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<th>Name</th>
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<td>Ashman, Robert F.</td>
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<td>Baker, John R.</td>
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<td>Yates, Robert D.</td>
<td>University of Alabama</td>
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**STAFF MEMBERS**

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<tr>
<td>Edda, Mac V., Jr. (in charge of course)</td>
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<td>Grant, Philip</td>
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<td>Robbun, Lionel I.</td>
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<td>Spratt, Nelson T., Jr.</td>
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**ASSISTANTS**

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<td>Fulton, Chandler</td>
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<td>Love, David</td>
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<td>Albert Ashman</td>
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(Sept. 10)

Zoology Department  
Univ. of Connecticut  
Storrs, Connecticut  
(Sept. 1)
WOODS HOLE EMBRYOLOGY COURSE - 1959

LECTURE SCHEDULE
June 16 - July 25

June 16 - 20
Tuesday  
Teleosts I (Saunders)

Wednesday  
Teleosts II (Saunders)

Thursday  
Fertilization; Echinoderms I (Spratt)

Friday  
Echinoderms II (Spratt)

Saturday  
Annelids and Molluscs I (Grant)

June 22 - 27
Monday  
Annelids and Molluscs II (Grant)

Tuesday  
Coelenterates I (Sussman)

Wednesday  
Coelenterates and Ctenophores (Sussman)

Thursday  
Ascidians I (Eda)

Friday  
Ascidians II (Eda)

Saturday  
Sponges I (Rebhun)

June 29 - July 4
Monday  
Sponges II (Rebhun)

Tuesday  
Growth and development: Definition and introduction (Eda)

Wednesday  
Isolation and recombination of blastomeres in relation to egg organization (Spratt)

Thursday  
Cytoplasmic particles in development (Rebhun)

Friday  
Regeneration in invertebrates (Fulton)

Saturday  
No lecture

July 6 - 11
Monday  
Inductive specificity (Saunders)

Tuesday  
Phenotypic variation in microbial systems I (Sussman)

Wednesday  
Phenotypic variation in microbial systems II (Sussman)

Thursday  
Class picnic

Friday  
Nucleo-cytoplasmic relations (Rebhun)

Saturday  
Aspects of protein formation in chick embryos (Heinz Hermann)

July 13 - 18
Monday  
Growth, cell division and DNA synthesis (Grant)

Tuesday  
Development of the cornea (A. J. Coulombre)

Wednesday  
Development of muscle (Love)

Thursday  
Morphogenesis of the vertebrate limb (Saunders)

Friday  
Aspects of plant morphogenesis (T. Stonier)

Saturday  
Enzyme regulation: a model for cell differentiation (Grant)

July 20 - 25
Monday  
Amoeboid movement (R. D. Allen)

Tuesday  
Cell and tissue stability: cellular ecology (Spratt)

Wednesday  
Morphogenesis at the macromolecular level (Eda)

Thursday  
The mammalian blastocyst (Oecilia Luttwak-Mann)

Friday  
Student reports

Saturday  
Student reports
The material available for study and experimentation will be mainly eggs and developmental stages of Fundulus, the killfish or brackish-water minnow. *F. heteroclitus* (and *F. majalis*) will be available throughout the period of study, and possibly other species may come into the laboratory. The egg of Fundulus is a demersal, or non-pelagic egg.

If available, the eggs of the cunner or chogset (*Tautogolabrus adapersus*) will be studied as an example of the pelagic egg type.

Since Fundulus eggs are in better condition and more abundant during June and the early part of July, it is a good plan to commence experiments with them as soon as possible. Cultures of Fundulus of various ages will be available on the central water table at all times.

1. **Equipment** – In addition to the usual items of glassware, the following will be found useful in handling eggs and embryos: hair-loope (available in laboratory) watchmaker’s forceps (available at Supply Dept.; N.B. – Do not allow these to corrode; they must be wiped free of sea-water after each use); depression slides (1.7-1.8 mm. depression). Solutions for preservation of embryos and special reagents for cytochemical techniques will also be available.

11. **Technique of Preparing and Handling Material:**

A. **Fundulus**

1. Although fish are usually brought to the laboratory in mixed lots of males and females it is advisable to segregate the sexes to prevent spawning. Males and females should be placed in separate tanks until needed and after stripping removed to a discard tank. The sexes of both species of Fundulus are easily identified. The mature female, *F. heteroclitus*, is pale olive in color and usually possesses no distinct bars or spots, although the young females have indistinct, dark, transverse bars on the sides. The dorsal fin is non-pigmented. The adult male of this species is a dull, dark-green, the sides bearing narrow, ill-defined transverse bars composed of silvery spots. The dorsal fin possesses black pigment arranged in a mottled pattern. The body markings of *F. majalis* are more conspicuous. The pale olive female has a pattern of heavy black longitudinal stripes on the sides and a non-pigmented dorsal fin. The sides of the slightly darker male bear a dusky broad, dark, transverse bars. The black patch on the dorsal fin is striking.

2. **Procurings Gametes**

Both eggs and sperm are procured by stripping. The fish is held firmly with the left hand while gentle pressure is applied to the abdomen using the thumb and forefinger of the right hand. As these fingers are pushed towards the anus, the pressure forces out the gametes. If the fish is held against the light while stripping, the eggs may be seen passing through the oviduct which runs along the anal fin.

3. **Preparation of Cultures**

Strip eggs into a clean finger bowl which has been moistened with sea water. **+** stand for 5 to 10 minutes. Neither
TELEOST DEVELOPMENT

eggs in 1/4 to 1/2 inch of sea water. Keep bowl covered with glass plate. Do not allow eggs to clump or accumulate in one spot. Label each lot with the exact time of fertilization. Change the water daily.

4. Methods of studying eggs

The eggs should first be studied in the condition in which they are spawned, but for many purposes it is desirable to prepare them for microscopic study as follows; roll the eggs on a piece of filter paper or paper towel until the jelly and the outer fibres are removed, leaving the surface of the chorion smooth and clean. The same procedure should be followed for day-old stock cultures in order to prevent clumping of the eggs.

For experimental work eggs are usually examined uncovered in shallow depression slides or Syracuse dishes and manipulated with hair loops. For laboratory study where eggs are to be observed over long periods of time and specific orientation is desired, either of the following methods is suggested: Place the eggs in sea water in culture slides having a 1.7 to 1.8 mm. depression (slightly less than the diameter of the egg). The egg may now be rotated by moving the cover slip. If these special slides are not available eggs may be placed in a drop of sea water on an ordinary glass slide and covered with a very thin, flexible sheet of mice. Water is then withdrawn with lens paper until capillary attraction causes a pressure on the egg and it may be rotated as in the previous method.

5. Permanent Total Preparations

Fix the eggs in Stockard's solution. This 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.

6. Preparation of Embryos for Sectioning

For fixation and embedding methods, consult instructor.

