gonads and remove them to fresh sea water. Often the stems are covered with debris or parasites in which case they should first be washed in running sea water.
1935

Regeneration in Tubularia

It is advisable to begin an experiment in the evening as the stems regenerate in about 36 hours and observations may be made during the day. With a sharp scissors cut off the stems of Tubularia at the point of attachment and sort out the stems according to length. Select stems of 30 - 50 mm. and use these for cutting into halves. Shorter stems can be used intact for experiments b and c. Do not handle stems with forceps as this will crush the stem. Use a bent glass rod for transferring stems from one dish to another. Keep the preparations as cool as possible, placing the finger bowls in running sea water if necessary.

a. Comparison of regeneration of distal and proximal halves.

Select stems about 20 - 30 mm. in length and cut off the hydranths with a few millimeters of the stem. Do not cut the hydranth at its base as the regeneration of this region is somewhat retarded. Cut the stems into equal halves and keep in separate finger bowls. Change water once a day. Observe how the hydranth differentiates in situ and not as an outgrowth. 18 - 24 hours after cutting the primordium of the oral and basal tentacles can be seen through the perisarc. Observe the circulation within the stem. After 30 hours take frequent observations recording the time for the emergence of the hydranth. Compare the number of distal and proximal hydranths present. After 60 hours compare the average rate of regeneration for distal and proximal halves.

The proximal end of the stems may also form hydranths. Record the time and compare. Some stolons may form at the proximal ends. Record number.

b. The action of the distal regenerating end upon the proximal end of a stem.

Select 30 stems and cut to make pieces 8 mm. long. Divide into 3 lots, A, B and C. In A ligature the stem at the distal end with a thread by tying it firmly. In B ligature the stems in the middle and keep C for control. Compare the rate of regeneration of the proximal hydranth in A, B, and C.
THE DEGENERATION OF THE MEDUSA

1935

Bougainvillea

The gonophores are borne singly or in clusters on the main stem and branches. New buds destined to become medusae are scattered throughout the colony so there is no arrangement of buds according to age. Examine buds showing successive stages of development and understand how a medusa is formed.

When mature the medusa becomes detached and swims away. The sexual organs are not developed when they are liberated. Study a mature medusa and note the position of proboscis, radial and circular canals, velum, tentacles and eye spots. A knowledge of the structure of this medusa will aid in understanding the development of the medusae of other forms.

Obelia

The gonosomes are recognizable by their large size, being several times as large as a hydranth. Examine specimens and see if there is any definite point of origin.

A gonosome of this form consists of an enlarged transparent covering, the gonangium, with a blastostyle extending through it from base to tip. The outer end, or tip, of the blastostyle forms an enlargement that in the mature gonosome forms a plug to the gonangium.

On the blastostyle are borne the gonophores which become free medusae that escape as they are detached through the opening usually closed by the enlarged end of the blastostyle. Examine the gonophores on a blastostyle and see which are the older. Determine the general structure and position of the tentacles of one of the older gonophores.

With needles open the gonangium of a well matured gonosome and examine the gonophores that are released. If any are practically mature they may show swimming movements. Some of the stages of development will be liberated by teasing and may be studied.

Mature swimming medusae which have been set free normally can usually be found in a dish of sea water in which the hydroid colonies have been kept over night. A peculiarity of the medusa of obelis is the way the bell is inverted when the medusa is at rest, so the manubrium sticks out like a handle from the center of the convex, although the subumbrellar side. Watch the swimming movement and see how this is reversed. Examine the structure and compare it with Bougainvillea. The velum will be found to form only a narrow somewhat lobed membrane near the bases of the tentacles.

The medusa of O. geniculata has 24 tentacles when liberated while that of O. commissuralis has 16. Both forms may be in the Laboratory. Neither have the gonads developed when liberated.
Pennaria (This form will be in better condition in July)

Gonophores bud off around the lower portion of the hydranth. They consist of slightly reduced medusae, with rudimentary tuft-like tentacles. These medusae are set free early in the evening, and swim feebly for a few hours. The gonads are always ripe when the medusae are liberated, and are shed into the water almost immediately. Eggs are fertilized in the water and reach the planula stage within 24 hours.

Gonophores of one sex only will be found on a single colony. The female gonophores when nearly mature contain four to six pink opaque eggs of varying sizes, while the male gonophores are whitish. Tease apart a gonophore of each type and examine the eggs and spermatozoa.

Set aside male and female stems in a fingerbowl over night. Next day remove the stems and look for free swimming medusae and for eggs in various stages. Follow the latter as far as possible. By changing the water at least once a day, the metamorphosis of the planula may sometimes be observed. More sexually mature gonophores are found about two weeks later in the season.

Campanularia

The gonosomes are similar in appearance to those of Obelia. Each consists of a transparent gonangium, blastostyle extending from base to tip with gonophores budding from it. Unlike Obelia the medusae are not set free and never attain any considerable development. The shape of the younger ones is somewhat marked by the brown pigment contained. Each female gonochore contains a very large irregularly shaped egg which undergoes development and reaches the free swimming planula stage before it is released from the gonangium.

Select a gonosome showing eggs in the basal gonophores. Study with different powers on a slide with cover.

Select another gonosome with planulae showing near the tip and study. With needles open the gonochore of such a gonosome and liberate the planulae. Study them for arrangement of cilia and movements.

Select gonosomes with very well matured planulae. They should be two or three times as long as broad and show movements inside the gonangium. Liberated such planulae with needles and place them in watch glasses (not more than two or three to a watch glass) with sea water, cover the watch glasses and set aside for future examination. Such specimens should attach to the watch glass in a few hours (4 to 10). Each should 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 sea water should be changed at least twice a day. Study the successive stages as development proceeds from the planula. This can be done from time to time as laboratory work permits.

The male gonosomes are similar to the female but the gonophores contain sperm which when liberated from the mature specimens becomes active upon coming in contact with sea water.
Tubularia

Gonosomes form long racemes or clusters growing from the region between the two circles of tentacles on a hydranth. Male and female gonophores differ somewhat in color. This species has no evident radial or circular canals in its gonophores.

