EMBRYOLOGY COURSE

THE STAFF
1943

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ASSISTANT

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Rockford College
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EMBRYOLOGY CLASS

1943

Aronson, Stanley M.  
City College of New York

Banks, Mary Elizabeth  
Washington University

Cole, Elsie L.  
University of Wisconsin (M. A., Heidelberg College)

Hopkins, Alice  
University of Rochester

Jenkins, Janet R.  
Wheaton College

Lehman, Gene  
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Loose, Marian  
Columbia College

Lowenhaust, Marian  
Washington University

Mekeel, Amy Grace  
Cornell University (Ph. D., Cornell)

Satke, Jack  
Washington University (M. D., Washington University)

Travis, Dorothy  
George Washington University

Wahlert, Mary Rita  
Washington University (B. S., Fontbonne College)

Wilde, Betty Lee  
Rockefeller Institute for Medical Research (B. A., Maryville College)

Wolf, Thlama H.  
Washington University (A. B., Harris Teachers' College)
**EMBRYOLOGY COURSE**

Schedule for 1948

Full Moon, June 18th

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**Groups**

1. Cyclopa in Fundulda (G. Lehman) Hamburger, Mittel
2. Regeneration in Tubularia (Travis, Loomis) Barth
3. Isolation of Blastomeres in Versa (Hopkins, Cole, Wilde) Costello
4. Twinning, exocoelaculation, centrifugation in Arbacia Hamburger
**Lectures in Embryology Course 1945**

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EMBRYOLOGY

Development of the Teleost

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

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

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

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

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

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

3. THE OVUM. To study unfertilized eggs they should be obtained from a female that has been kept in fresh water for about 20 minutes. Strip and keep the egg in dilute sea water (70% fresh water 30% sea water) to retain the morphological characteristics observable at time of extrusion. Note details of structures of the unfertilized mature ovum. These include yolk plates, oil drops, protoplasm, membranes, micropyle, etc. (The micropyle must be observed before removal of chorionic jelly.) If immature ova are present compare these with mature ova.
4. **FERTILIZATION.** Note exact time of fertilization and be prepared to study immediate changes. Note time of change of yolk plates; of formation of perivitelline space. What are your conclusions in regard to the rapidity of the activation of the egg? If practicable find micropyle on unfertilized egg. Inseminate and note spreading of fertilization reaction from locus to micropyle.

5. **FORMATION OF THE BLASTODISC.** Note the gradual accumulation of the protoplasmic cap. This is the blastodisc or germ disc. Compare polar and lateral views. Polar bodies may be more advantageously studied in pelagic eggs. What is the relation of the pole of the ovum to gravity? How does this compare with the condition in the frog egg; with the chick egg? Do any processes take place in the unfertilized egg similar to those in the fertilized egg?

6. **CLEAVAGE.** Watch for the appearance of a groove in the surface of the blastodisc - the indication of the first cleavage plane. This usually occurs from two to three hours after fertilization. The rate of development varies with the temperature. Note the geometric and time relations of the subsequent cleavages. Do the cleavage planes divide the entire ovum? The entire blastodisc? During interkinesis the nuclei are sometimes visible. Distinguish between central and marginal cells. Follow cleavage carefully to the 32 cell stage. Note irregularities. When do horizontal cleavage planes first appear? Does the blastodisc increase in size or alter in form?

    Note that cleavages continue for a considerable period without much change in form from that of the original blastodisc. This is called the period of the high blastula (See Oppenheimer '36 for chronological terms). When does the change of form to the "flat" blastula occur?

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

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

8. **THE GERM RING AND THE EXTENSION OF THE BLASTODISC.** (18 to 48 hrs) subsequently to the nucleation of the periblast note the change in form and size of the blastoderm (blastula). Soon the margin of the disc appears relatively thicker (germ ring) due both to an actual thickening and to the thinning of the central part of the disc. The germ ring can best be observed in F. majalis. During the next few hours the germ ring grows completely over the surface of the yolk mass. The uncovered portion of the egg is the blastopore.
The final covering of the yolk or the closure of the blastopore occurs after the first stages of the formation of the embryo. Under favorable conditions the beginning of gastrulation may be observed in the appearance of a slight indentation at the edge of germ ring at a time when the yolk is about 1/4 covered. Staining with neutral red may help in identification of germ ring. (Use 1 or 2 drops to a sycaruse dish of sea water.)

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

   a. While the germ ring is extending around the yolk, the formation of the embryo takes place. Its first indication is a cellular thickening known as the embryonic shield, caused by a more active movement of cells in one part of the germ ring.

   This formation is initiated when the blastoderm has covered from one quarter to one third of the surface of the yolk. By the time that the blastoderm has covered about one half of the yolk the embryonic shield has become a bluntly triangular area extending from the margin of one portion of the blastoderm to near the center of the blastoderm. The embryonic shield can most readily be identified when seen in profile. As the blastoderm spreads over the surface of the yolk the embryo grows rapidly in length.

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

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

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

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

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

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

   b. The brain and sense organs. Find in early stages the optic vesicles, lens, neuromeres, mid-fore- and hind brain regions and trace the development into cerebrum, optic lobes, cerebellum, medulla, etc. (Drawings at twenty-four hour intervals will give a good record of development of parts.) Study the development of the eye, olfactory pit and otocyst. How is the lens of the eye formed?
c. The circulatory system: Note extra embryonic body cavity; formation of pericardium; first blood vessels; (and especially their mode of formation from wandering mesenchym cells); first action of the heart; form and position of heart. Compare course of circulation on 4th and 6th days. Illustrated by diagrams.

d. Mesenchymal cells. Note wandering mesenchymal cells especially abundant beneath posterior end of embryo on 2nd and 3rd days. Can you distinguish various types? By successive observations at brief intervals the change in form, migration and division of these cells may be noted.

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

e. Note first appearance of fins, of the urinary vesicle or bladder (a bilobed outgrowth of the hind gut), the liver posterior to left fin bud.

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

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

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

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

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

### I. General Reference Works.

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<tr>
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<tr>
<td>Brachet, A.</td>
<td>1935</td>
<td>Traite d'embryologie des vertébrés, 2nd ed.</td>
</tr>
<tr>
<td>Hertwig, O.</td>
<td>1906</td>
<td>Handbuch der vergleichenden und experimentellen Entwicklungslehre der Wirbeltierel</td>
</tr>
<tr>
<td>Oppenheimer, J. M.</td>
<td>1936</td>
<td>Historical introduction to the study of teleostean development. Osiris, 2, 1936.</td>
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### II. SPECIAL REFERENCES ON MORPHOLOGY OF DEVELOPMENT

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<tr>
<th>Author(s)</th>
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<tr>
<td>&amp; Whitman, C.O.</td>
<td>1891</td>
<td>Some points in the development of the toadfish. Jour. Morph. 5</td>
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<tr>
<td>Clapp, C. M.</td>
<td>1899</td>
<td>The lateral line system of Batrachus tau. Jour. Morph. 15.</td>
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<tr>
<td>Cunningham, J. T.</td>
<td>1885</td>
<td>On the relations of the yolk to the gastrula in Teleostean and in other Vertebrate types. Q.J.M.S.6.</td>
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<tr>
<td>Wilson, H.V.</td>
<td>1891</td>
<td>The Embryology of the Sea Bass (Serranus atrarius) Bull. U.S. Fish Com. 9.</td>
</tr>
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</table>
III. REPRODUCTIVE CYCLE AND BREEDING HABITS


IV. CIRCULATORY SYSTEM


V. EXPERIMENTAL WORK.


Clapp, C. M. 1898. The relation of the axis of the embryo to the first cleavage plane. Biol. Lect. M.B.L.


Hybridization Experiments with Fundulus heteroclitus

Fundulus heteroclitus may be readily crossed with Fundulus majalis and the mackerel (Scomber scombrus). Embryos can be gotten from reciprocal crosses of F. heteroclitus x F. majalis which live until about the time of hatching. With the mackerel, however only one combination is viable, Fundulus ♀ x Scomber ♂, and these may develop to advanced embryonic stages.

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

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

References

Bancroft, F.W.

Jodrich, H.B.

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


Perrill, Alice
Cyclopa and other abnormalities of the head may be obtained by treating Fundulus embryos with alcohol early in development (Stockard '10). Eye abnormalities alone can be obtained by treatment with MgCl₂.