B. Cunner and other Pelagic Eggs

Whenever possible, observations should be made on pelagic eggs as well as on the demersal eggs of Fundulus. Many structures such as the germ-ring, embryonic shield, and Kupffer's vesicle will be easier to see in the former because they have fewer oil globules and a less-granular protoplasm. The formation of polar bodies may also be seen in this type of egg. Pelagic eggs are far more sensitive, however, in their oxygen requirements, so require careful handling.

Cunners should be caught the same day as needed; females are ordinarily obtained only after 12M. The male has a somewhat brighter green color and can also be distinguished by its bright red cloacal lining epithelium. Milt is stripped into a large finger bowl which contains sufficient sea water to cover the bottom. Eggs are stripped into a separate bowl containing a small amount of sea water. It will prove helpful to use a cloth towel or work-gloves for holding the fish while they are being stripped, because they are extremely slimy and have sharp spines in the dorsal fin.
then dilute with sea water and decant into smaller finger bowls, or pour into a cylinder or Erlenmeyer flask and add sufficient sea water to fill. Good eggs will float on the top and collect principally at the edge of the meniscus. They should be pipetted off and placed in covered finger-bowls containing 1/4" of clean sea water, and set in the sea water table where they will keep cool.

Only glass-clear eggs are suitable for study; if the eggs show the slightest opacity they are either immature or dead. If bits of tissue are clinging to the egg, it is immature and should be discarded. The perivitelline space appears immediately after fertilization; time should not be wasted in observing eggs which do not develop such a space within a few minutes. For observing the formation of polar bodies, the 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 5 to 10 minutes after fertilization as small clear beads on the surface of the blastodisc. Cleavage is rapid, occurring approximately once every 20 minutes at a temperature of 16 to 18°C. The cleaving eggs are crystal-clear; there are no obscuring oil droplets; and the nuclei appear as pinkish objects for a short time between divisions. If neutral red is used for outlining the cleavage spindles, it should be extremely dilute. Too much stain will stop cleavage or make the pattern irregular.

Cunner embryos may be kept alive for several days if care is taken not to crowd them and the water is changed frequently. It is suggested that not more than 3 to 6 eggs be placed in each finger-bowl, that they be transferred to clean sea water morning and night, and that the bowls be covered and kept in the sea water table. Opaque (dead) eggs should always be removed. The embryos usually hatch within 4 or 5 days after fertilization.

111. Observations on Normal Development of Fundulus and Suggestions for Experimental Procedure

The normal development of Fundulus heteroclitus has been described by Oppenheimer (137) and Solberg (138). Reprints of these papers will be available for individual use. The papers of Sumner (104) and Wilson (119) are also highly recommended, for comparative purposes.

Attention is called below to some special features of the various developmental stages, and some suggestions for utilizing them in experimental procedures are included. Because of the variety and interest of the various procedures available, it is suggested that individuals personally observe the results of others' experiments. Individual students may wish to apply procedures of special interest to them to the teleost material available. Special equipment or reagents necessary can be made available to a limited number of the class in such cases.

Laboratory Records - These will very largely be a matter of individual choice. Detailed records should, of course, be kept of all experimental results.

A. Reproductive Cycles, Oogenesis, and Spermatogenesis

Newman (108) has described the spawning behavior and sexual dimorphism in Fundulus. The seasonal cycle in the gonads has been studied by Mathews (138). Details of the histology and histochemistry of the ovary can be found in Guthrie (125, 128), and Marza, Marza, and Guthrie (137). Ovarian and oviducal eggs may be obtained by dissection.
The Gametes and Fertilization

1. Unfertilized Eggs - Strip the eggs from a female into diluted sea water (70% fresh water, 30% sea water). Note the details of egg structure, such as the cortical alveoli, oil drops, membranes, and micropyte. The latter structure must be observed on eggs which have not had the chorionic jelly removed. At first it will be difficult to see, but with practice it can be found after the egg has been properly oriented. Note the presence of immature ova among the mature eggs.

2. Sperm - Examine a drop of sperm suspension under a cover slip with high power. Details of the structure of the testis and seasonal changes in the gland may be found in Mathews ('38).

3. Fertilization - Prepare a culture of fertilized eggs following the procedure outlined in Section II, 4, 3. Record the exact time of insemination (do this for all cultures you prepare). Transfer eggs to a depression slide immediately, and follow the activation processes, including the fading of cortical alveoli, and the formation of the perivitelline space. How is the perivitelline space formed?

After some practice has been gained in the manipulation of the eggs in the depression slides, place some just-extruded eggs in the depression, put the cover slip over them, but allow room to insert a drop of sperm suspension. Locate the micropyte on an egg, and, without moving the cover glass, introduce some sperm and try to observe the entrance of a spermatozoon through the micropyte. Proper illumination is quite important for this observation. Note cortical changes in immediate vicinity of micropyte (Wessels and Swartz '53).

Experimental procedures with gametes.

a) Hybridization - Reciprocal crosses can be made between Fundulus species (particularly F. majalis and F. heteroclitus), and, if available, between Fundulus and other genera. The papers of Bancroft ('12), Morris ('14) and especially Newman (15, 17, 18, etc.) should be consulted. Guthrie ('25) has studied the cytology of cross-activated eggs of Teleosts.

b) Yamamoto ('44) studied the effects of centrifugation on the activation processes in Oryzias, particularly with reference to the formation of 'cortical alveoli' and the perivitelline space. It has been noticed (Forsthoefel, '51; Shaver, '51) that low-speed centrifugation of fertilized, uncleaved eggs will produce duplications, complete or partial, in a low percentage of cases. Moreover, some recent experiments (Trinkaus and Groves, in press) indicate that the position of the egg relative to gravity normally plays an important part in determining the position of the embryonic shield. Centrifugation of eggs at various times after fertilization is a simple and useful procedure for studying the organizational lability of the early embryo.

c) Yamamoto noted the intervention of the Ca ion in the 'cortical breakdown' reaction in Oryzias. The important events following activation and the possible role of Ca ion may be studied with the use of Ca-free sea water.

4. Formation of the Blastodisc: Note the gradual accumulation of the protoplasmic cap. This is the blastodisc or germ disc. Observe this in polar and lateral
views. What is the relation of the egg to gravity? How might the teleost egg be described, with reference to relative location of cytoplasm and deuteroplasm, before and after activation?

5. Cleavage: The first cleavage furrow usually appears on the surface of the blastodisc within 2 or 3 hours after fertilization (depending on the temperature). What type of cleavage is this? Follow carefully the time sequence and geometric relations to the 32 cell stage. Note any irregularities. Do the cleavage planes divide the whole blastodisc? The whole ovum? Distinguish the central and the marginal cells. When do horizontal cleavage planes first occur? For a time, the blastodisc does not appreciably alter its original size and form. This is the period of the high blastula. When does the change of form to the flat blastula occur?

Observe carefully the uncleaved protoplasm around the margins of the blastodisc, and note the difference between circular and radial cleavages. The nuclei of this marginal uncleaved area, called the periblast, are easily visible. The syncytial area at the border of the blastodisc is the marginal periblast; that beneath the blastodisc is the central periblast (notice that the latter is a contradiction in terms). If observed closely it can be seen that the nuclei of the marginal row of cells become free of cell outlines, continue their divisions and migrate into the marginal periblast. Important physical and chemical properties have been attributed to this syncytial layer (Courvier, '41; Lewis, '49; Devillers, '50; and Trinkaus, '51). Consult Oppenheimer ('36a) for details of the cleavage pattern in Fundulus.