Examine a male gonophore. Notice its attachment, shape, the condition of its distal end and the position of the sperm surrounding the dark red manubrium. Open a ripe male gonophore and examine the motile sperm.

Examine a female gonophore. Notice its shape and look for tentacles at its distal end. They are usually represented by four short blunt processes. Occasionally one or more may be somewhat elongated and tentacle-like. Examine the space around the manubrium. In it embryos are developed to the Actinula stage. Open some female gonophores and study the freed larvae. The younger are flattened with marginal blunt processes. At first these are not symmetrical in outline. Later radial symmetry is attained and the larva grows to the actinula stage which has mouth and tentacles, with a rounded aboral body that later becomes attached.

Place a few actinula in watch glasses of sea water and examine two or three times a day. They will not advance far without feeding which would require more care than can be given them in this course.

Budendrium

The gonosomes are strikingly different in the male and female sex. Those of the female consist of tufts of gonophores (the medusa is very degenerate) attached about the stem, each gonophore being bright orange in color. The male gonophores appear slightly pink, and are found in a series of four or more on stems which radiate off from the hydranth like spokes in a wheel.

The eggs are fertilized within the female gonophore and develop to the planula stage when they are liberated. Tease out gonads from different colonies, and study all stages found.

Gonothyraea

An interesting form for comparison or if there is sufficient material for detailed study. The medusae develop within the gonangium as in Obelia. When mature they remain attached to the end of the blastostyle, outside the gonangium for several days. Within the body of the medusa the eggs are fertilized and develop to the planula stage, when they are set free, and the medusa drops off.

If time permits study Clava, Hydractinia or Sertularia. The last is especially interesting because of a special brood pouch or acrocyst, containing embryos in various stages.
 Gonionemus (Not available until the latter part of July)

Select large specimens, showing swollen gonads. Place in finger bowls, or preferably in flat-bottomed glass dishes, of which the bottoms are covered with clean slides. Place in the dark (desk drawers are a good place) about 3 P.M., and leave for 60-75 minutes. Eggs are normally laid about 7 P.M. but in above way their deposit may be hastened. They will stick to slides sufficiently to permit easy manipulation and examination under the microscope. Watch for:

1. 1st division about 60-70 minutes after fertilization.
2. 2nd division about 50 minutes later.
3. Succeeding divisions appear at about 50 minute periods. Specimens left over night to lay normally should furnish blastula and gastrula stages. How is the gastrula formed? A ciliated, flatworm-like larva (planula) develops in about 12 hours.

If slides bearing developing eggs are kept in flat glass dishes containing sea water, the hydra like larva will be found within two or three weeks. Study stained specimens of these later stages.

Aurelia

Select an adult specimen showing granular material entangled on the oral "arms". Tease off some of this material on to a slide with a drop of sea water, and examine for ciliated embryos. They will prove to be in different stages of growth some spherical, others oval, fully formed planulae. How does the gastrula and planula differ from others studied?

Place a considerable number of planulae in two or three clean watch glasses, and set aside for further study on subsequent days. Their gradual change in form, acquisition of tentacles, and elongation into the sessile scyphula stage can be easily followed.

The various stages of the metamorphosis of the scyphula, through its strobilation phases, the liberation of ophyrae, the metamorphosis of those last into the adult morphology should be worked out on material furnished. In the ophyrae, note the homology of sense-organs with tentacles, the mode of origin of gastric filaments and order of appearance and branching of the radial canals.
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ECHINODERM EMBRYOLOGY

The study of the typical development of echinoderms will include observations on the following forms: A) the starfish (Asterias), B) the sand dollar (Echinarachnius), 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.

It is essential that material be conserved. This is especially true in the case of the starfish. Ripe individuals have gonads in each of the rays. One of the arms may, therefore, be removed and the gametes which issue from the gonad may be made to serve a large number of students. When one arm is removed from an animal, that animal should be placed in a special container in order that, should it be a male, the sperm shall not infect females opened in the same way. The same care in conservation should be used with Echinarachnius and Arbacia as well.

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) Echinarchnium: 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 purplish 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 gonads. The sex glands of the male are grayish white; those of the female are maroon-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. B. 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 micropyle. 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. B. 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 Echinarchanthes and Arbacia? Sketch. When the eggs are mature they may be fertilized.

3. Fertilization. A.B.C.

In order to avoid pathological polyspermy in the eggs studied a dilute sperm suspension should be used in making the insomiations. This may be assured by the following method. Add one drop of 'dry' sperm to 25 c.c. of sea-water. To a mass of eggs in approximately 50 c.c. of sea-water add 6 to 7 drops of this suspension. This concentration is sufficient to ensure fertilization of all of the mature ovum 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 insomiation. Mount a few of the eggs in India ink and observe. Make sketches.

The activity of the spermatozoon 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 gametes. For this reason it is desirable to study those changes in proportions 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 motile? Your sketches should be an accurate record of the events of cleavage showing the relation between successive divisions, the relationships 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 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 Echinometra. 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., 100 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 inseminations? It should be the same in every case.

Cross-fertilization.

Prepare eggs and sperm of the starfish and sea-urchin. Insinate 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 coccolith fluid. What effect do these solutions have on the eggs? On the sperms? Insinate eggs in these solutions as described above. Now insinate eggs in sea-water and, after ½, 1, 2½, 5, 10 and 15 minutes, add to the solutions of coccolith fluid. Does the presence of coccolith 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 uninseminated 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/30,000, 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 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? Report 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 egg? 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 first 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
- 30 cc. " "
- 20 cc. " "
- 55 cc. m/2 NaCl₂
- 70 cc. " "
- 80 cc. " "

In these solutions eggs fuse or agglutinate. Leave in the solution 8 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.