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

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

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

References

Adelmann, H. B. (Good Bibliography)
1936. The problem of cyclopia.

Stockard, C. R.
1921. Developmental rate and structural expression. Amer. J. Anat. 28. (Good Bibliography)

Child, C. M.
1941 Patterns and Problems of Development. pp. 270, 287.
EMBRYOLOGY OF THE SQUID

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

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

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

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 is closely applied to it.

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

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

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

Make a drawing of the egg and the chorion.

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

II. FERTILIZATION AND MATURATION

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

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

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

1. Penetration of a sperm through the micropyle.

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

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

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

III

CLEAVAGE

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

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

IV. Time Table of Development.

There is considerable variation due to temperature differences, and the following schedule gives a rough approximation of the time at which certain stages are reached.
Time after fertilization  

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

V.  

**Spreading of the Blastoderm.**

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

VI.  

**Organ Formation.**

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

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

1) **Shell gland at dorsal pole.**

2) **Mantle primordium, an ectodermal concentric fold beneath the shell gland.** (fig. 2).
On the anterior side:

3) Mouth
4) Eye primordia - ectodermal invaginations

On the posterior side:

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

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

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

Observe:

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

On the posterior side:

3) The formation of the siphon by concrescence of the anterior siphonal folds. The posterior siphonal folds continue as ridges to the anterior side.
4) The anus, between the gill primordia.

Note the further growth of other primordia; contractions of the yolk sac, etc.

Old stages (before hatching; Chart figs. 5 and 6)

1) The eye stalks are very prominent. They contain the primordia of the optic and cerebral ganglia, the so-called "white bodies", also a central mass of yolk.
2) The lens. The inner sector which is formed by the outer part of the optic vesicle (different from Vertebrates) is clearly visible as a club shaped rod extending into the eye vesicle.
The mantle has overgrown the anus and gills. It is contractile. In oldest stages it is beset with Chromato.hores. Note different types and colors: observe their contraction and expansion. They are equipped with muscle fibers and are innervated.

The statocysts lie close together.

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

The branchial hearts will be found at the basis of the gills, and the systemic heart between them. All three pulsate.

The rectum and ink bag.

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

Observe the locomotion of an old embryo after it has hatched.

VII

The Spermato.hore.

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

Study the intact spermato.hore.

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

2) Rinse with distilled water several minutes.

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

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

OBSERVATIONS

1) The opaque sperm mass in the center.

2) The flask-shaped cement body in front of it.

3) The spiral filament in front of the cement body.
4) The outer tunic is the outermost layer of the entire envelope.

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

6) The middle tunic may be slightly swollen.

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

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

9) The inner membrane is a very delicate structure between middle membrane and spiral filament.

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

The evaginated inner tunic and outer membrane will form the sperm reservoir after the explosion. The sperm reservoir is closed at one end by cement from the cement body and open at the other end (see under 10). The sperm, mixed with a gelatinous mass, will ooze out slowly in a cloud; this will continue for hours or days. All other structures are left behind after explosion.
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W. K. Brooks</td>
<td>1880</td>
<td>Development of the Squid. Memoirs Boston Soc. of Nat. Hist. (Good figures of all stages)</td>
</tr>
<tr>
<td></td>
<td>1919</td>
<td>II. The spermatophore; its structure, ejaculation and formation. J. of Morphology, vol. 32. (Both papers are recommend for collateral reading; consult figures in #II for details of the structure of the spermatophore).</td>
</tr>
<tr>
<td>E. Korschelt</td>
<td>1892</td>
<td>Entwicklung des Darmkanals und Nervensystems der Cephalopoden. Festschrift für Leuckart (good figs. of development of intestine)</td>
</tr>
<tr>
<td>E. Korschelt</td>
<td>1936</td>
<td>Vergleichende Entwicklungsgeschichte der Tiere vol. 2 pp. 968-1009. (Contains Bibliography)</td>
</tr>
<tr>
<td>A. Naef</td>
<td>1928</td>
<td>Die Cephalopoden, vol. 2; Embryologie. Fauna e Flora del Golfo di Napoli. (Complete series of figures of development of Loligo on plates I-VII; seriation of stages Consult particularly plate 7, figs. 4 and 4a for newly hatched squid. Note that Naef describes the Mediterranean species, L.vulgaris, which develops more slowly than L. P.)</td>
</tr>
<tr>
<td>S. Ranzi</td>
<td>1931</td>
<td>Sviluppo di parti isolate di embrioni di Cefalopodi, Pubbl. della Stazione Zool. di Napoli, vol. II.</td>
</tr>
<tr>
<td></td>
<td>1931</td>
<td>Duplicitas cruciata in embrioni di Cefalopodi ibid. vol. II.</td>
</tr>
<tr>
<td>S. Watase</td>
<td>1891</td>
<td>Studies on Cephalopods. I. Cleavage. J. of Morphology vol. 4. (See figures)</td>
</tr>
</tbody>
</table>
FERTILIZATION

Use only the pipettes at the stock dishes to obtain gametes. Carelessness will result in contaminating the stock of unfertilized eggs with spermatozoa. The stock dish of eggs will be kept at the front of the room, the stock dish of spermatozoa, at the rear. Care must be taken to avoid inseminating heavily. Too many spermatozoa often cause polyspermy which results in abnormal cleavage and development. Polyspermic eggs of some forms develop more rapidly than normally fertilized eggs. The polyspermic eggs of Nereis, however, usually fail to cleave.

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

Nereis

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

After becoming familiar with the unfertilized egg, inseminate by adding a drop of freshly prepared sperm suspension. Stir the egg at once by a circular movement of the dish and observe changes. The first changes will begin a few seconds after insemination. At 21°C, the following schedule applies to 50% of a population of eggs (time after fertilization). 1st. polar body, 45 minutes; 2nd polar body, 60 minutes; 1st cleavage 95 minutes (ref. #22). The laboratory will probably be warmer than 21°C, so that development will proceed more rapidly. There are many changes in the egg to observe before the first polar body forms. Observe and record the following: laboratory temperature, time of breakdown of the germinal vesicle, time of final penetration of sperm head through membrane, time of first polar body formation, time of second polar body formation, time of cleavage. The time schedule in the descriptive text below should hold approximately if the air temperature of the laboratory is about 24°C.
If possible, arrange to observe two or more eggs which are touching immediately after fertilization. 2 - 3 minutes after fertilization they will begin to be pushed apart by transparent jelly secreted by the eggs external to the vitelline membrane. By 20 minutes, the zone of jelly around each egg will be as wide as the egg diameter. The margin of the jelly can often be made out by observing super-numerary spermatozoa and other particles at the edge of the jelly. 5 or 6 minutes after fertilization the vitelline (fertilization) membrane will be noticeable due to the formation of the narrow perivitelline space upon jelly extrusion. At 7 or 8 minutes, the entrance cone begins visibly to form. Find an egg showing a profile view of the entrance cone and the sperm which is to enter. From 3 to 12 minutes or longer, the sperm is clearly visible outside the vitelline (fertilization) membrane at the tip of the conspicuous entrance cone. In the course of the next 8 to 10 minutes, the vitelline membrane is indented slightly at its point of contact with the entrance cone. This tends to obscure the sperm from view to some extent, and at about 20 minutes after fertilization the egg wrinkles, becoming distorted and quite irregular in somewhat amoeboid fashion. The entrance cone has already flattened considerably, but is still present, and although the sperm is partially obscured from view, the entrance of its head into the egg is not completed until some time later (ref. #12, 15, 16). The final penetration of the sperm head thru the membrane, leaving the middle piece and tail outside, may also be observed. At about 30 minutes, the egg rounds up again, but as the time approaches for 1st polar body formation, the egg elongates in a direction perpendicular to the polar axis. If no eggs lie so that the forming polar body is on the horizon, the dish should be shaken. The 1st polar body may form at about 36-40 minutes, and it lies in the space between the egg and the vitelline membrane. This space is wider in the region of the animal pole than elsewhere. The second polar body often forms at about 50 minutes and commonly does so immediately under the first polar body, which is thus lifted into the perivitelline space. At perhaps 80 minutes the eggs will begin to divide into two unequal blastomeres. Observe 2nd and 3rd cleavages also, if time permits. The 3rd division, from 4 to 8 cells, produces 4 micromeres by spiral cleavage (ref. #24).