Experimental Analysis of Early Development: Certain simple techniques can be utilized on a rather large scale without a great deal of previous experience with the material (a, b, c). Procedures which require manipulative skill and special precautions might be attempted by those willing to spend proportionately greater amounts of time (d, e, f).

a) Sensitive periods in the morphogenesis of various organ systems: By the application of various inhibiting agents at various stages and studying the developmental effects one can often get valuable indications of the course of determination of various organ primordia. Many agents in the past have proved useful in such studies; lithium and magnesium (Stockard, '06, '09), anesthetics such as alcohol and chloroform (Stockard, '10a), and low temperature (Stockard, '21). These experiments may be readily repeated.

b) Axial gradients: Teleost material has proved useful for studies of the relation of gradients of metabolic activity to later differentiation (Child, '45). Using the differential reduction by the blastoderm of various vital dyes one may make suggestive correlations of this sort. What is the significance of these correlations?

c) Relation of respiratory activity to morphogenesis: Enzyme inhibitors with a high degree of specificity are important tools in studies of the relation between metabolic activity and morphogenesis. Do morphogenetic movements of gastrulation depend upon oxidative metabolism, e.g. cytochrome system? Oxygen consumption may be inhibited in Fundulus eggs with Na azide monochloacetate or parachloromercuribenzoate.

d) Germinal localization: Oppenheimer ('36a) has described the technique of vital dye marking as applied to the teleost embryo. A general review of experimental work showing the developmental capacities of the teleost blastoderm will be found
in Oppenheimer ('47). A summary of the procedure for vital staining of fish blastoderms will be found in Rugh, Manual of Experimental Embryology, p. 396. For studies on morphogenetic movements in which greater precision is desired the carbon marking technique is superior. The application of the carbon marking method to the study of surface behavior in the Fundulus blastoderm has been described by Trinkaus ('51), and by Brummett ('54).

a) Analysis of prospective potency: Defect experiments have been described by Nicholas and Oppenheimer ('42). This type of experiment should be tried only after experience in the manipulation of embryos. The most practicable procedure is through the chorion, since dechorionation without injury to the blastoderm takes considerable practice. Destruction of blastomeres in the 2-4 cell stage is probably easiest. Those that wish to attempt dechorionation will find techniques described in Nicholas ('27) and Trinkaus ('51, p. 272-273).

b) The role of yolk in morphogenesis: The effects of yolk removal have been described by Morgan (193). He describes a simple technique, but one which is difficult to control. Explantation of isolated blastoderms has been described by Oppenheimer ('36a), Devillers ('49), Tung, et al. ('45), and Trinkaus and Drake ('55).

6. The Germ Ring and Extension of the Blastodisc (18-48 hours)

After the formation of the flat blastula the margin of the blastodisc begins to thicken, with the formation of the germ ring. The embryo must be manipulated with the hair-loop and observed in a variety of positions to appreciate this process (the germ ring is particularly well seen in F. mazilius). During the next few hours the germ ring spreads over the surface of the yolk mass. What is happening to the periblast during this period? The uncovered portion of the yolk is the blastopore. Staining with neutral red will facilitate observation of the details of germ ring and embryo formation. Add 1 or 2 drops of stock solution to a Syracuse dish of sea-water.

Some of the mechanics of amphioly in the teleost have been considered by Lewis ('49), Devillers ('51b), and Trinkaus ('51). These papers may be consulted for a description of the process and the results of experimental analysis.

The activities of isolated cells and small groups of cells from gastrulation and pregastrulation stages may be studied in vitro after dissociation by trypsin or versene. Instructions for the use of these agents and for culture procedure may be procured from the instructor.

Potency and regulation during gastrulation stages

a) Nicholas and Oppenheimer ('42), Hoadley ('28), and Luther ('36, '37) performed experiments on destruction of various portions of the germ-ring and early neural 'keel'. This type of experiment takes practice and skill, and should be attempted only by those willing to spend time learning this technique. Consult the papers noted above for details. (See Oppenheimer, '47, for complete bibliography.)

b) In connection with the centrifugation technique suggested in 5, above, the formation of the germ-ring in embryos developing from centrifuged eggs should be carefully studied, particularly for the appearance of supernumerary organization centers.
7. Formation of the Embryo: (24–36 hours)

While the blastoderm is spreading over the yolk the embryonic axis is being established, the first indication being the embryonic shield, a thickening caused by a convergence of cells toward one part of the germ-ring.

The morphogenetic movements at this time have been described by Oppenheimer (136) for Fundulus, and in great detail for Salmo, the trout, by Pasteels (136). His (140) review should be consulted for a comparison of gastrulation movements in vertebrates. (See also, Vanderbruck, '36.)

By the time the blastoderm has moved about half way over the yolk, the embryonic shield has become a bluntly triangular area extending from the margin of one portion of the blastoderm to near the centre of the blastoderm. The shield should be studied from all angles, particularly in profile view. Make a series of observations in optical sagittal 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 circumference of the germ-ring?

Several other features will become apparent at this time. The notochord will be seen. Study it in longitudinal and transverse optical section. What are its anterior and posterior points of termination? The somites also become visible. How many of them are there at the time of closure of the blastopore? Kunfér's vesicle, a large clear structure near the hind of the embryo will also be seen at this time.

3. Later Development

Embryos of 2, 3, 4, 5, and 6 days should be studied. Changes in the nervous, circulatory, and other systems can be conveniently recorded in chart form.

Later stages are best studied after removal of the chorion, which is much more easily accomplished at this time than at the earlier stages. The following features should be especially observed and followed chronologically:

a) Somites: Follow their growth and increase in number on successive days.

b) Nervous System: Observe the appearance and development of the optic vesicle, lens, neuromeres, fore-mid- and hind-brain, (trace development of the cerebrum, optic lobes, cerebellum and medulla). Note especially the development of the eye, olfactory pit, and otocyst.

Histological details of organ formation, especially the sensory structures will be found in Jones ('39).

c) Circulatory System: As in all vertebrates, the circulatory system in Fundulus exhibits a great deal of variation in its development. Note especially the extra-embryonic cavity, formation of the pericardium, and the first blood vessels, formed from wandering mesenchyme cells. (The papers of Stockard, '05, '15, on the origin and development of mesenchyme and vascular epithelium, are of interest here.)

Note the formation and position of the heart, and the times of its first action (Armstrong, '31, has studied heart function in Fundulus in connection with its innervation).

d) The pigmented melanophores are especially striking. Follow their distribution and appearance both in normal stages and is some Fundulus hybrid (cf. especially Bancroft, '12). The embryonic origin of pigment cells in the teleost is still uncertain, and definitely open to investigation.
e) Further details of development can be obtained from Oppenheimer ('37). Note and record the appearance of fins, the urinary bladder (from what structure is it an outgrowth?), and the liver.