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. Protosplasmic 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 MgCl₂ which whitens the embryonic area. Find embryos which show:

a) the 5 embryonic rudiments

b) the nauplius stage with the first 5 pairs of appendages. Are any biramous at this time?

c) later stages having 6 or more pairs of appendages. In these the stomach, ventral fold, dorsal shield, telson, and ganglia may be found.

d) the zona and Megalopa stages (from cultures prepared by the instructor). Quiet the larvae with Na₂CO₃ or other anaesthetic. Under the high power, details of muscles, the compound eyes, the contractile heart and intestine can be observed.
Crustacea

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 egg. Protoplasmic 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 HgCl₂ which whitens the embryonic area. Find embryos which show

a) the 5 embryonic rudiments
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 stages the stomodeum, ventral fold, dorsal shield, telson, and ganglia may be found.
d) the Zoea and Megalops stages (from cultures prepared by the instructor). Quiet the larvae with MgSO₄ or other anaesthetic. Under the high power, details of muscles, the compound eyes, the contractile heart and intestine can be observed.
Crustacea

The Barnacle, Balanus eburneus.

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 entire. Place it in a watchglass of seawater 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; e) the nauplius after the first and second molt.

In the nauplius notice the appendages, the labrum the median eye, paired ganglia or brain, the digestive tract.
Literature List on Crustacea


Binford, Fertilization in Menippe. J. Morph. 1913, 24

Brooks, Embryology and Metamorphosis of Macroura.

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


Korschelt and Heider, Textbook of Embryology.

MacBride, Textbook of Embryology.

Muller, Fur Darwin.
Spiral Cleavage in Crepidula

Note the Crepidula shells on the Hermit Crab shell. The flat species is C. plana; the arched one is C. fornicata. With a heavy knife pry off a shell and observe how the egg capsules are attached. The small, light yellow capsules contain the earlier stages in development: the large, deep yellow or mud-colored ones have much older embryos and larvae.

Because the small cells which lie over the four basal macromeres are almost transparent, the eggs must be stained for study. They are mounted whole, not sectioned. A low power ocular and high power objective are useful.

All stages in development from the formation of the first polar body up to the late gastrula may be found. Slides marked A have early stages up to about 8 cells; those labeled B carry development to about 32 cells; gastrulae are in Slide C. Make a careful study of the two maturation divisions, the growth in size of the sperm nucleus until it fuses with the egg nucleus, the first cleavage.

In the later stages (4, 8, 12, 16, 20, 24, 25 cells) the origin of the newly formed blastomeres can be determined by the mitotic figures.

The endoderm cells in the gastrula stage are still large and few in number. The opening of the blastopore can be seen.

Methods for preserving and staining Crepidula eggs are given in Conklin, Karyokinesis and Cytokinesis, p. 6.
FERTILIZATION

General Instructions.

Dishes containing unfertilized eggs and sperms are at opposite ends of the room. Use only the pipettes in the dishes.

To fertilize a batch of eggs, take up a few unfertilized eggs from the stock dish and place them in a Syracuse watch glass full of sea water. Then add a single drop of sperm suspension. In sea water the sperms quickly lose their fertilizing power. Do not use a sperm suspension that is more than 15 minutes old.

For microscopic study, put the eggs on a plain slide and support the coverglass with a bit of thin paper. It may be necessary to add a drop of water occasionally.

1. The Unfertilized Egg.

Note the large nucleus or germinal vesicle, the oil globules, yolk spheres, and the cortical layer.

2. Fertilization Phenomena.

Make a preparation of fertilized eggs. Put some on a slide adding a drop of thick Chinese Ink. Notice the extrusion of the jelly from the cortical layer, the change in the appearance of the latter, the germinal vesicle, and the point of attachment of the sperm.

In this preparation or a fresh one made without any ink find an egg which shows the sperm in profile view. Watch the changes which take place,—the development of the Sperm Cone.
and its subsequent behavior. It may be necessary to add a drop of water occasionally to prevent the preparation from drying.

Make a fresh preparation of eggs which have been fertilized for about 30 min and watch the extrusion on the polar bodies, and the first two or three cleavages.


When first removed from the worm the eggs show the large germinal vesicle; it quickly disappears and the first maturation division proceeds as far as the metaphase, stopping at this point until fertilization occurs.

After insemination notice the elevation of the vitelline membrane, the extrusion of the polar bodies (Unusually large) and the first two cleavages. Record the changes in shape of the egg at this time and the behavior of the Polar Lobe.

**Time Table**

<table>
<thead>
<tr>
<th></th>
<th>1st P.B.</th>
<th>2d P.B.</th>
<th>1st Cleav.</th>
<th>2d Cleav.</th>
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<tbody>
<tr>
<td>Nereis</td>
<td>45</td>
<td>55</td>
<td>90</td>
<td>120 min.</td>
</tr>
<tr>
<td>Chaetopterus</td>
<td>20</td>
<td>40</td>
<td>60</td>
<td>90</td>
</tr>
</tbody>
</table>

Note; When polyspermy occurs these processes are much more rapid.
| Literature List on Fertilization, Cleavage, etc. |
|---------------------|------------------------|
| Lunar Periodicity   | Fox; Proc. Roy Soc. Lond, B., 1924 |
| Maturation          | Cowdry, Cytology: Wilson, The Cell. |
| Fertilization       | Lillie, Problems in Fertilization; (full literature list) |
| Cleavage            | Conklin, Crepidula. J. Morph. 1897, 13 |
|                     | Wilson, Nereis, J. Morph. 1892, 6. |
|                     | Wilson, Cell Lineage. W. H. Lectures, 1898. |
| Historical          | Balfour, Comp. Embryology, Vol. 2 |
|                     | Whitman, Clepsine, Q.J.M.S. 1878, 18. |
CREPIDULA VELIGER

Study a veliger under the microscope and compare in all respects with the veliger of the Lamellebranch, Cumingia.

I.

In a contracted veliger see the coiled shell.

II.