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

**Nereis: Exaggerated Entrance Cones:**

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

Nereis: Centrifuged Eggs:

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

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

Breeding habits of Nereis limbata

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

CHAETOPTERUS:

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

When the egg is taken from the female it contains a large immature nucleus (germinal vesicle), as does the Nereis egg, but unlike the egg of Nereis, it spontaneously undergoes partial matura tion when placed in sea water, even if not fertilized. A number of species of eggs partly mature when they enter sea water and Pasteel (ref. #21) has shown that this is dependent upon the presence of Calcium in the sea water.
Chaopterus eggs develop quite rapidly. If eggs are fertilized just after the partial maturation in seawater has been completed, they develop as rapidly as eggs inseminated 12-15 minutes earlier when first placed in sea water (ref. #2) (time counted from fertilization): 1st polar body 14.5 minutes; 2nd polar body, 27 minutes; "pear" shaped stage, 46 minutes; polar lobe bulge, 52 minutes; cleavage with polar lobe attached, 53 minutes; completed cleavage with polar lobe resorbed into one blastomere, 62 minutes; 4 cell stage, 32 minutes. If the laboratory air temperature is about 24°C, development will be more rapid, and about at the rate indicated in the descriptive text below.

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

If eggs are now lightly inseminated and stirred, a few sperm may be seen adhering to the egg almost immediately. Within 5 or 6 minutes, the vitelline membrane may be seen to separate from the egg surface, after which time it is called the fertilization membrane. It is not conspicuous and does not elevate much above the egg surface. In 10 or 12 minutes, the eggs, which had become almost spherical after fertilization, are seen to elongate in an axis perpendicular to the polar axis. This is preparatory to formation of the 1st polar lobe at about 12 minutes. In this division the egg thus assumes approximately the shape of a blastomere, although the polar body is a vestigial cell. After the 1st polar body forms, the egg again rounds up (13-19 minutes), but it elongates again in the same manner to form the 2nd polar body at perhaps 23 minutes. The 2nd polar lobe often forms under the first, which is thus pushed away from the surface of the egg. The egg again rounds up (26-30 minutes). The egg pronucleus may now sometimes be seen migrating toward the center of the egg, and occasionally the sperm pronucleus may also be noted. At 30 minutes, the clear zone has extended from the polar region to the equator of the egg, and at 37-40 minutes a typical "cleavage" stage is reached. The polar bodies lie at a position adjacent to where the stem attaches to the pear, and the bulge of the egg for the polar lobe begins to widen suddenly at the anti-polar end of the egg (40-43 minutes). When the polar lobe is fully developed, however, it corresponds to the stem end of the "pear", and the polar bodies are opposite.

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

CONCENTRATION AND ACTIVITY OF SPERMATOZOA:

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

CUMINGIA:

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

Special Projects:

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

1. Fertilization of Platynereis. Collect Platynereis from the
Carradatta Wharf floating stage at the right phase of the moon. Com-
pare L. E. Just's papers on breeding habits and fertilization. Com-
pare your observations with those on Nereis limbata.

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

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


Further references relating to laboratory work:


Lunar Periodicity

SPECIFIC INTERACTING SUBSTANCES OF EGGS AND SPERM

Fertilizin, a substance which may be obtained from the eggs of certain marine invertebrates, has the property of agglutinating specific spermatozoa. Another substance, called antifertilizin since it neutralizes the sperm agglutinating property of fertilizin may be obtained from sperm. This sperm antifertilizin appears to be identical with an egg antifertilizin which, in the case of the sea urchin, may be obtained from jellyless eggs. It has been shown (Tyler, 1941) that these substances are an aid to fertilization. Whether or not they play an essential role in this reaction is uncertain.

Preparation of fertilizin from Arbacia eggs.

Prepare a concentrated suspension of washed Arbacia eggs in a nyrosue dish or finger bowl. The suspension should contain 25-50% eggs by volume. Cover the dish and set it aside for an hour or more then test it for fertilizin. In the meantime, proceed with the experiments below, using the fertilizin supplied by the instructor. This sample of fertilizin was prepared by extracting eggs in acid sea water.

Properties of fertilizin.

Mix two or three drops of 2% (1 drop dry sperm in 10 c.c. sea water). Arbacia sperm suspension with an equal volume of fertilizin. At the same time mix equal volumes of the sperm suspension and sea water. Throughout these experiments it will be absolutely essential to prepare accurate controls for every test. Note microscopically the sperm agglutination reaction and its reversal. Repeat the test and observe microscopically. What other effect upon sperm does fertilizin have upon sperm?

Effect of fertilizin upon reversed sperm.

Mix two drops of sperm with two drops of fertilizin. After reversal of agglutination add a third drop of fertilizin to reversed sperm and at the same time add one drop of fertilizin to sea water control. If the reversed sperm agglutinates, repeat the experiment using more fertilizin in the initial treatment.

Absorption of fertilizin by sperm.

Add 5 c.c. of concentrated (25-50%) sperm suspension to 5 c.c of fertilizin solution, mix well in a 15 c.c. centrifuge tube. At the same time prepare a control (5 c.c. fertilizin + 5 c.c. S.W.). Set the two tubes aside for an hour or more, then centrifuge down the sperm and test the supernatant fluid and the control for fertilizin.

Arbacia sperm antifertilizin.

Antifertilizin is prepared by cytolizing spermatozoa and removing the insoluble material from the suspending medium. Heat a
few c.c. of 25% sperm to 100°C. in a water bath, and filter off, or centrifuge down the insoluble sperm mass (method of Frank). Since antifertilizin is heat labile a more satisfactory method of preparation involves breaking up the cells by the freezing-thawing method. If dry ice is available freeze a few c.c. of a 25% sperm suspension, set aside to thaw, then remove the insoluble sperm mass from the fluid by filtration or centrifugation.

Effect of antifertilizin on egg jelly and fertilizin.

Mix two drops of antifertilizin with two drops of freshly washed eggs. Examine microscopically for egg agglutination, and microscopically for egg jelly membranes. Test the fertilizin neutralizing property of antifertilizin by mixing a drop of fertilizin and one of antifertilizin and after allowing time for neutralization add test sperm suspension. Compare with appropriate control. If neutralization of fertilizin is not evident, repeat, using a larger proportion of antifertilizin. Repeat these experiments, using egg antifertilizin supplied by the instructor.

"Univalent" Arbacia fertilizin.

Sea urchin fertilizin may be inactivated by heat, enzymatic digestion, x-radiation and ultra violet irradiation. Such inactive fertilizin, although it will not agglutinate sperm, will nevertheless render sperm non-agglutinable by normal untreated fertilizin. Sperm treated with such inactive, "univalent" fertilizin is thus equivalent to "reversed" sperm. A quartz mercury arc will be set up in the laboratory. Caution: do not expose the eyes to the irradiation of the tube. Test samples of fertilizin which have been exposed to the ultra violet light, and their unirradiated controls for sperm agglutination. Test the irradiated fertilizin for inhibition of sperm agglutination by untreated fertilizin. (Add untreated fertilizin to sperm which have been previously treated with irradiated fertilizin.)

Effect of Nereis fertilizin on specific sperm.

If Nereis are available, prepare a few c.c. of fertilizin solution from eggs following the procedure outlined for Arbacia. Make up a dilute (1-2%) active Nereis sperm suspension. Nereis sperm are very sensitive to CO2, therefore it is essential that test sperm suspensions be very fresh. Note the striking autoagglutination reaction of fresh active sperm in dilute suspension. Mix such active sperm with the fertilizin. Do the agglutinates break up spontaneously as in the case of Arbacia? Can they be broken up by mechanical agitation? Compare fertilizin agglutination with the autoagglutination in this respect.

Fertilizin of Asterias.

Asterias spermatozoa in the concentration usually employed for agglutination tests are only weakly active and do not react appreciably to fertilizin. By special treatment the sperm may be made to react with fertilizin. Mix a drop of isotonic hen's egg white with two drops of 1-2% Asterias sperm. Note the activity of the sperm.
Add a drop of Asterias fertilizin. Is the resulting agglutination permanent or spontaneously reversible? Note the type of clumps formed.