9. Hatching:

Armstrong ('36) and Milkman ('54) have studied the mechanism of hatching in _E. heterocyclus_. Note and record the time of hatching of normal cultures, hybrids, and experimentally treated embryos.

Embryos may be more easily studied after hatching by anesthetization with chlordane.

10. Experimental Procedures with Later Stages:

Nicholas and Oppenheimer ('42) performed many extirpation experiments on later stages (Oppenheimer stages 20-23 are especially favorable). These stages are much easier to decapsulate than earlier ones. Aseptic conditions are important in cutting down mortality of embryos. Autoclave facilities can be arranged for those wishing to do this type of work. The following are examples of experiments that are practicable:

a) Remove _pectoral fin_ anlage as it appears (ventral and posterior to ear vesicle - Stage 20).

b) Unilateral extirpation of eye. This, and other extirpations, can be accomplished by suction. Draw up the eye into a small-bore pipette, and cut it off at the base.


d) Removal of small bits of nerve cord (Stages 17-20). Remove only about two somite lengths of nervous tissue, and as little adjacent tissue as possible.

f) Transection of spinal cord of motile stages (Stage 23). The nerve cord can be transected, without removal of tissue, at different levels. Note behavior patterns of later embryos (Stage 26).

g) Grafting of tissue to pericardial sac to study morphogenesis when isolated from normal tissue environment.

h) Culturing organ anlagen in nutrient media (cf. Trinkaus & Drake, '55).

i) Behavioral studies (a la Coghill) and mechanism of hatching (Milkman, '54).
Bibliography

DEVELOPMENT OF THE TELEOST

The references below represent only a small part of the very extensive literature on the normal development of, and experimental procedures performed on, teleost material. Important older literature is cited in some of the papers below.

General References

Rugh, R., 1948 Experimental Embryology, A Manual of Techniques and Procedures (pp. 360-418 contain useful summaries of many of the procedures noted above and descriptions of development of several teleost species).

1. Normal Development (Stage Series) of Fundulus and Other Teleosts


11. Reproductive Cycles, Gésenesis and Spermatogenesis


111. The Gametes and Fertilization

V. Cleavage and Early Stages


Devillers, Ch., 1947. Explantation in vitro de blastoderme des Poissons (Salmo, Esoc) EXPERIATIA, 3, 71.


Gemmall, J. F., 1912. The teratology of fishes, Glasgow (73pp.).


VI. Formation of the Germ Ring and Early Embryo

Brummett, Anna Ruth, 1954. The relationships of the germ ring to the formation of the tail bud in Fundulus as demonstrated by the carbon marking technique. J. Exp. Zool., 125, 447-480.


VII. Formation of the Embryo


VIII. Later Stages


IX. Origin and Migration of Pigment Cells


Arteria mesaxoidea
Echinoderm Development

Early phases of development of various Echinoderm species have been followed during the study of fertilization and activation. The student should spend the next two days in a thorough study of the normal development of three species: Arbacia punctulata (Sea urchin), Echinarchnus parma (Sand dollar) and Asterias forbesi (or A. vulgaris) (starfish). For a study of the larval stages it will be necessary to continue observations, intermittently, during the third and fourth days. If material is available the relatively transparent eggs of Strongylocentrotus may also be studied with profit.

Refer to the preceding section on Fertilization for methods of obtaining the gametes and accomplishing successful artificial fertilization and activation of the eggs of the three species to be studied. Fertilize a batch of eggs of each of the species at the beginning of the laboratory period. These may be studied comparatively by keeping a table of "cleavage times" and are to be used for the study of gastrulation and larval formation on the second and third days of observation. Detailed study of early cleavage stages should be done with a second batch of eggs for each species separately and consecutively.

It will pay off to take special precautions to avoid over-crowding and over-heating of eggs in the sea water dishes. Have no more than a single layer of eggs on the bottom of a finger bowl and keep the temperature of the sea water below 25°C for best results. Temperatures above 30-32°C are lethal for Arbacia eggs.

**Arbacia punctulata**

**Cleavage:**

The rate of cleavage and development in general are dependent upon the temperature of the sea water. The following figures give the average time for the first three cleavages (Fry, '36):

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

Different batches of eggs vary slightly (1-2%) in average cleavage time and, while within a batch of eggs most will develop at the average rate, some may vary by about 10%. These differences may still be apparent during larval stages.

The plane of the first cleavage is meridional (in the polar axis) and divides the egg into two equal sized blastomeres. The furrow of the first cleavage appears at the animal (upper or apical) pole of the egg and spreads rapidly (ca. 2 minutes at 24°C) to the vegetal (lower or buccal) pole. The egg then passes into a resting...
stage (lasting ca. 25 min. at 24°) before the onset of the second cleavage (Brooks, 1890, K. Dan, '54).

The plane of the second cleavage is also meridional but at right angles to the first, the furrows appearing at the animal poles of each of the two blastomeres and rapidly spreading (4-5 minutes at 24°) to the vegetal poles. This results in the formation of four equal sized blastomeres. A resting stage then follows.

The plane of the third cleavage is equatorial or horizontal (at right angles to the polar axis). Each of the four blastomeres divides into an upper and lower blastomere of equal size. In another species of sea urchin, Paracentrotus lividus, the plane of the third cleavage is occasionally sub-equatorial.

The student should follow these early cleavages carefully. Note the relationship of the hyaline plasma layer to the cleavage furrows (see Dan and Ono, '52). It will also be interesting to attempt to follow the movements of the orange-red, cortical granules in relation to the early cleavages (see Dan, '54).

At the fourth cleavage the upper four cells divide meridionally forming eight equal sized cells called mesomeres. The lower four cells divide unequally and horizontally forming four larger cells called macromeres and below them, at the vegetal pole, four small clear cells called micromeres. Prior to the fourth cleavage (according to Morgan, 1894, as early as the four cell stage) the orange-red pigment migrates away from the vegetal pole and this accounts for the absence of pigment in the micromeres. Micromere formation in arctica under certain conditions (e.g. mechanical disturbance) may be suppressed, 1, 2, 3 or no micromeres being formed but this does not impair further normal development (Horstadius, '37).

At the fifth cleavage the eight mesomeres divide equally and horizontally forming two tiers of cells termed animal 1, \( (an_1) \) and animal 2, \( (an_2) \). The four macromeres divide meridionally forming a tier of eight cells. The four micromeres also divide. Thus a 32-cell stage is attained.

At the sixth cleavage the \( an_1 \) and \( an_2 \) cells divide more or less radially. The macromeres divide horizontally forming two tiers termed vegetal 1, \( (veg_1) \) and vegetal 2, \( (veg_2) \). The eight micromeres also divide. In the 64-cell stage five layers or tiers of cells may be distinguished. From the animal to vegetal pole these are: 16 \( an_1 \), 16 \( an_2 \), 8 \( veg_1 \), 8 \( veg_2 \) and 16 micromeres. In later cleavages these layers become increasingly difficult to distinguish.