Organs of the Veliger

When the veliger expands its velum the following organs may be seen.
1. A bilobed contractile velum with cilia about its curved edges.
2. Two darkly pigmented Eye spots.
3. A pair of small tentacles projecting from the front of the velum.
4. The mantle a loose collar free on the dorsal sides.
5. A large curved organ, the foot, below the velum.
6. A pair of large otoysts containing otoiths within the base of the foot.
7. The Gills in the mantle cavity. (Finger like filaments extending forward and obliquely.) Not always clearly visible.
8. A pulsating Heart sometimes two hearts are present, one larval, one permanent.
9. A coiled Intestine in the right side of the body and a large bilobed Stomach. There are two liver lobes. One is located in the apex of the shell, the other lies more centrally.
10. The Mouth may be seen as a large ciliated opening situated between the foot and the velum. Where is the Anus?
11. How much of the digestive tract is ciliated?
12. Cerebral Ganglia at base of tentacles, beneath the eyes.
13. Pedal Ganglia near the otoysts.
14. Are there indications that the veliger is a feeding animal? Feed a suspension of Chinese ink.
15. Sometimes a pair of larval kidneys is visible below and at the sides of the velum. These are very prominent humps in some species.
16. How does a veliger differ from a trochopore? What changes would make a snail of the veliger? What becomes of the velum? How does it differ from the lamellibranch veliger?
17. How many or what proportion of the invertebrate embryos studied move and feed by means of cilia?

III.

Origin of the Organs

If time permits study younger embryos of Crepidula in which the organs are first recognizable as humps. Note their relation position and shape. Trace them until they become functional organs. See series of figures by Conklin, "Embryology of Crepidula" Journal of Morphology 1897.
EMBRYOLOGY OF CUMINGIA TELLINOCIDES

I SPawning

Cumingia will usually spawn readily within thirty or forty minutes after being placed in bowls of sea-water. Observe the spawning through the dorsal siphon. (Keep sexes separate, one specimen per dish.)

II

PERFILIZATION

1. Study the unfertilized egg under high power microscope. Is there a vitelline membrane and germinal vesicle? What has become of the latter? Structure of the yolk?

2. Fertilize some eggs in a watch crystal and see if there are prompt changes such as were observed in the Nereis and fish eggs. Is there any observable change, such as the formation of a fertilization membrane or protoplasmic movements?

III

MATURATION AND CLEAVAGE

Supplement your observations on fertilization and maturation by watching changes in the egg of Cumingia from insemination until first cleavage;

1. Polar bodies. Observe the extrusion of the polar bodies. What is their relation to the vitelline membrane? (First one 10 to 12 minutes, 2nd 25 to 30 minutes.) The polar bodies of Cumingia are interesting because of their size and they should be observed.

2. Fusion of the Pronuclei. You should be able to see the fusion of the pronuclei and formation of the first cleavage astor.

3. Cleavage. Study the egg through the first three cleavages. Are the first two blastomeres equal? Are all blastomeres of the eight celled embryo equal? Note carefully their exact position in reference to each other. The cleavage is spiral no doubt, but what makes it less easily recognizable than in Crepidula or Nereis?
TIME SCHEDULE TEMPERATURE 20 to 22°C.

Time schedule:
- First polar body 8-15 minutes
- 2nd polar body 25-35 minutes
- 1st cleavage, approximately 50-60 minutes
- 2nd cleavage, 1 hour and 10 minutes to 1 hr. and 40 minutes
- 3rd cleavage, 1 hour and 35 minutes to 2 hrs. and 5 minutes

Note: Other cleavages at intervals of about twenty-five minutes. (The rate is affected by temperature.)
The lowest figures of the above table are the rate, at 22°C. The highest figures at 20°C. If interested, compare the rate of cleavage of the Cumingia egg at 18° and 22°C to see the remarkable difference.

GASTRULATION

The embryo becomes a swimming gastrula in 6 to 9 hrs. Pass it by or spend only a few minutes in its study. Is there a recognizable blastopore and archenteron? Perhaps you can recognize shell gland and blastopore in a nine-hour embryo.

V.

THE TROCHOPHORE

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 light 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. A digestive tube is developing however.
5. Is the yolk equally distributed or is one end lighter?
Draw side and polar views in outline, showing ciliation.
6. Can this larva be distinguished from the annelid trophi-
phore? Compare with your drawings of the trophophore of Hydroides. What is the significance of such resemblances?
EMBRYOLOGY OF CUMINGIA TELLINOIDES
VI.
THE VEILIGER

The trophophore begins its transformation into the veliger in from eighteen to twenty-four hours. In the following study observe:

1st. What changes have taken place in converting the trophophore into the veliger and 2d, learn the characteristics of the lamellibranch veliger.

I. General characteristics of the veliger:-

Place some cumingia veligers, 2, 3, and 4 days old, in a watch glass or depression slide and study with the low power of the microscope, as they swim freely in the water, or as they stop momentarily at the edge of the water, the following are distinguishable (Use the binocular microscope if available.).

1. A bivalve shell, similar to that of the adult.
2. A swimming organ bearing Cilia called a velum.
3. Shape of body from various points of view.
   Is hinge line distinguishable?
4. Position of velum in reference to the shell and mantle lobes. What is the relation of the velum to the prototroch of the trophophore?

Draw edge and side views of the veliger larva.

II. Detailed structure of the veliger:-

Make several mounts of Cumingia veligers including those 2, 4, 6 and 8 days old. Before adding the cover glass add a few shreds of finely divided lens paper to entangle them. All these have practically the same structure but some show one thing and some another more distinctly. It is therefore desirable to study all of them simultaneously. It will be necessary to find more or less quiet individuals with velum extended. Contracted individuals will not do.
EMBRYOLOGY OF CUMINGIA TELLINOIDES

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. What is the coelom in Mollusca?

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. Is the stomach also drawn back at the same time? Make out the muscle fibres which retract them. These are best developed in the oldest veligers, but are visible only when the velum is extended.
The Embryology of Chiton Apiculata

I.

External features of the adult Chiton.

Examine an adult animal to familiarize yourself with its external features.