References


Just, E.E., 1930. The present status of the fertilizin theory of fertilization. Protoplasma, 10: 300-342


Tyler, A., 1941. The role of fertilizin in the fertilization of eggs of the sea urchin and other animals. Biol. Bull., 81: 190-204

Echinoderm Embryology

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

Material

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.

Ripe individuals of the starfish 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 to avoid contamination of eggs and sperm. The same precautions should be used with Echinarchnium and Arbacia as well.

In Echinoderms, 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. Rinse well in running sea-water from the tap. 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 "dry". When eggs cease to flow from the female gonad, the fleshy part should be removed and the ovary 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 purple in color) as described above for the starfish. C) sea urchin: after washing the animals, puncture the soft tissue at the peristome with a sharp pair of scissors and cut around the equator exposing the gonads. The sex glands of the male are grayish-white; those of the female are
maroon-red. After rinsing 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 them in a finger bowl full of sea-water. After approximately five minutes strain through cheese cloth and 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 cytoplasm of the egg 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? 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 pva are not mature. Examine eggs from each of the forms. These 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. Record by means of sketches the changes observed. 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 Echinarachnius 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 inseminations. This may be assured by the following method.
Add one drop of 'dry' sperm to 25 cc. of sea-water. To a mass of eggs in approximately 50 cc. of sea water, add 6 to 7 drops of this suspension. This concentration is sufficient to ensure fertilization of all of the mature ova in a good batch of eggs. As soon as the sperm is added the water containing the eggs should be agitated in order that the sperm may be as evenly distributed as possible. Place some of the eggs on a slide immediately after removal and observe the changes which take place in the eggs. Note the gross changes here. The penetration of the sperm and the elevation of the membrane may be better observed by another method of insemination. Mount a few of the eggs in India ink and observe. Make sketches.

The activity of the spermatozoa and the rapidity with which the sperm enters the egg and the accompanying changes take place varies directly with the temperature of the medium and hence the gametes. For this reason it is desirable to study these changes in preparations which have been chilled to such an extent that it takes place slowly. Make preparations as follows. Chill eggs and sperm in sea-water by the use of an ice bath. Mount a few of the eggs on a slide under a cover glass and add a little of the sperm suspension at one side. Observe constantly until sperm appears 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 cc. 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, A, B, C.

One result of completed 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? Compare the rate of cleavage in the three types of eggs. 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
5. Gastrulation. A. B. C.

Gastrulation is foreshadowed in the blastula by a flattening of one of the poles (Which?). The cavity of the blastula or segmentation cavity may now be called a blastocoel. Do you find any loose cells within this cavity after invagination is initiated? What are they? Where do they come from? What cells are concerned in the invagination? The secondary cavity formed by the invagination is the gastrocoel or archenteron (cultures of from 24 hrs. to 120 hrs.). Note the two lateral diverticae 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. Organogeny 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 stomodaeum. 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 Echinarachnius. 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.
1. Exogastrulation in Arbacia

It was discovered by Forrest (1892) that when the early stages in sea-urchin development were submitted to solutions of lithium the gut often failed to invaginate and the blastula formed took on the characteristics of endoderm. Since this time numerous agents have been used to produce this effect in echinoderms and some other forms.

All stages in exogastrulation and entodermalization can be obtained in Arbacia by submitting the fertilized eggs in the one-cell stage to 10 cc. of 0.54 M LiCl plus 1.0 cc of sea water.

Place 2-3 drops of concentrated eggs in the lithium solution. Examine at the end of 10-12 hours and compare with controls in normal sea water from the original batch of eggs. Remove a portion of the eggs from the lithium solution to sea water using care to transfer as little as possible of the lithium solution.

At end of 24 hours again examine the larvae in the lithium solution and transfer a portion to sea water. Compare with controls. Examine also those larvae that have been transferred to sea water 12 hours previously.

At 10-12 hour intervals for the next 72 hours examine and make drawings of the lithium, larvae and controls. Note carefully the effects of initial and continuous exposure to lithium. Sketch all degrees of entodermalization and recovery. What happens to the apical region of the larvae?

References.


Note: For complete lists of references see Lindahl and Child.
CENTRIFUGATION AND MEROGONY IN ARBACIA

By enormously increasing the gravitational forces it is possible to separate more or less completely the contents of the egg of Arbacia into layers of substances according to their specific gravities. The layers in the sea urchin egg, after strong centrifugation, are from light to heavy: (1) oil, (2) homogeneous watery layer with high protein content, (3) mitochondria, (4) yolk, (5) pigment. The nucleus is thrown into the lighter regions near the centripetal end. Continued centrifugation will cause the egg to break into hyaline and pigmented portions. The pigmented portion, although without a nucleus can be fertilized and will develop with only the sperm nucleus. This procedure is known as merogony. Also uninucleated fragments can be stimulated to cleave through the use of parthenogenetic agents.

Examine the centrifuge and understand its mechanisms. Fill the small centrifuge tube with 0.95 M sucrose. Now with a finely drawn pipette add a quantity of unfertilized Arbacia eggs to the sucrose solution and insert firmly in the arms of the centrifuge. Centrifuge at high speed for 10 minutes. Remove the sugar and suspended eggs to a syracuse watch dish of sea water. Wash with sea water by drawing off the sugar solution and adding sea water. Note the appearance of the centrifuged eggs and egg fragments under the microscope. Draw eggs of various shapes and various fragments. Eggs do not always separate exactly at the center so the fragments will contain various proportions of the different substances.

Add a few drops of dilute sperm suspension to the stratified eggs and fragments. Note the time and regions of membrane elevation. Watch the cleavage pattern. Study and sketch the developing fragments in various stages. •

If time permits artificial parthenogenesis may be attempted on the stratified eggs and egg fragments.

References


Note: Use above for other references on centrifugation and merogony.
3. ARTIFICIAL PARthenogenesis IN ARBACIA

The fact that many eggs can be stimulated to develop without
the sperm has attracted the attention of many investigators. One
of the most important of the earlier workers was Jacques Loeb whose
formulae for the treatment of eggs to initiate development are in
current use.

In order to dilute the activating solutions as little as
possible "dry" unfertilized eggs should be used. They may be
obtained in the following manner: Wash the sea urchin thoroughly
under the fresh water tap. Also see that your hands and instru-
ments are well rinsed in fresh water. With your scissors cut car-
fully around the test just outside the peristome using care not to
injure the ovaries. Remove peristome and lantern and pour out any
coeilocomic fluid. Place the animal on its spine on the aboral sur-
face in a clean finger bowl. If ripe, eggs will be shed through
the gonopores in a concentrated mass and free of perisomic fluid.

Single treatment. Place a drop of dry eggs in the bottom of
a watch dish and flood with 2.5 N NaCl. Watch the second hand
of a clock and at 10 second intervals remove portion of the
eggs to 250 cc. of normal sea water. Six different exposure
periods should be adequate. Check carefully the exposure period
best suited for membrane elevation without cytolysis. Vary the
exposure time by 2 seconds intervals on either side of this period
for 100 % membrane elevation and cleavage.

Double treatment. Place a drop of dry eggs in the bottom of
a watch dish and flood with a solution of 2.8 cc of 0.1 N butyric
acid plus 50 cc. of sea water. Remove eggs from this solution to
sea water at 20, 30, 40, and 50 minute intervals. After 15 min-
utes in sea water remove the eggs to a solution of 8.0 cc of 2.5N
NaCl plus 50 cc. of sea water. After 20 minutes in this hypertonic
solution return to sea water. Watch for membrane elevation and
cleavage. How far will larvae obtained by parthenogenetic treat-
ment develop?

References.

Heilbrunn, L. V. 1915. Studies in artificial parthenogenesis. II.

Just, E. E. 1922. Initiation of development in the egg of Arbacia.
1. Effect of hypertonic sea-water in producing membrane

1939. Basic Methods for Experiments of Eggs on Marine

Loeb, J. 1913. Artificial parthenogenesis and Fertilization.


Note: Use above for additional references.
4. Fertilization: (Arbacia recommended.)

2. Concentration of spermatozoa.

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

b. 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 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.

c. Cross-fertilization.

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

d. Effect of agents in solution on fertilization.

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

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

a. 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 5th) take place in several batches of eggs. Make records of as many temperatures as possible and compute the temperature coefficient for the process.

b. Effect of osmotic pressure on development.