On the basis of staining with vital dyes (e.g. Nile blue sulfato) Horstadius ('39) has shown for Paracentrotus that \( an_1 \), \( an_2 \) and \( veg_1 \) cells form the ectoderm of the pluteus larva (see below).
veg_2 cells form the gut endoderm and part of the mesoderm (coelomic pouches, secondary mesenchyme), the micromeres form the remainder of the mesoderm (primary mesenchyme) which produces the skeleton of the larva. Sketch the various stages through the sixth cleavage.

**Blastula:**

At the eight cell stage there is a very small central cavity which enlarges, as cleavage continues, to form the blastocoel, this is filled with a fluid which, presumably, is mostly sea water plus colloid (see Dan, '52). About 6 hours after fertilization a smooth-surfaced, spherical young Blastula is formed, the wall of which is one cell thick. Cilia soon develop on the surface and the blastula is rotated by their action within the fertilization membrane. At about 10 hours post-fertilization the blastula hatches out of the fertilization membrane. It has been shown (Kopac, '41) that the blastula releases a "hatching enzyme" at this time that weakens and dissolves the membrane sufficiently for the blastula to break through.

A small tuft of long cilia develops at the animal pole of the blastula which is the forward end when it is swimming. At the base of this apical tuft the blastula wall is thickened, forming the apical plate. At the vegetal pole the blastula wall soon becomes flattened, forming the vegetal plate. Meanwhile, the micromeres located in the approximate center of the vegetal plate region have begun to migrate as individual cells into the blastocoel, forming the primary mesenchyme which later forms the skeleton. There is evidence that the micromeres continue to divide during this "ingression" process. Sketch early and late blastula stages.

**Gastrula:**

At about 20 hours after fertilization the cells at the vegetal pole invaginate to form a blind tube, the archenteron. Contrary to older views, there is no true involution (gradual turning in to form walls of the archenteron of a relatively large number of cells in the surface of the blastula) and no decrease in volume of the blastula. There is actually an increase in volume during gastrulation. Invagination is apparently brought about by a fairly uniform stretching in an animal-vegetal direction of the whole veg. disk, i.e., the vegetal plate (Horstadius, '39). This is a point which interested students might check by studying and measuring a series of fixed blastula and early gastrula stages and by attempting to stain the vegetal plate with vital dyes. Note also the gradual lifting or wrinkling of the hyaline layer at the vegetal pole of living early gastrulae (see Dan, '52).

The archenteron reaches the opposite wall of the blastocoel in about 5 hours. The gastrula contains about 1,000 cells and its outer wall as well as the wall of the archenteron has a single layer of cells. The primary mesenchyme cells form a ring around
the blastoporal end of the archenteron. Secondary mesenchyme and later, coelomic sacs are budded off from the tip of the archenteron. Draw beginning and completed gastrulae.

Prism:

At the completion of gastrulation the tip of the archenteron bends to one side of the gastrula which becomes flattened over an area extending from the animal pole nearly to the blastopore. This is one of the first signs of bilateral symmetry, the flattened side of the gastrula toward which the archenteron bends representing the ventral side of the embryo, the other flattened, blastoporal side representing the posterior or anal surface of the embryo. Another early indication of bilateral symmetry consists in the aggregation of primary mesenchyme cells into two groups, one at each of the postero-latero-ventral angles of the prism-like gastrula. Each group secretes a triradiate spicule, the first rudiments of the skeleton.

Where the tip of the archenteron touches the ectoderm the latter bends inwards to form the stomodeum. An opening, the larval mouth, later develops between the stomodeum and the adjacent tip of the archenteron. The archenteron becomes divided by two constrictions into oesophagus, stomach and intestine. The apical tuft disappears, a ciliated band surrounds the oral area, the embryo begins to elongate in the dorso-ventral axis and the direction of swimming changes so that the ventral (oral) side is forward. Draw a prism larva.

Pluteus:

After about 48 hours the embryo enters the pluteus stage which is fully developed at the end of the third day. The original apical plate grows out in a ventral direction to form the oral lobe which includes the stomodeum and anterior part of the oesophagus. Two short outgrowths, the oral (antero-lateral) arms are formed on the oral lobe and, at the anal side, two longer anal (aboral or postoral) arms grow out in the same general direction. The original triradiate spicules form skeletal rods which extend into the oral arms (oral rods), the anal arms (anal rods), dorsally through the body (body rods) and laterally (ventral transverse rods). The rods are each made up of three or four parallel parts joined by cross bars. Different species of sea urchins differ in this regard, so the structure of the skeletal rods is a useful characteristic in hybridization studies. The embryo continues to elongate in the dorso-ventral direction and becomes pointed at the postero-dorsal end where the body rods meet. The axis running thru oesophagus, stomach and intestine becomes J-shaped. The stomach expands to form a spherical structure that fills a large part of the body of the pluteus and sphincter muscles connect it with oesophagus and intestine. The two coelomic sacs extend posterolaterally from the oesophagus. That on the left side becomes larger and later acquires
a dorsal opening called the pore canal. The right coelom buds off cells to form the madreporic vesicle but otherwise remains rudimentary. The left coelom undergoes extensive later development in the formation of the structures of the adult sea urchin. These changes do not occur until the second week when metamorphosis begins in properly fed larvae and will not be studied here. It should be mentioned, however, that the adult organs are built up in and around a structure termed the Echinus rudiment which is formed by the fusion of an invagination (amniotic invagination) of the ectoderm on the left side with the mid-portion (hydrocoel) of the left coelom. The left side of the pluteus becomes then, the future oral face of the adult. Draw a 3 day old pluteus in postero-ventral and side view. Some students may be interested in trying to rear plutei through early metamorphosis (2-3 weeks).

**Asterias forbesii**

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

**Cleavage:**

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

**Blastula:**

Eventually (10-12 hours post fertilization) the cells arrange themselves as an epithelial wall enclosing the blastocoel. The surface cells acquire cilia, and the blastula rotates within the vitelline membrane. The two polar bodies are still visible, either attached to the animal pole, or detached from the embryo. The embryo hatches in the late blastula stage. Note that the cells at the vegetal pole are thicker than those at the animal pole. Note also that there is no "ingression" of cells at the vegetal pole, there being no primary mesenchyme comparable to that found in Arbacia. Mesoderm formation in starfishes occurs after gastrulation, i.e.,
after formation of the archenteron.

Gastrula:

Early gastrula: The vegetal pole area thickens and flattens and invagination begins. The blastopore is destined to become the anus. The larva elongates along the animal-vegetal axis. Note the large space between the archenteron and the outer wall of the gastrula.

Middle and late gastrula: The gastrula becomes pear-shaped ca 20 hours after fertilization. The blind inner end of the archenteron becomes thin-walled and expands. From this end mesenchymal cells wander out into the blastocoel. In a slightly later stage, two outpocketings of the distal end become distinct, the primordia of the coelomic sacs. At the same time, the archenteron bends towards one side which is the future ventral side. This is the first sign of the change of radial into bilateral symmetry. Note the ciliation in the archenteron, and that the archenteron extends only about halfway from the vegetal toward the animal pole.