1. Note the number of valves or segments of the shell. Which is largest? Is there a head?

2. Note that the mantle extends beyond the shell valves all the way around.

3. On the ventral side note proboscis or head vesicle. The mouth is situated near its center. The anus is at the opposite end.

4. The foot is long and narrow and occupies the greater part of the ventral surface. Is it distinctly separated from the proboscis? Is the foot muscular? Function?

5. Between the foot and the mantle along the sides of the body note numerous gills. Examine them with hand lens. Note their relation to the mantle.

6. The paired oviducts open into the mantle chamber. The eggs are expelled beneath the posterior end of the mantle, the posterior edge being raised locally roof-like.

II.

Extrusion of the Eggs

1. To obtain eggs place several mature chitons in a crystallization dish of fresh sea water late in the afternoon (6 or 7 P.M.) Males and females may be placed in the same dish since there is no danger from polyspermy. They should begin to spawn shortly after dusk.

2. As far as has been observed the eggs are laid during the months of June, July and August between seven and eleven o'clock in the evening. Watch the extrusion of the eggs in two clouds. There is also lunar periodicity. Although eggs are obtainable at any time they are shed abundantly between full moon 3rd quarter or two or three days preceding full moon until 3rd quarter. A single individual may continue to expel eggs for an hour or two and sometimes intermittently for four hours. (Spawning season approximately June 20 to September 1)

III.

Cleavage of the Eggs.

1. Place eggs in a watch glass or depression slide and watch for the first segmentation which occurs after one hour and forty minutes to one hour and fifty minutes. Are the first two blastomeres equal or unequal or variable? Draw. Is the bristly covering of the eggs a vitelline membrane or a chorion?
2. Follow cleavage to the eight cell stage noting the relative positions assumed by the blastomeres. In the eight cell stage note four macromeres A,B,C,D, and the first quartette of micromeres a,b,c,d. Are the macromeres all alike or can cell D be distinguished by its larger size as in many molluscs and annelids? Is the spiral arrangement in evidence? Draw.

3. The further cleavage shows no unusual features. It is of the spiral type and there are four quartettes of micromeres. The first three quartettes give rise to the ectoderm, nervous system and stomodaeum while the fourth quartette becomes part of the endoderm along with the macromeres. There is one exception to this in that the cell 4d of the fourth quartette becomes the mesoderm as is the case in other molluscs and annelids, this need not be followed now. See Heath's figures. The gastrula develops powerful prototrochal cilia after 12 hours and hatches as a trophophore in 25 to 30 hours, i.e. breaks from the chorion and swims.

IV

The Swimming Larva or Trophophore

Note: If the larvae swim too rapidly you may successfully entangle them in a few shreds of lens paper or slow them down with carbon dioxide.

1. Examine the youngest swimming larvae (30 to 35 hrs. old) and note shape, position of prototroch and apical tuft of cilia.

2. What is the means of propulsion? What seems to be the function of the apical tuft? How used?

3. Is the movement straight forward or in spiral course? Does the body rotate upon its longitudinal axis also? How many embryos so far examined seem to move in this way? Do any adult animals also swim in a similar manner? Is there any mechanical explanation?


5. Can you find the mouth or anus or digestive tract? (See older larvae)

6. Is the trophophore at this stage radially symmetrical or are there indications of bilateral symmetry? Watch for further indications of it in older embryos. Make a careful drawing of the trophophore, on a large scale, showing all observed structure and correct outline. Label fully.

7. Study older embryos, three days old, in the same way and note changes in size or shape. The mouth and archenteron have become visible because of the reduction of yolk. The larvae continue to become less opaque as they grow older. The larval eyes are also plainly visible. Does the digestive tract seem to be complete or incomplete?
Later stages in development and metamorphosis

1. In embryos five or six days old look for the development of the shell. Compare with the shell of the adult. Where does it first make its appearance?

2. Note the developing foot on the side opposite the shell. Is it contractile? Are any other parts contractile?

3. The large anterior lobe is called the head vesicle. Note its shape and thickness. Where are the eyes, in reference to it? Is the prototroch upon it, or back of it? What is the extent of the digestive tract at this time?

4. Is the larva now bilateral or radial in symmetry? What part of the larva is still radial?

5. Do these older larvae ever attach to the bottom and creep upon the foot? Draw an older larva showing correct shape from two views. Emphasize features that are different from the younger one. Label fully. Is this larva a trochophore or a veliger?

6. About the eighth or ninth day the prototroch is shed and the embryo settles permanently to the bottom and creeps. Watch the metamorphosis, if material is available. What changes take place during this metamorphosis? Are the lateral eyes still present? Does it now resemble the trochophore or the adult? Note shell, foot, mantle and head vesicle. What does the head vesicle become? Where is the mouth located now? What becomes of the prototroch? Draw the metamorphosed embryo.

Note: Larvae have metamorphosed under laboratory conditions in from seven to twelve days. The finest lot of young Chitons obtained during four years study metamorphosed seven days after the eggs were spawned. This lot contained about two hundred individuals. The usual time is nine days. From the time of metamorphosis rapid growth begins and if kept washed clean they develop readily in running water in the laboratory. It has been observed that many embryos grow very little until after metamorphosis although they usually begin to feed before that time.

VI

Problem. Secure the eggs as suggested and place them in finger bowls of sea water. Change the water every few hours until the larvae hatch. Remove the larvae with medicine dropper to a fresh bowl of sea water. Change twice daily in this way until they metamorphose (7 to 9 days). Draw daily to show changes, including growth of shell and foot. At what age do they first settle to the bottom and creep? Is the foot functioning before metamorphosis? What chief changes in form accompany metamorphosis?
EMBRYOLOGY OF HYDROIDES, HEXAGONUS

I. SPAWNING

The annelid Hydroides will spawn immediately after being removed from its calcareous tube.

Place several in finger bowls or shallow dishes of sea water, keeping sexes separate. (one worm per dish).