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

c. Modification of cleavage pattern.

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

6. DEVELOPMENT OF ISOLATED BLASTOMERES (ARBACIA).

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

7. DEVELOPMENT OF FUSED EGGS.

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

\[
\begin{align*}
45 \text{ cc.} & \quad \text{sea H_2O} & 55 \text{ cc.} & \quad \text{m/2 NaCl}_2 \\
30 \text{ cc.} & \quad " & 70 \text{ cc.} & \quad " \\
20 \text{ cc.} & \quad " & 80 \text{ cc.} & \quad "
\end{align*}
\]

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.

6. ECTODERMIZATION OF ECHINODERM EGGS.

Remove the ripe eggs from a sea urchin or sand dollar to sea water using all possible care to prevent insemination. Wash with several changes of sea water and test a few for fertility. Now using a medicine dropper and with a minimum quantity of sea water transfer approximately 200 of the unfertilized eggs to the following solutions in clean finger bowls:

(a) Normal sea water.
(b) Calcium-free sea water.
(c) 20 cc. of 0.54 M NaCNS plus 100 cc. sea water.
(d) 20 cc. of 0.54 M NaCNS plus 100 cc. Ca-free sea water.

After 12 hours in these solutions run the eggs through several changes of sea water and fertilize in sea water with a drop of dilute sperm suspension. Examine for fertilization membranes. Cover the finger bowls and, if possible, leave on a water table.

Examine the developing larvae under the microscope in depression slides at 12-hour intervals following fertilization. Compare the controls and tests. What is the effect of the thiocyanate on viability, cleavage, gastrulation, etc.? Explain. What proportion of the larvae show the effects of the agent? Sketch representatives of the cultures at each observation.

9. ENTOCERMIIZATION OF ECHINODERM EGGS.

Place 200-300 fertilized sea urchin or sand dollar eggs in 200 cc. of M/30 LiCl. (This solution is made up by diluting a 0.54 M solution of LiCl with normal sea water.) Arrange 6 controls in normal sea water. Keep in covered finger bowls on a water table if possible. At the end of 4, 8, and 12 hours examine and remove a portion of the larvae to normal sea water for further development.

Examine the lithium-treated and control larvae at approximately 12-hour intervals for the next three days. What is the effect of early treatment with lithium on invagination? On mesenchyme formation? What happens to the presumptive entoderm? Ectoderm? Explain. Sketch larvae in varying stages of inhibition and recovery.
10. **THE EFFECTS OF CA-FREE SEA WATER ON DEVELOPMENTAL PATTERN**

Place 200-300 fertilized sea urchin or sand dollar eggs in Ca-free sea water (Distilled water, 10,571 cc.; NaCl, 307 gm.; KCl, 8 gm.; MgSO₄, 66 gm.; NaHCO₃, 2 gm.). Run controls in normal sea water. Observe at 4, 8, 12 and 24 hours after fertilization. At each observation remove a portion of the eggs to normal sea water and study at 12-hour intervals. Sketch representative forms.

Note the effects of early treatment with Ca-free sea water on cleavage, formation of mesenchyme, oral lobes, anal arms, symmetry, amount of skeleton, etc. Note the fusion of some of the larvae. What are the effects on fields, patterns, etc. of such fusions. How would you explain the action of Ca-free sea water?

11. **SUSCEPTIBILITY GRADIENTS IN AREACIA AND ASTERIAS**

Activity gradients in the developing organism have indicated a mechanism of correlation and integration which is of a dynamic nature. Regions of greater physiological activity may exert a control (dominance) over regions of lower activity. In uncleaved eggs the animal pole commonly shows a greater rate of activity than the vegetal pole. As development proceeds secondary regions of activity appear probably as a result of physiological isolation from a primary gradient system. Regions of high activity are more susceptible to toxic agents than are regions of lower activity. They die faster and, on death, cytolize or disintegrate thus marking their position.

Place a quantity of unfertilized eggs, blastulae, gastrulae, and plutei or bipennaria in watch dishes. Remove as much of the water as possible and flood with sea water solution of 0.01 M, 0.005 M, and 0.001 M KCN. The watch dishes should be covered. At intervals of 30 minutes observe under the low power of the microscope noting regions of disintegration in the eggs or larvae and the comparative susceptibility of larvae in different stages of development. It is often advisable to follow the process of disintegration through a single egg or larva. Sketches made during the different periods in the disintegrating process are advisable. This experiment may take several hours.

Gradients in reduction of vital dyes parallel the disintegration gradients. Eggs or larvae stained lightly with Janus green (1 part to 100,000 - 200,000 sea-water) for a few minutes and sealed under a coverslip with vaseline will reduce the dye to a brilliant red when the oxygen is diminished. The most active regions, if uninjured by the dye, will become red first. This experiment requires considerable care in staining and is optional. Only clear or lightly pigmented eggs and larvae are suitable.
References


BIBLIOGRAPHY ON
ECHINODERM DEVELOPMENT

I. NORMAL DEVELOPMENT

Goveri, 1901. Th. Die Polaritat von Oocyte, Ei und Larve

Dawydoff, C. Embryologie comparee des Invertébrés. Paris
1928.

Jenkinson, L. W. 1911. On the origin of the polar and
bilateral structure of the egg of the sea urchin. Roux
Arch. vol. 32.

Korschelt, E. 1936. Vergleichende Entwicklungsgeschichte
der Tiere. Fischer Jena.

London. 1914

Wilson, E. B. and Mathews, A. P. 1895. Maturation,

II. METAMORPHOSIS

Bury, H. 1895, Metamorphosis in Echinoderms. Quart.

Hoto, 1938, Metamorphosis of Asterias pallida Journ.

Grave, Caswell, 1900. Embryology of Ophiocoma echinata

Horstadius, Sven, 1926. Entwicklung von Astropecten.

McBride, E. W. 1896-1914. Several papers on development
and metamorphosis of Ophiuridae, Echinidae and Crinidae
in Quart. Micr. SC. (V ol. 38, 42, 51 and 59.)

Mortensen, Th. 1936. Contributions to the study of
development and larval forms of Echinoderms. I and II

III. EXPERIMENTAL

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

Schleip, W. 1929. Die Determination der primitiventwick-
lung Leipzig, p. 542.

Some other fundamental papers:
A. **FERTILIZATION**


A general study on the cytological aspects of fertilization may be found in


B. **POLARITY OF EGG AND ORIENTATION OF EMBRYO.**


Morgan, T. H. and Lyon, E. F. 1907. *The relation of the substances of the egg separated by strong centrifugal force, to the location of the embryo."


C. **HYBRID DEVELOPMENT**


von Ubisch, L. Keimblatthimären. 1933. Naturwiss. Vol. 21

D. EXPERIMENTAL EMBRYOLOGY


Hörstadius, Sven. 1939. The mechanics of sea-urchin development, studied by operative methods. Biological Rev. Vol. 14. (This is a comprehensive review in English of Hörstadius' work).


Child, C. M. A contribution to the physiology of exgastrulation in echinoderms, Ibid., 1936b.


Child, C. M. Patterns and Problems of Development. Chicago. 1941.


Cell Lineage
Maturation, Fertilization and Cleavage in Crepidula

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

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

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

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

Details of the formation of the first three quartettes of micromeres and the cells derived from the 1st and 2nd quartettes can be found on slide B. Find examples of the 3, 12, 16, 20, 24, on 25 cell stages. (Slides A + B contain all stages up to 25-cells).

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

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

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

1. Obtaining the material:

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

II. Staining:

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

III. Mounting:

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

Alternative Method.

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

1. Fix for 30-120 minutes in Mayer's Micro-sulphuric.
2. Wash in 35, 50, 70% alcohol. Leave in latter until y. color ceases to come out.
3. 50, 35% alcohol, to water (5 min. each).
4. Stain in Conklin's haematoxylin (1 part Dolefield's to 4-5 volumos dist. water, to which 1 drop of micro-sulphuric fixative is added for each 10 cc. of the diluted stain.) for 5-10 minutes.
5. Wash in water, dehydrate 5 min. in each alcohol; 10 min. in 95%; 2 changes of absolute alcohol; xylol.
6. Mount in thick balsam with supported coverglass.
Cell-lineage References

1. General:

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

2. Special:

Child, C. M. 1900 Arenicola, Sternapsis. Arch. f. Entwickl. 9
Weath, H. 1900 Development of Ischnochiton. G. Fischer, Jena.
Embryology of Annelida

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

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

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

I.