Dipleurula Larva:

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

Fully formed Dipleurula (early Bipinnaria) larva: This larva represents an early larval type common to Asteroidia, Echinoidea, Ophiuroidea and Holothoidae (see Korschelt, vol. 1, p. 499, or Sell, 1948). Study carefully a ventral, dorsal and lateral (preferably left) view.

Observe the following:

Shape of larva. Notice convexity of ventral side and mouth opening underneath the overhanging oral lobe. Compare with the flattened oral lobe of Arbacia.

Locomotion. What part of the larva is directed forward in swimming?

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

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

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

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

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

**Fully formed Bipinnaria Larva:**

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

**Arms.** Identify unpaired median dorsal, paired antero-dorsal, postero-dorsal, postero-lateral, postoral and preoral arms. See Gemmill, plate 18, fig. 7, and MacBride, p. 465.

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

**BrachioIaria and Metamorphosis:**

Study ventral and lateral views. Consult the excellent figures in Gemmill, plates 19 and 20.

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

**Intestine**

Coelom, in different stages of subdivision

Disk, or developing starfish, on left side.

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

**Echinarachnius parma**

The eggs of this Echinoid are almost twice as large as those of Arbacia and consequently offer some advantages as experimental material. Although early development of the sand dollar is not markedly different from that of Arbacia the student should nevertheless compare the corresponding stages: early cleavage, blastula, gastrula and pluteus. Can you distinguish animal, vegetal and micromere tiers of cells? Is primary mesenchyme formed prior to archenteron formation? Is there a folding or wrinkling of the hyaline layer at the vegetal pole during gastrulation? Does the archenteron proliferate secondary mesenchyme from its tip? Is the volume of the gastrula larger or smaller than that of the blastula? Compare the plutei of the sand dollar with corresponding plutei of Arbacia. What obvious differences do you see?

**Experimental Procedures with Echinoderm Embryos**

Since the most thorough and extensive experimental studies have been done using various species of sea urchins the student may be interested in repeating some of the same experimental procedures with Asterias and Echinarachnius eggs. A few types of experiments are listed below.

1. **Experimental analysis by operation:** Development of isolated blastomeres (easiest); development of isolated animal and vegetal halves (more difficult); development of recombinations of animal and vegetal layers (extremely difficult); combination of operation and chemical agents.

2. **Experimental analysis through the effect of agents:**
   Physical: Centrifugation; development of light and heavy halves and quarters; development of whole centrifuged eggs; combination of centrifugation and chemical treatments.
Chemical: Effect on development of: LiCl, MaSCN, iodosobenzoic acid, proteolytic enzymes (trypsin, chymotrypsin, ribonuclease), sodium azide, etc.

3. Gradient studies: redox gradients as demonstrated with methylene blue, Janus green, Nile blue sulfate or Neotetrazolium chloride.

For the above, or any other experimental procedures which members of the class might wish to try, special directions, reagents and equipment will be made available by the instructors.
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Representative molluscs and annelids commonly used for experimental and descriptive embryological studies will be supplied. Our initial laboratory work will be concerned with descriptive development of type forms - or forms which are readily available at this time. It may be well to remind students that cleavage in these forms is characterized by partial rotation of the micromeres - so-called spiral cleavage. The spiral pattern is not observed with equal ease in all of the forms, thus it is obscure in eggs of Spisula, but very clear in those of Crepidula, etc. In addition to the precise pattern of cleavage the eggs and early embryos of molluscs and annelids are noted for their lack of ability to regulate. When blastomeres are isolated, under most circumstances they continue to develop as though still part of the whole embryo. They exhibit a mosaicism (or determinative cleavage) which has frequently been contrasted with the regulative (tendency of parts to form a whole) behavior of the eggs of other forms. Finally a structure which is unique to these eggs (and a few related forms) is encountered. This is the polar lobe, a large mass of non-nucleated cytoplasm which frequently resembles a blastomere. The polar lobe forms and retracts in synchrony with the first few cleavages.

The handling of the eggs and embryos of molluscs and annelids, like those of other marine forms, requires a reasonable degree of care (see Just, 1939, Basic Methods for Experiments on Eggs of Marine Animals). The eggs are sensitive to adverse conditions and in order to avoid high temperatures, evaporation, etc., a stock batch of eggs or embryos should be kept in a covered finger bowl or sten- dor dish on the sea water table. Samples of the embryos may be taken out at intervals for microscopic observation. Glassware should be thoroughly washed to avoid contamination.

For observing rapidly moving larval stages it is necessary to immobilize them without causing excessive harm. Polyvinyl alcohol may be used for this purpose (1 part polyvinyl alcohol to 1 part sea water containing the larvalae). This solution is very viscous and slows down larval movement without apparent damage. A 1:2 solution of 0.1% Chlorobutanol (MS 222) may also be used.

**EMBRYOLOGY OF MOLLUSCA**

*Spisula solidissima* (Pelecypod)

1. **Obtaining Eggs.** Remove hinge of the clam, breaking the ovary and forcing the eggs to exude into a beaker of filtered sea water. Decant 4 times in the first half hour, leave the heavier more mature eggs on the bottom of the beaker. Add two drops of a sperm suspension (one drop of dry sperm in 10 ml. sea water) to eggs contained in 40-50 ml. of water in the beaker. Limit egg concentration so that there are no more than 20-25 in a low power field since they are very susceptible to overcrowding. Dry ovaries and testes may be kept overnight in a refrigerator. Washed eggs may be kept for 4 to 6 hours.

2. **The unfertilized egg:** Obtain a sample of unfertilized eggs in a stenodor dish and transfer to a depression slide for study. When shed, the eggs are irregular in shape due to pressure within the ovary, but they become spherical on standing. They are small (56 micra) and the center is almost completely filled by the enormous germinal vesicle with its prominent nucleolus. Note the thin layer of clear cortical cytoplasm and the densely packed yolk. Unless eggs are inseminated,
they will maintain this appearance for many hours. The eggs are fertilizable until the germinal vesicle breaks down, although the capacity for normal fertilization and development is impaired with long standing.

3. Fertilization and Naturation: Fertilize eggs and immediately transfer a sample to a depression slide. A few minutes after insemination the outline of the germinal vesicle starts to become indistinct, and in 15-20 minutes there is only a lighter area in the center of the egg, marking its former position. A thin fertilization membrane is raised, but this is not lifted far from the egg surface, and it is best seen in the region of the polar bodies or spanning the cleavage furrows. The first polar body forms shortly after germinal vesicle breakdown, and the second polar body follows directly beneath the first. Both polar bodies are usually formed within 30-35 minutes after insemination. Note the position of the polar bodies, for they mark the plane of the coming cleavage.

4. Early cleavage: About 50 minutes after insemination two nuclei will be visible, the male and female pronuclei. Can you see them approach and fuse? Cleavage occurs about 15 minutes after they first appear. The first cleavage is unequal, and the first two blastomeres differ greatly in size. The second cleavage follows in about half an hour. In the case of the larger cell, cleavage is again unequal, resulting in one larger cell and three smaller ones. The following cleavages are rapid, perhaps only ten minutes intervene between the 4 and 8 cell stages. The cleavage is undoubtedly of a spiral type, but this characteristic is more difficult to detect than in Crepidula because of the size differences of the blastomeres.