II. THE OVUM

Study the unfertilized egg

1. Color?
2. Germinal vesicle present?
3. Yolk abundant or light?
4. Vitelline membrane relatively thick or thin?
5. Size?

III. FERTILIZATION AND MATURATION

Add a few drops of sperm suspension to a dish of eggs. Are the spermatozoa active before they are placed with the eggs? Do they become more active after an hour in sea water? Compare with the sperm of other species as to vigor of movement.

1. Are there observable changes in the egg at the time of insemination? viz. fertilization membrane or internal changes? How soon does the germinal vesicle break down?
2. Mount some eggs which were fertilized before class hour and observe the extrusion of polar bodies. Is there a change in the shape of the egg as the polar bodies are formed?

IV. CLEAVAGE

1. Study the egg during the first three cleavages.

Are the first two blastomeres equal or unequal?

Is a large cell, Cell D, distinguishable in the four or eight cell stages?
2. At what stage of development does the larva hatch and begin to swim? At what stage does it become ciliated and begin to rotate?

3. The nine hour larva is a good gastrula and its study is desirable if time permits. (Twelve hours is too late) Study also the process of gastrulation in Hydrodones (7 to 10 hrs.) Is there true invagination? Compare with Nereis. What is the nature of gastrula in Nereis?

TIME SCHEDULE

Fertilize some eggs and fill in the time schedule for the early cleavage. Record the temperature. If interested, compare the rate at 20° and 22° C.

1st polar body
2d polar body
1st cleavage
2d cleavage
3rd cleavage
Swimming larva, (5-6 hrs. Blastula)
Gastrula (9 to 12 hrs.)
Trochophore 20 hrs. to two weeks. (Best ones for study 2 to 5 days)

VI.

THE TROCHOPHORE

Mount trochophores two, three, and five days old in a light suspension of Chinese ink and compare them as follows:—

(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 Methylen Blue if desirable)

1. Shape of the trochophore larva.
2. Apical tuft of cilia. What is its function?
3. Equatorial band of cilia called the prototroch. Determine its exact distribution as seen in side and polar views.
VI. THE TROCHOPHORE, CONTINUED

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? By what means do they swim?

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 (Proteonephridium) may be seen similar in position to that shown in Hatcheck's figures. It appears as a slender cord extending diagonally from oesophagus to intestine.

9. Body cavity or primary body cavity surrounding the digestive tract. Is this the same as the cleavage cavity or is it a coelom? How much mesoderm is visible at this stage?

10. A large vacuolated cell called the anal vesicle at the posterior end.

Draw the trochophore as seen in side and polar views.

VII.

METAMORPHOSIS AND DETAILED STRUCTURE OF THE NEREIS LARVA

The formation of the segmented worm from the trochophore is best seen in a form like Nereis. Note the formation of the first three segments. What part of the later larva is represented by the trochophore? At what point are new segments added?
With these questions in mind study Nereis larvae two, three, five and seven days old.

Note:-

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

2. The digestive tract consisting of stomodaeum, *mid intestine* and proctodaeum. When and from what do the stomodaeum and proctodaeum form?

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

4. Two or 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* and *tentacular cirri* on head vesicle.

8. *Anal cirri* and *anal flagellum* at posterior end.

9. How many of these structures can you recognize in the one segmented larva?

10. The young Nereis trochophore very soon goes into three segmented condition but no further segments are added until about two weeks have elapsed. Where is the elongating region? What part of the adult worm does the trochophore represent? See Wilson's figures.

11. At what age do the three segments form? Is there a true trochophore at any time?
THE GASTRULA OF ANNELIDS

Study Nereis embryos 16 hrs. and 30 hrs. old from polar view showing macromeres and overgrowing cotoderm. These may be regarded as the gastrula. Is there a gastrula cavity (arcenteron) in this species? What type of gastrulation is this? The prototroch should also be visible in these larvae. Is there an apical tuft and apical plate? How is gastrulation accomplished in annelids? Compare such forms as Hydroides, Nereis and Podarke. Refer to papers by Hutchek, Shearer, Wilson, Mead, and Treadwell.

IX

CIRRHATULUS GRANDIS

Some worms never develop an actively swimming trochophore but instead the trochophore like embryo grows promptly a worm body of several segments.

I.

Method

a. Place several of these worms in a crystallization dish of sea water (first wash off the adhering mud) and allow them to spawn together. After an hour or two remove the eggs to a dish of clean sea water, and watch the progress of development for three or four days.

II.

Development

1. Study the 12 and 24 hour embryo. Is it a trochophore?
   a. Note the apical tuft.
   b. The equatorial ridge: Is a well-developed protroch visible?
   c. Is the larva motile?
   d. Is it ciliated?
2. Study briefly one, two, three and four day embryos with low power and without a cover glass. How soon does the worm body form? Note the delay in going beyond the three segment stage. This is not true of all annelids.

3. Study the four day embryo more in detail:
   a. Are mouth and complete digestive tract visible?
   b. Are the prototroch and apical tuft still present?
   c. By what means is locomotion accomplished?
   d. Are parapodia or setae present?
   e. What part of the worm is formed from the prototroch?

What are the most evident changes that take place in the metamorphosis of the trochoïd into the worm?

Is the metamorphosis of other worms studied similar or different?

4. Some annelids have a true trochophore larva which later goes to a three segmented condition. Other annelids omit the trochophore and develop at once three or more segments. The trochophore may be considered typical.
EMBRYOLOGY OF BUGULA

The polypoza Bugula flabellata and Bugula turrita are abundant in the Woods Hole region. See living colonies on the demonstration table.

I. METHOD OF OBTAINING LARVAE

The larvae of these species are developed in special brood pouches or ovisacs. They issue from the colonies early in the morning (5 to 9 A.M.) as free swimming larvae. Collect several mature colonies late in the afternoon and place them in a large jar of sea water. The larvae may be found swimming at the surface the following morning. Transfer several to a watch glass or depression slide with a pipette and study with a binocular or a compound microscope.