The Trophophore of Hydroides.

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

Observe:

1) Shape of the trophophore.

2) Apical tuft (several long cilia, probably functioning as a sense organ).

3) Apical organ, a thickening of ectoderm at the animal pole; a nerve center and probably the primordium of the cerebral ganglia.

4) The prototroch, an equatorial band of large cilia. In older trophophores, two rows of cilia will be found; a row of short cilia anterior to the large cilia. The prototroch is the most characteristic structure of the larva, and gave it its name. It is always anterior to the mouth (prooral). It consists of a few large prototroch cells which become pigmented in older stages.
5) The metatroch (para troch), a circular band of cilia in the middle of the posttrochal hemisphere.

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

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

8) Two statocysts on the ventral side.

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

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

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

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

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

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

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

Draw lateral and polar views.
II.

Metamorphosis of the Nereis Larva.

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

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

Observe:

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

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

Identify all structures found in A. In addition observe:

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

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

Notice the change of shape and the gradual demarcation of the first 3 segments.
Observe the following new features:

1) Sense hairs at the apical pole.

2) Additional eye spots; number variable.

3) Mesotrochal ciliary bands at the boundaries of the segments.

4) Parapodia with parapodial cirrhi appearing on segments 2 and 3.

5) Tentacular cirrhi on the head.

6) Stomadaeum, stomach, intestine become distinct.

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

D. Late Stages of Metamorphosis. 4 days and older.
(Wilson. Fig. 92)

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

Observe:

1) Frontal antennae, tentacular cirrhi, palpi, all on the head.

2) Anal cirrhi.

3) Two large and several small eyes.

4) Jaws inside of the oesophagus; they can be protruded. The animal is carnivorous and rapacious.

5) Study the finer structure of the setae, and their motility. (muscles at their bases).

III.

The Trochophore of Sabellaria.

This larva shows very long bristles which have probably both a sensory and a protective function. Study trochophores, 3 days and older (consult D. P. Wilson '29, and Novikoff '38).

Observe:

1) Stiff apical cilia in the place of the apical tuft.

2) The prototroch consists of 3 rows of cilia, and shows a gap on the dorsal side.

3) The neurotroch in the midventral line.
4) One eye on the left side. More eye spots develop later.

5) The hood, a fold overhanging the mouth.

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

IV.

Gastrulation and Formation of the Trochophore in Hydroides.

Prepare your own cultures (see p. 1). Gastrulation by invagination occurs approximately 7-10 hrs. after fertilization. Consult the figs. in Shearer ('11) and Hatschek ('86).
<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Child, C. M.</td>
<td>1900</td>
<td>Early development of Arenicola. Roux' Archiv.</td>
</tr>
<tr>
<td>Lillie, F. R.</td>
<td>1902</td>
<td>Differentiation without cleavage in the egg of the annelid Chaetopterus. Roux Archiv I.</td>
</tr>
<tr>
<td></td>
<td>1906</td>
<td>Observations and experiments concerning the elementary phenom. of embryonic development in Chaetopterus (Formative stuff) Jour. Exp. Zool. III.</td>
</tr>
<tr>
<td>Shearer, C.</td>
<td>1911</td>
<td>Development and structure of the trophophore of Hydroides, Quart. J. Micro. Sci. vol. 56.</td>
</tr>
<tr>
<td>Wilson, E. B.</td>
<td>1892</td>
<td>Cell lineage of Nereis. Journ. of Morph., v.6</td>
</tr>
<tr>
<td>Wilson, E. B.</td>
<td>1898</td>
<td>Considerations of cell lineage and ancestral reminiscences. Ann N. Y, Acad. Sci. vol. 11</td>
</tr>
</tbody>
</table>

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Studying the typical Veliger larva of Crepidula fornicatea. Obtain material by breaking the animal from the substrate to which it is attached. You will find the yellowish eggs (enclosed in transparent capsules) attached to the substrate or in the shell of the mother. Tease the embryos out of the capsules. Obtain swimming larvae in different stages, particularly old ones with the yolk resorbed. They are transparent and show the inner organs. Consult Conklin (197) figs. 80-82 and the textbooks of Parker-Haskell vol. I (1928) fig. 557 on p. 734, or MacBride figs. 263 ff. On pages 301 ff, or Korschelt, 1936, vol. 2, pp. 881-891. Study dorsal, ventral and lateral views. Mouth and foot are on the ventral side, the anus is on the right side.

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

II. EARLY STAGES OF THE VELIGER LARVA.

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

Literature:
Lamellibranchia (Pelecypoda)

III. The Veliger Stage.

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

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

1. The general shape of the body with straight hinge line and semicircular shell.
2. The ciliated velum and apical tuft.
3. The digestive tract consisting of oesophagus, stomach and intestine. Is the latter coiled or straight?
4. Position of mouth with reference to the velum. Also position of anus.
5. Ciliation of the digestive tract. How much of it is ciliated? What is the mode of feeding? Add Chinese ink to the water.
6. The primary body cavity is a rather large space seen above and below the digestive tract. It is not a true coelom but perhaps a persistent cleavage cavity.
7. Extending from the hinge line to the velum are several strands of muscle fibres.

IV. The Trochophore Stage.

Study the Trochophore stage of Cumina (12-17 hrs. after fertilization) and its transformation into the Veliger larva. (13-24 hrs. after fertilization).

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

1. Observe the general shape in side and polar views.
2. Position of the band of cilia.
3. Apical tuft?

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

Draw side and polar views in outline, showing ciliation.

1943

BIBLIOGRAPHY

Experimental Embryology of Annelida & Mollusca

TEXTBOOKS

Huxley & de Beer
Morgan, T. H.
Needham, J.
Schleip, W.
Weiss, F.

1942. Biochemistry and Morphogenesis. MacMillan

Special References

Conklin, E. G.
Costello, D. P

1941. Several abstracts on isolation experiments of blastomeres of Nereis. Anot. Rec. V. 78. Suppl., 5 p. 71, 132, 133

Crampton, H. E.
Hoerstadius, Sven.
Lillie, F. R.
Morgan, T. H.

<table>
<thead>
<tr>
<th>Author</th>
<th>Year</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilson, E. B.</td>
<td>1904</td>
<td>Experimental studies in germinal localization. 1. The germ regions in the egg of Dentalium. 2. Experiments on the cleavage mosaic in Patella and Dentalium. J. Exp. Zool., 1</td>
</tr>
<tr>
<td>Yatsu, N.</td>
<td>1904</td>
<td>The development of egg fragments in Annelids. Roux' Arch, f. Entw., 117</td>
</tr>
<tr>
<td>Zeleny, Ch.</td>
<td>1904</td>
<td>Experiments on the localization of developmental factors in the Nemertine egg. J. Exp. Zool., 1</td>
</tr>
</tbody>
</table>
A comprehensive survey of developmental processes in the tunicates would include examination not only of eggs and embryos but also of metamorphosing larvae, various types of vegetative reproduction, and regenerating forms. Because these developmental forms must be constantly related to the structure of larvae and adults, it is necessary that the student should first of all review in a standard text the anatomy and life history of the common types of tunicates.

A. SIMPLE ASCIDIANS

DEVELOPMENT TO THE TADPOLE STAGE.

Styela. (Old name Cynthia)

Conklin's classic description of the development of Styela eggs (1905) provides the morphological background for the experiments (Conklin '31, Tung '34, Daloz '38, Rose '39, Tung, Wu, Tung '41, etc.) which are actively shaping our conception of the organization of Tunicate eggs and embryos. Conklin's figures should certainly be referred to during the following studies.

a. Methods

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

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

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

b. Mature Unfertilized Egg.

Sketch the mature unfertilized egg, which should show the following:

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

(2) Small spherical inner follicle cells ("nurse cells") between chorion and egg itself. They contain yellow granules.

(3) Peripheral layer of egg, a clear layer containing minute yellow granules.

(4) Central part, consisting of gray yolk platelets.

(5) Germinal vesicle, a very large eccentrically placed mass.