4. Time table of development: There is much variation in the developmental rate depending on temperature and other environmental conditions, but the following tables (Schenker, 1941) will give some idea of the chronology at 25°C. Times are recorded from insemination:

<table>
<thead>
<tr>
<th>Time</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 min</td>
<td>Germain vesicle reaction</td>
</tr>
<tr>
<td>30 min</td>
<td>Polar bodies formed</td>
</tr>
<tr>
<td>50 min</td>
<td>Pronuclei visible</td>
</tr>
<tr>
<td>1 hr. 5 min</td>
<td>First Cleavage</td>
</tr>
<tr>
<td>1 hr. 35 min</td>
<td>Second cleavage</td>
</tr>
<tr>
<td>5 hrs.</td>
<td>Swimming blastula</td>
</tr>
</tbody>
</table>

5. Later development: (The figures of the development of Dreissensia (Neisenheimer, 1900) will prove helpful).

Gastrulation and early Trochophore stages: Remove samples 4-9 hours after insemination. If forms are moving too rapidly, add a drop of polyvinyl alcohol. In the younger stages, note that the smaller, more rapidly dividing ectodermal cells are spreading over the larger, yolk-filled endodermal cells. This type of gastrulation is known as endoblastically. The uncovered region is the blastopore. When the larva starts to swim (5-6 hours after insemination) a plate of large cells which will form the shell gland is visible on the future dorsal surface. Internally, two large dark cells, the mesodermal teloblasts are often visible. By 9 hours the embryos have lost their somewhat barrel-shaped and are pyramidal, the expanded base of the pyramid being the region in which the velum will form. The cilia are not marked at this time. The shell gland is visible as a conspicuous concavity on the dorsal surface. By 12 hours the shell gland will have evaginated and this concavity will no longer be visible. The cilia of the velum and the apical flagellum will be visible at this stage.
Young veligers: Obtain samples of cultures about 18-19 hours after insemination. Note:
   a. General Shape.
   b. The two-valved shell with its straight hinge line. How much of the body is enclosed by the shell?
   c. The animal flagellum, telotroch, and the long cilia of the developing velum.
   d. The stomadeal invagination on the ventral side, just below the velum. The proctodal invagination appears later (23 hrs.)
   e. The internal structures are difficult to recognize at this time, for a large dark mass of undifferentiated endoderm and mesoderm cells fills most of the post-velar area.

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1941 Experimental studies upon the egg cells of the clam, Mastra solidissima, with special reference to longevity. J.E.Z.

66:461
Chaetopleura apiculata (the Chiton) (Amphineura)

The species is dioecious, but there is no way of distinguishing the sexes externally.

Suggestions for Observation: Because of the large size and opacity of the egg, the early development is best studied by mounting egg samples in depression slides. Moving larvae can be mounted in a dilute solution of Janus Green for observation.

1. Obtaining eggs: Breeding begins about June 20 and is abundant from July 10 to August 20. Eggs are usually spawned from 8-11 P.M. Few, if any, individuals spawn during the first night in the laboratory. To obtain eggs and sperm remove 30 to 40 individuals from their shells and place them in sea water in a large finger bowl. Keep aerated sea water running through the bowl during the day. In the late PM wash the animals and dish free of sediment and leave the dish undisturbed on a table. Animals may begin to spawn at about 8 PM or thereafter and may continue for an hour or so.

2. Study of Normal Development.
   The Unfertilized Ovum: The spherical egg measures from 180 to 190 micra in diameter and appears opaque due to the large amount of yolk. Although internal processes can not be seen in living eggs, sections show that the ovum is usually in the process of developing the first maturation spindle when it is shed. Surrounding the egg is a tough, bristly chorion. When the eggs emerge from the oviduct they are embedded in a viscid jelly-like secretion which spreads over the bottom of the dish in a thin film.

3. Fertilization and Cleavage: There are no visible changes at the time of fertilization; a fertilization membrane is not raised and the egg does not change shape. Two transparent polar bodies are given off but no polar lobes are formed. The first noticeable change occurs shortly before first cleavage when there is a slight flattening of the egg at the animal pole. The first cleavage furrow (1 hr. 40 mins. to 1 hr. 50 mins. after insemination) divides the egg, in most cases, into equal blastomeres. In a small percentage of cases, one blastomere is perceptibly larger. The second cleavage is at right angles to the first, and again in some cases the D cell is slightly larger. The cells of the first quartette of micromeres given off by the axiolotrophic third cleavage are distinguishable from the larger macromeres. The further divisions follow the regular pattern of spiral cleavage. Four quartettes of micromeres are given off. The first three give rise to the ectoderm, nervous system, and stomach, while the fourth quartette (except for the 4d cell) becomes part of the endoderm along with the macromeres. The 4d cell gives rise to the mesoderm as well as endoderm.

4. Time Table of Development: The following record, procured from a batch of eggs developing at 23-24°C is offered as a rough outline of developmental rate. Metamorphosis is supposed to occur early in this batch, the usual time being from 7-12 days. Time is recorded from the time of insemination:

<table>
<thead>
<tr>
<th>Event</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st polar body</td>
<td>30 min.</td>
</tr>
<tr>
<td>2nd &quot;</td>
<td>55 min.</td>
</tr>
<tr>
<td>1st cleavage</td>
<td>1 1/2 hours</td>
</tr>
<tr>
<td>2nd &quot;</td>
<td>2 &quot;</td>
</tr>
<tr>
<td>3rd &quot;</td>
<td>2 &quot; 40 min.</td>
</tr>
<tr>
<td>gastrulation</td>
<td>ca. 13 hrs.</td>
</tr>
<tr>
<td>beating cilia</td>
<td>14 hrs.</td>
</tr>
</tbody>
</table>
5. **Later Stages of Development and Metamorphosis**

A. **Young Trophophores 40-60 hrs. old:** These larvae are propelled through the water by the beating of a band of powerful cilia (the prototroch). The body rotates on its longitudinal axis and the course followed is a spiral. Crowned the posttrochal hemisphere (the head visicle) is a clump of very long cilia (the apical tuft) which is apparently sensory in function. The two lateral, reddish-brown larval eyes give a certain amount of bilaterality to the otherwise radial organism. Although the mouth may be visible just below the prototroch, the other regions of the digestive tract are obscured by the yolk mass.

B. **Older Larvae (3-4 days):** There is an elongation of the body, especially of the post-trochal hemisphere. The mouth and the archenteron are now visible due to the reduction in the quantity of yolk. The anus plates are beginning to appear on the dorsal surface. Note the contractile foot that develops on the ventral surface just posterior to the mouth. Locomotion is still by way of the prototrochal cilia, although older larvae may creep along by means of the foot.