II. STRUCTURE OF THE LARVAE

The larva is nearly spherical and measures .18mm in diameter. Several external structures are visible:

1. What is the color of the larva?

2. At the anterior end is the apical organ surrounded by a circlet of rigid cilia.

3. At the posterior end there is a circular depression which appears in side view as a notch.

4. Extending from the posterior depression forward on one slide, as far as the equator, is a lateral groove.

5. In the anterior extremity of this groove there is a tuft of long flagella which beat back and forth in an antero-posterior direction.

6. What part of the larva is ciliated?

7. The larva of Bugula turrita has two pairs of eye spots, that of flabellata has none visible. Note their position in reference to the groove and longitudinal axis.

8. Some species have a digestive tract. This larva has none.

9. The posterior depression contains an inverted sac. (Internal sac) It however is not visible externally.

III. BEHAVIOR OF THE LARVA AND METAMORPHOSIS

1. By what means does the larva swim?

2. Is the swimming in a straight line or a spiral?
III. Continued

BEHAVIOR OF THE LARVA AND METAMORPHOSIS

3. Is the swimming continuous or interrupted by momentary attachments?

4. By what means does the larva attach temporarily?

5. Is it positive or negative in its response to light? Observe several two, four, and six hours after liberation. Is the first reaction to light reversed after some hours? (Positive to negative?)

6. From 11 A.M. to 3 P.M. the larvae are becoming permanently attached. The process of fixation may be observed in a watch glass under a binocular microscope or even under a compound microscope. It is accomplished quickly and is an extraordinary spectacle and well worth an hour of effort to see it take place. The sequence of events is briefly as follows:

1. The larva seeks a shadow and attaches momentarily by the lateral groove.

2. It suddenly extrudes a large inverted sac which becomes the holdfast. (See paragraph 9 Section II Page 1.

3. This organ grasps the support and adheres permanently. The lateral groove releases its hold.

4. The larva thus attached begins promptly to elongate. The cilia continue to beat for half an hour but finally cease.

5. Observe an attached larvae 4, 8, 12, 24, and 48 hours after attachment.

6. Observe colonies 4 to 6 days old. What is the mode of budding and development of the colony?

PROBLEM

Collect colonies of B. flabellata and of B. turrita and secure the larvae by the method suggested above. Compare the larvae of the two species.

Transfer larvae to tender dishes or finger bowls of sea water and study their behavior until they attach. Then place the dishes in running sea water and observe the growth of the colony by budding. Observe daily and draw to show the rate of growth.

See "Journal of Morphology" for June 1930.
Before beginning work on this group, review the anatomy of an adult Ascidian, if it is not already familiar to you. Parker and Haswell vol. II and Sedgwick Vol. III are adequate for this purpose but more detailed and better illustrated references are Delage and Heronard vol. 8, or the monographs on Ascidia and Betryllus issued by this Liverpool Marine Biological Society.

Simple Ascidians --
Development to the tadpole larva

Styela

Eggs and spermatozoa are shed normally shortly after sundown, but usually animals must be isolated in fingerbowls of sea water and remain undisturbed for at least twelve hours before that time. Eggs shed normally are much to be preferred for study of development since all are mature, and capable of development. Like several other Ascidians Styela ordinarily is self sterile, i.e. eggs will not develop if sperm from the same individual only are present. If single individuals are isolated in separate fingerbowls and allowed to shed, development can be started at any time within an hour or two by simply mixing the gametes from several dishes together.

A small number of developing eggs can be secured at any time during the day by excision of the gonads from mature animals. Remove the test from several good sized animals and cut the orange gonads into a fingerbowl containing a little sea water. Mince the gonad masses with scissors or needles and fill the fingerbowl with water. Stir the water rapidly and remove the coarse debris from the center with a pipette. Eggs in all stages of growth will be found floating in the water. Those which are fertilizable will begin their development within the next 2 to 15 minutes.

The development of Styela eggs furnishes the classic example of determination of the various organs of the embryo in the undivided egg following fertilization, as shown by Conklin's description. Comparison with Conklin's figures should be made as the various stages are observed. The mature unfertilized egg should show the following:

(a) Chorion, within which are (b), test cells (small spherical cells) surrounding the egg itself. (c) The periphery of the egg shows a clear layer containing minute yellow granules; the central part,
(c) gray yolk, eccentrically placed within which is a very large
(c) germinal vesicle.

Carefully watch for rearrangement of egg substance which follows within 2-3 minutes after eggs have been cut from gonads (i.e. immediately on fertilization.) The clear yellowish peripheral layer streams to the lower pole, over the yolk, followed by the clear protoplasm from the animal pole. The gray yolk seems to rise to occupy the upper pole, save for such space as surrounds the
maturation spindle complex. Soon the yellow substance migrates to one side of the lower hemisphere where it assumes crescentic form, the broad middle of which is located at the posterior end of the future embryo, while the horns of the crescent band around the egg on its right and left sides. Immediately above the broadest part of the yellow crescent the clear cytoplasm collects. The position of the forthcoming embryo is now marked. The yellow crescent is the posterior end. The animal pole, where the germinal vesicle lay, is the ventral side, while the vegetal pole, where the spermatozoon entered, is the dorsal side. If the yellow pigment is not visible, try more specimens. Examine eggs with the diaphragm on the microscope wide open.

The pigment is segregated in cells that develop into mesoderm. The cleavage to 32 cells should be followed accurately, and the destination of the material of each cell worked out. Later study the gastrulation, formation of the blastopore, its conversion into the neurenteric canal; and the changes in form as the tadpole larva develops. Under normal conditions the embryo with its numerous differentiated organs hatches in about 10 hours.

Molgula

From specimens of Molgula start eggs developing, using the same method as for Styela. Watch the cleavage up to the gastrula. If developing eggs are isolated in the watch crystal, tadpoles and young stages in metamorphosis may be obtained. No pigment is present in these eggs, but otherwise development is the same as in Styela. Molgula is self-fertile.

Amaroecium tadpoles and their metamorphosis.