C. Post-Fertilization Rearrangements.

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

Carefully watch for the rearrangement of egg substances starting within 2-6 minutes after fertilization. The clear yellowish peripheral matter streams to the lower pole over the yolk, followed by the clear protoplasm from the animal pole. If the yellow pigment is not visible, try a more brightly colored egg. Use only daylight for illumination, and have the diaphragm on the microscope wide open. Roll the egg around by moving the cover slip with a needle. The yellow inner follicle cells may also be migrating; watch the egg cortex.

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

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

D. Cleavage.

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

First cleavage to 2 cells after 40 minutes.
2nd " " " 8 " " 30 "
3rd " " " 16 " " 20 "
4th " " " 32 " " 20 "
5th " " " 54 " " 20 "
6th " " " 112 " " 20 "
7th " " " 218 " " 20 "
8th " " " 218 " " 20 "

To neural plate stage, 2 more hours.
Fully formed tadpole 12 hours after fertilization.
Tunicates

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

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

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

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

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

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


Four cells at the anterior border of the embryo just below the equator (A6.2, A6.4, A6.2 and A6.4), and two just above the equator (a6.5 and a6.5) will give rise to the notocord and neural plate.

All the rest of the cells are ectodermal.

(6) Later cleavage, gastrulation, neurulation. The gastrula passes through disc-shaped, saucer-shaped and cup-shaped stages, starting with the 7th cleavage. As it finally becomes egg-shaped, the gastrula's blastopore, found at the small hind end, becomes T-shaped, the stem of the T bordered by the yellow mesoderm-mesenchym cells.
-4- Tunicates

The cells overhanging the crossbar of the T-shaped blastopore constitute its dorsal lip. They overgrow it, finally engulfing the yellow cells which from then on are only seen dimly through the translucent ectoderm.

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

Molgula.

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

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

Start some Molgula eggs developing, and follow the cleavage through gastrulation. If developing eggs are isolated in a watch glass, tadpoles and young stages of metamorphosis are obtainable. Tadpoles are fully formed in 8 hrs. (Conklin '05a) (Grave '26).

B. COLONIAL ASCIDIANS.

1. AMAROCUUM: TADPOLES, METAMORPHOSIS, EPICARDIAL BUDDING.

(a) Methods.

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

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

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

If colonies are kept in a shrouded aquarium the shedding can be postponed until a more seasonable hour. Swarms of active tadpoles usually appear within fifteen minutes of bringing ripe colonies out of the dark. About a third of the tadpoles will emerge within half an hour, if illuminated first at nine o'clock in the
morning; if the colony is kept dark until mid-afternoon about three-quarters of them will emerge within half an hour. Nearly all of them will commence metamorphosis within an hour of being shed.

b. Early stages.

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

c. Tadpole structure and Behavior.

Watch Ammococium tadpoles swarming in a dish of sea water. What are the reactions to light and gravity? How do they propel themselves? Place a few tadpoles on a slide in a small drop of water and study their anatomy under the microscope. (Grave '20, '21.)

Not all structures can be seen in a living tadpole. Note the thick test with contained test-cells, the adhesive papillae, the atrial siphon (near the tail), the oral siphon (usually larger) and the sensory vesicle between the two siphons. The small pigment cup with lens is a light-perceiving organ, and there is a small round black static organ also within the sensory vesicle. In the tail, note the muscle cells and notocord.

Some Ammococium tadpoles have been fixed in Bouins and stained with borax carmine. In one of these stained tadpoles, identify the pharynx with its visceral clefts, the central yolk mass, the intestines and the nervous system. (Grave '21). Sketch a tadpole, showing structural details seen.

d. Metamorphosis.

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

e. Later Stages.

Observe and sketch metamorphosed Ammococium individuals which have been growing for four days or so after attachment. They are fastened to watch glasses which have been stored in framos under water. Gently flush debris from them at the tap, and avoid tipping off their cover of water. After making your records you will return the specimens, still living in their watch glasses, to the framos they came from.
Tunicates

Examine the specimens under low power first, for orientation. If they are growing upright they may be flattened out by gently lowering a cover glass on them. (If grown on cover slips, turn it over.

At one end of this animal are the atrial and oral siphons, at the other is the post-abdomen, with the heart at its tip. Watch the heart for periodic reversal of beat. Identify the epicardium, a usually pigmented strand of tissue running throughout the post-abdomen from pharynx to heart region. It is an agent in asexual reproduction and colony formation.

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

f. Epicardial Budding.

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

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

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

2. BOTRYLLUS: TADPOLES, METAMORPHOSIS, ATRIAL BUDDING.

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

a) Structure of Tadpole.

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

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

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


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

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

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

a. Week-Old form.

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

The first blastozooid bud in Botryllus is single, all the later ones are in symmetrical pairs. The same organ structures may be seen in all the individuals, notwithstanding their diverse embryology, with the minor exception that the oozooid does not develop gonads. Thru rearrangement of individuals, the completed colony shows a common atrial pit in the center, with separate pharyngeal openings at the periphery.
Tunicates

For the anatomy of the zooids and structure of various stages in bud development compare the living preparation in the watch glasses with fixed-stained-mounted preparations that are available. These were made from a colony that was nearly transparent when living. Most colonies develop so much pigment in the tunic that whole mounts are useless. Each bud consists at first of a disc, then a sphere. The sphere extrudes sex cells at one or both sides and becomes partitioned into three vesicles, the lateral ones forming at real chambers, the middle one the pharynx. Later stages show differentiation of the rest of the organs from the pharynx-vesicle. (Berrill, '41).

3. PEROPHORA: SEPTAL BUDDING.

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

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

Organogenesis takes place in the inner vesicle, which is derived from mesenchyme (Berrill '35). This method of budding is distinguished as the septal type.
1942

BIBLIOGRAPHY

GENERAL WORKS AND COMPARATIVE EMBRYOLOGY OF
PROTOCHORDATES

Conklin, E. G.

Dalq, A.
1938. Form and Causality in Early Development. Cambridge.

Dawydoff, C.

Hardman, E. C.
1924. Botryllus. Liverpool Marine Biological Committee Memoirs, I:

Hardman, W. A.
1899. Ascidia. Liverpool Marine Biological Committee Memoirs, I:

Korschelt, E.

Seeliger, O.

Willey, A.

TUNICATE EMBRYOLOGY

Berrill, N. J.
1931. II. Abbreviation of Development in the Molgulidae. Ibid., 219 (B):281-346.
Chabry, L.

Cohen, A. & Berrill, W. J.

Conklin, E. G.
1931. The development of centrifuged eggs of Ascidians. Ibid., 60:1-120.

Kingsley, J. S.

Kowalevsky, A.

Morgan, T. H.

Rose, S. M.

de Selys-Longchamps et Damas, D.

Tung, Ti-Chow

TUNICATE ASEXUAL REPRODUCTION

Berrill, N. J.


Frien, P.

Caullery, M.

Kowalevsky, A.

Pizon, A.

Ritter, W. E.

de Selys-Longchamps

Spek, J.

TUNICATE REGENERATION, ETC.

Berrill, N. J. and Cohen, A.

Brien, P.
Driessch, H.  

Huxley, J. S.  


TUNICATE TADPOLES AND THEIR METAMORPHOSIS

Grave, C.  


Grave, C. and Ricci, P. A.  

Grave, C. and Riley, G.  

Grave, C. and Goodbridge, E.  

Nowalsky, A.  
1892. Beiträge zur Bildung des Kanteils der Ascidien. Mem. Acad. St. Petersbourg., 38:

Nast, S. O.  

van Beneden, E. and Julin, C.  

CRUSTACEA

Litinia. (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 vermillion 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 some 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. I troplasmic 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 by reflected light under low power after the eggs have been placed in strong MgCl₂ which whitens the embryonic area. Find embryos which show:

a) the 5 embryonic rudiments (examine by reflected light)

b) the nauplius stage with the first 3 pairs of appendages. Are any biramous at this time?

c) later stages having 5 or more pairs of appendages. In these the stomodeum, ventral fold, dorsal shield, toison, and ganglia may be found.

d) the Zoaea stage (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.

Hippa

If Hippa females are available, remove and examine eggs as for Litinia. Cleavage stages are more frequently found for this form.

Balanus eburneus (The Barnacle)

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 sea water and tease out a few eggs with a needle.

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

Study the cleaving egg up to the 16-cell stage. These eggs are delicate; usually the same lot cannot be watched through all of these cleavages. If they die, find other eggs in the stage that you need.
Later stages of interest are: a) gastrula; b) the 3 segment stage; c) the 5 segment stage; d) the nauplius just hatched; e) the nauplius after the first and second moult.

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

**Lepas**

If goose neck barnacles are available, remove the blue egg lamella from the body by breaking open the shell, and observe as for Balanus. During development the blue pigment in the eggs changes thru violet to pink in the nauplii.

**Literature List on Crustacea**


Brooks, Embryology and Metamorphosis of Macroura.

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


Korschelt and Heider, 1899. Textbook of Embryology II.


Müller, F., 1864, Für Darwin.


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

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

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

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

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

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


COELENTÉRATES.

Introduction to Hydrozoa

a) Sensitiveness of the Material.

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

b) Types of Life History Illustrated.

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

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

c) Order of Study

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

Laboratory Procedure

A. Study of Forms with Perfect Medusa

Examples: Bougainvillia, Obelia, Podocoryne.

Characteristic life history: Zygote shed from medusa; Development to plithula larva; Metamorphosis to polyp; Asexual Multiplication of polyps by budding, which produces a colony; Medusae formed by special buds, in a gonosome (Obelia) or separately (Bougainvillia); Shedding of medusae, which mature slowly as separate individuals before forming eggs or sperm.
BOUGAINVILLIA. (June, July, August; not always available in June)

The gonophores are borne singly or in clusters on the main stem and branches, and in this genus develop into complete medusae. The medusa-buds are scattered irregularly throughout the colony, there being no orderly arrangement according to age. Select buds that show various stages of medusa development and mount them under cover slips, and study their unfolding structure. Draw off water from under the cover slip with absorbent paper to produce a slight pressure on the buds. Sketch three stages in medusa development.

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

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

OBELIA (June, July, August)

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

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

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

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

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

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

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

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

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

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

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

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

Sketch the three types of polyps and show several stages in medusa development. Gonads can be made out along the radial canals of the swimming medusae, very immature but sexually distinguishable. The asexual colony gives off either male or female medusae, not both.

**B. Study of Forms with Imperfect Medusae.**

_Examples: Pennaria, Tubularia._

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

**Pennaria.** (July, August, September; begins to ripen middle of July.)

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

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

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

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

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

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

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

**TUBULARIA.** (June, July.)

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

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

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

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

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

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

C. Study of Forms with Degenerate Medusae.

Examples with blastostyle inside gonotheca: Campanularia, Gonothyrea.

Examples with naked gonophores: Hydractinia, Eudendrium.

Life Histories:

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

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

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

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

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

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

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

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

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

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

GONOTHYREA. (July, August).

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

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

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

HYDRACTINIA. (June, July, August)

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

a) Gonosomes and Gonophores:

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

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

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

b) Cleavage and Development.

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

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

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

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

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

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

Illustrations of cleavage in Bunting '94, Beckwith '14; of later planula development in Teissier '27; of developing gonosome in Goette '07 and '16.

**EUDENDRIUM.** (July, August).

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

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

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

Development of Scyphozoa.

AURELIA OR CYANEA. (April - June)

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

Select a number of active planulae and place them in clean watch glasses of sea water for further study on later days. Their gradual change in form, attachment to the bottom, acquisition of tentacles and elongation into the sessile scyphula stage can be easily followed. The resemblance of the scyphula to a simple polyp is obvious. Attachment plus the formation of 2 to 4 tentacles occurs on the second day, as does the development of an open mouth. There are 3 tentacles at 4 days, 16 at 2 weeks, 24 at 1 month. Sketch the scyphula in side view and in top view.

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

Aurelia life history illustrated in the texts of MacBride '16, Korschelt '36 and Hyman '40.
BIBLIOGRAPHY FOR COELENTERATES

MONOGRAPHS, KEYS, SPECIAL WORKS

Brooks, W. K.

Frey, B.

Goette, A.

Hargitt, C. W.

1903. XIV. The Scyphomedusae. Ibid., 37:331-345.


Hargitt, G. T.

Hyman, L. H.

Kingsley, J. S.

Mayer, A. G.

Nutting, C. C.

Sumner, F. G. et al
A. GENERAL EMBRYOLOGY, DESCRIPTIVE

Allen, Carrie M.

Beckwith, Cora J.

Benoit, P.

Bunting, Martha

Doflein, F.

Goette, A.

Grönberg, G.

Hargitt, C. W.


Hargitt, G. T.

Lowe, E.

May, A. J.

Smallwood, W. M.
Smallwood, W. M.

Teissier, G.

Teissier, L. and G.

Wulfert, J.

B. SPECIAL TOPICS - HERMAPHRODITE GONOPHORES, NORMAL AND EXPERIMENTAL

Bunting, Martha (listed above)

Föyn, B.

Goto, S.

Margitt, C. W.

C. SPECIAL TOPICS - SYNCHRONOUS SPAWNING AND ITS RELATION TO LIGHT

Baker, E. G. S.

Ballard, W. W.

Brooks, W. K. (Listed above)
Hargitt, C. W. (Listed above, 1900 and 1904)
Hargitt, G. T. (Listed above, 1909)
Mayer, A. G. (Listed above, 1910)

Murbach, L.

Rittenhouse, S.

Sigerfoos, C. F.

Smallwood, W. M. (Listed above, 1909)

Torrey, H. B.

D. SPECIAL TOPICS - ISOLATION OF BLASTOMERES

Beckwith, Cora J. (Listed above)

Hargitt, C. W.
1900. (Listed above)


Maas.

Teissier, G. (Listed above)

Torrey, H. B. (Listed above, 1907)

Zoja, R.

E. SPECIAL TOPICS - REGENERATION AND AXIAL GRADIENTS

REGENERATION FROM CELLULAR AGGREGATIONS

Beadle, L. G. and Booth, F. A.


Foy, E. B. (Listed above)


REGENERATION FROM NON-DISSOCIATED CELLS


Child, C. M.  1941. Patterns and problems of development. U. of Chicago Press. (Contains references to all Child's papers)


1924. IV. The quantitative relations between current density, orientation and inhibition of regeneration. Ibid., 39: 357-380.

Morgan, T. H.

Morse, M.
1909. The autotomy of the hydranth of Tubularia. Ibid., 16:172-182.

Rössler, H. C.

Reed, Florence

Torrey, H. B.

Zwilling, E.

PAPERS ON SCYPHOZOA

Friedemann, O.

Hargitt, C. W. and Hargitt, G. T.

Hein, W.

Korschelt, E.

MacEride, E. W.

Percival, E.
REGENERATION IN TUBULARIA

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

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

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

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

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

Theoretically Tubularia is a solitary form but practically so many individuals grow together that a dense tangled mass usually results in the older forms. Young short stems are the best for experimental work and can be obtained from floats and rocks in swift current. In general it is best to collect your own stems. Since the stems need running water and a low temperature 18 C they do not keep well in the laboratory. In nature the hydranths drop off about the end of July and the stems remain dormant until the water cools down in the fall. At Woods Hole the stems appear in mid June and can be used until August. However, since the waters of Cape Cod bay on the North shore are much colder, Tubularia may be obtained from the North end of the canal throughout August.

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

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

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

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

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

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

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

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

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

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

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

EXPERIMENTAL CONTROL OF POLARITY

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

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

Select stems about 25 mm in length and cut off the hydranth together with a few mm. of the stem as before. Then cut the remainder into pieces about 5 to 6 mm. long discarding what is left. As these pieces are cut place them in separate dishes labelled distal, middle and proximal thirds. Continue 2 hr. observations as long as possible and take a final observation the second day at 48 hours and average the times, by totalling the total number of hours for each lot and dividing by the total number of stems. If some stems have not yet regenerated include those in the total number as they would regenerate if given time.
REFERENCES TO TUBULARIA (ALSO CORYMORPHA)

Child-1941 Patterns and problems of Development
Pages 313, 333, 345, 359, 372, 378, 413
Also Chapter 8 which is theoretical.

Barth 1940 Biological Reviews 15. Also Biol. Bull. 74;
Physiol. Zool 11; Biol. Bull. 78

Rose and Rose 1941 Physiol. Zool.