The small size of the larvae of Styela and Molgula makes them rather unsatisfactory for study of the internal organization. The compound Ascidian Amaroecium has a large tadpole easily secured from the parent colonies which are viviparous. The best tadpoles are secured soon after sunrise, between 6 and 7 A.M. They may also be secured by squeezing a mass of Amaroecium over a fingerbowl partially filled with sea water. If the bowl is filled up with water the coarser particles whirling near the top can be poured off. The tadpoles secured in this way do not swim immediately, and often fail to attach normally.

Note the swimming movements of the tadpole. How does it propel itself? What is its reaction to light? To gravity?

As soon as the tadpoles swim, place one in a drop of water on a slide in order to study the structure. For the behavior during attachment isolate several tadpoles each in separate drops of water in Syracuse watch-glasses. Cover the Syracuse dishes and observe the tadpoles several times during the next two days. The metamorphosis to the simple Ascidian condition takes place within a few hours after attachment, but this can be observed best at the age of two days to one week.
Structure. From a living tadpole certain structures only can be
made out. These are test, test cells, adhesive papillae, atrial
siphon nearer the tail, oral siphon (usually the larger), sensory
vesicle between the siphons. The small pigmented spot is the
light perceiving organ. In the tail, the muscle cells and nato-
chord are visible.

Fix several tadpoles in Bouin's solution for 10 minutes.
Wash in 60% alcohol. Stain in acid borax-carmine prepared as
follows: to 5 cc. of 70% alcohol add 1 drop of HCl and enough
borax-carmine to make a deep pink. Allow the tadpoles to remain
in this until they are a pale rod. Dehydrate and mount. From the
stained slide the pharynx, gill-slits, endostyle intestine, and
nervous system can be added to the drawing of the tadpole.

Earlier stages. From the debris in the finger bowl pick
out eggs undergoing development. Usually several stages can be
found.

Later stages of Amaroecium larvae which have been
developing for a week or two will be ready for class study. The
embryos are attached to the dishes and cannot be removed. Carefully
wash off debris without using force and keep dish filled with water.
Examine with low and high power of the microscope. The post-abdom-
inal region with the heart at the end is the first thing noticed.
Watch the heart. What happens that is different from the heart of
other embryos studied? Find the atrial and oral siphons. The
pharynx with its numerous stigmata opens into a short oesophagus
which connects with a round yellow stomach marked with muscular
bands. The intestine shortly after leaving the stomach turns sharply
and ends near the atrial siphon. The endostyle is a rod on the wall
of the pharynx and separates the two atrial pouches that surround
the pharynx and open to the exterior at the atrial siphon. Note
structure of the mouth.

Budding. This is accomplished by segmentation of the
post-abdomen. The pieces develop into separate zooids which slowly
move up and take positions about the parent. It occurs in laborato-
ry cultures about seventeen days after attachment of the tadpole.
A few demonstrations will be available for study with a hands lens.

Colony Structure. Take a slice from an Amaroecium colony
which has been narcotized with Epsom salts. Draw several zooids
to show the arrangement of the colony, internal structure of the
zooid, and the developing embryos.

Botryllus

Botryllus is another compound Ascidian and is found
commonly upon eel grass or old piles. The tadpoles can be obtained
if a few colonies are stripped from the grass and placed in a
finger-bowl. They may also be secured by squeezing a mass of col-
onies. If in the finger-bowl a few slides are placed up-right on
the side near the light, tadpoles will attach to them and undergo
metamorphosis. Besides the tadpole and early stages in metamorphosis
study the various stages that have been started from one to three week
previously. Understand the scheme of budding by which the colony is
formed. Budding in Botryllus is from the side (pallial) rather than from a branching stolon. The first bud is single, all the later ones are in symmetrical pairs.

Structure. The tadpole is not so large as that of Amareocium but it shows an interesting feature not present in that form. Just under the adhesive papillae are a group of accessory hearts folded up like the petals of a rosebud. In metamorphosis, they unfold and spread around the edge of the developing tunicate. As the colony grows these increase in number.

Day old form. Besides the accessory hearts, the two siphons, clype spot, pharynx with several stigma and yolk are present.

Three days old. The animal is clearer and larger, usually it lies so that the observer looks directly into the atrial and oval siphons. The large pharynx shows one row of stigmata around it. 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 atrium opening. From the stomach the intestine after turning to one side returns to open into the atrium. The pulsating heart and the circulation is of particular interest, especially the passage of the blood around mouth and pharynx. A new bud now appears at one side as an evagination of the atrium. A blood supply to the bud is furnished and the differentiating parts can be distinguished in older buds. From what germ layers do the buds apparently come?

Week old. In these forms the same general structures already studied can be easily seen. The pharynx has developed 4 rows of stigmata and the buds have 3 or 4 rows if they are well developed. Buds of the second and third order may be present.

Older stages. Various stages in the formation of the colony can be studied. In all the same structures will be seen. The chief interest in these stages is the relation of the different individuals to each other. A completed colony shows the common atrium in the middle with the separate pharyngeal openings surrounding it. Study the living specimens, then fix and stain several of different ages for further details of structure. Place slides bearing the animals in Bouin's fluid, transfer to 50% alcohol, then to water and finally stain 30 minutes in borax carmine. Then pass through the various grades of alcohol to sylol. If the animals do not clear, return to 100% for a few minutes. Mount in balsam under a cover slip.
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The glassware and supplies listed below and contained in Table No. have been received, to be returned clean and in good condition at the end of the session.

Date ______________________

Signed ____________________

8 finger bowls
5 saucers
1 large glass dish
6 pipettes (1 large mouth)
1 black, 1 white tile
5 small, 1 large glass plate
6 Syracuse watch glasses
6 watch crystals

4 clear, 2 black cellars
2 depression slides (1 white, 1 black)
1 piece mica
2 small brushes
1 felt pad
6 slides
vaseline
10 cover glasses
neutral red
methylene blue

Other reagents will be supplied as needed.