C. **Metamorphosing Larvae:** Metamorphosing larvae may be procured from the bottom of a culture dish. Note that the prototrochal and apical cilia are lost during metamorphosis, and that the larvae now creep about by means of a well-developed foot. The shell plates have increased in number, though the full set of the adult is not yet complete. The mantle (a fold of the body wall) develops just dorsal and lateral to the foot.

**REFERENCES**


Crepidula fornicata (Gastropod)

Due to the opacity of the living eggs, the details of maturation, association of the germ nuclei, and cleavage can best be studied from prepared slides. The eggs are not sectioned, but are whole mounts. Number one or zero cover glasses have been used, making it possible to examine the material under high power. Do not, under any circumstances, use oil immersion objectives on these slides. Conklin's two monographs (1897 and 1902) may be used for reference, or the photo copies of the plates from these papers.

Crepidula is a dioecious genus with the males fewer in number and smaller than the adult females. The spermatocysts mingle with ova before the egg capsules are formed around groups of eggs in the oviduct of the female. The mature females are sedentary, and the males locomotive, and at the breeding season or perhaps once for all, the females are visited and inseminated by these motile males. All of the ova produced by a given individual during a season are laid within a short space of time.

1. On the prepared slides, make a careful study of various stages in the two maturation divisions. In the same eggs find the sperm nucleus and note its approach to the egg nucleus. Note also the small antipolar lobe. Make drawings.

2. Study the first and second cleavages, noting the direction of the axes of the mitotic figures in the latter. The small antipolar lobe may be visible near the vegetal region of the furrow at the 2-cell stage. Make drawings.

3. Study in detail the formation of the first three quartettes of micromeres and the formation of the derivatives of the first and second quartettes. Indicate which divisions are dextrorotary and which are laevo rotary. Find and draw examples of the 8, 12, 16, 20, 24 and 25-cell stages. The last is difficult to find and it should be checked carefully. The nuclei may be identified by their size characteristics.

Some students, in their spare time, may wish to prepare their own slices of Crepidula maturation and cleavage stages. The following method is suggested:

A. Obtaining Eggs: With a heavy knife, loosen a Crepidula shell from its attachment. The egg capsules will either be attached to the substrate or to the foot of the female. 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. Remove the capsules, by means of forceps, to a watchglass of sea water. Tear open the capsules with a pair of needles to release the eggs. Discard empty capsules. Examine the eggs under the microscope to ascertain the stage. Dark-field illumination may be helpful. It is best to mix several batches of young stages for slide-making.

B. Fixation: (Do not use fixatives or other reagents in any dishes which are used for living materials. Confine these reagents to vials). Having freed the eggs, wash them by gentle rotary rinsing with a pipette, and then concentrate them in the center of the dish. Change the sea water two or three times. Then take up the concentrated eggs with a pipette, and drop them, with a small amount of sea water, into a vial three-quarters full of Kleinenberg's picric sulphuric fixative. The eggs should be fixed at least 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 acid from them, hydrated in 50%, 35% and washed thoroughly in 2-3 changes of water.

C. Staining: After washing with water, fill the vial with undiluted Mayer’s haem-alum, and stain for 5-10 minutes. For the polar 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.

D. 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 the 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 vials in which they have been 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.

4. The Veliger Larva of Cremidula

Study the typical Veliger larva of Cremidula fornicate. Obtain material by breaking the animal from the substrate to which it is attached. You will find the yellowish eggs (enclosed in transparent capsules) attached to the substrate or in the shell of the mother. Tease the embryos out of the capsules. Obtain swimming larvae in different stages, particularly old ones with the yolk resorbed. They are transparent and show the inner organs. Consult Cunklin (1897) figs. 80-82 and the text books of Parker & Haswell volume 1 (1928) fig. 657 on p. 734, or MacBride figs. 263 ff. on pages 301 ff. or Kur chelt volume 2 (1936) 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. Ciliated velum
2. Head vesicle (dorsal)
3. Eyes with lens (dorsal)
4. Foot (ventral); study it in lateral view
5. Statocysts (ventral) at the sides of the foot
6. Ciliated mouth (ventral) above foot
7. Oesophagus (dorsal), stomach and 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.
(There is no typical trochophore stage in Crepiaula. Study stages of direct transformation of the gastrula into the Veliger larva. (Conklin, 1897, figs. 77-79.) Observe the gradual development of the shell gland, shell, velum and foot.)

5. Crepiaula - Derivatives

1st quartette of micromeres:

a. all ectoderm cells of head vesicle
b. apical plate of ciliates cells
c. posterior cell plate
d. dorsal portion of functional velum and portion of first velar row on ventral side
e. supracesophageal ganglia and connectives
f. cerebro-pedal connectives
g. possibly the pedal ganglia
h. an apical sense organ
i. paired eyes

2nd quartette of micromeres:

a. larger part of velum
b. shell gland
c. at least part of the foot
d. larval mesenchyme from derivatives of 2a - 2c.

3rd quartette of micromeres:

derivatives lie wholly outside of velar area and form a considerable part of the lower hemisphere.

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Ilyanassa obs.leta (Gastropoda)

The Obtaining of Eggs and Early Stages: The best method of obtaining very early stages consists of removing egg capsules which have been deposited on glass plates by snails in aquaria. Eggs are visible at the oviducal opening (anterior median part of foot). After the egg is fastened to glass, the snail may be removed gently, and the capsule transferred to a watch glass of filtered sea water with a pair of fine forceps. Since snails seem to prefer to deposit eggs on the wooden sides of the tank, in preference to the glass, J. Oppenheimer suggests inserting sheets of glass over these wooden sides; these can be removed from the sides for inspection purposes without disturbing the animals.

The Opening of Capsules: (The stage of development of the eggs may be determined before removal from the capsule with the aid of a binocular microscope.) When the proper stage is found, pin down the side of the capsule furthest from the eggs with a dissecting needle. With another needle, tear off the section that is pinned to the dish, making sure that a very large tear is made. If gentle pressure is now applied to the capsule, the eggs will flow out of the mass of jelly. Make sure that all of the eggs are free of the capsule and that the capsule is rather well torn up before releasing the pressure, or else the thick jelly will rush back into the capsule, carrying the eggs with it. All of the operations must be carried out under water, for the eggs rupture on contact with air. The jelly dissolves in the water, and after a few seconds, the eggs will settle down to the bottom of the dish.

Cleavages: Approximate Time Table:

1st lobe 50 minutes (1st polar body coming off)
Disapp. of lobe 59 minutes (1st polar body off)
2nd lobe appears 1 hr. 12 min.
2nd lobe one 2 hrs. 12 min.
3rd lobe 2 hrs. 42 min.
1st cleavage 3 hrs. 42 min.
4 cells 4 hrs. 52 min.

Later Stages: Observe later stages of development through the veliger larva (there is no trochophore larva.)

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EMBRYOLOGY OF ANNEELIDA

Three forms are available for study - Nereis, Chaetopterus and Hyaloides. Cultures of advanced stages will be prepared by the instructors. Students should prepare their own cultures of early stages. It is suggested that at least two forms be chosen for observation of early embryonic development.

Nereis limbeta

The animals are obtained about an hour after sunset from the Eel Pond. They exhibit lunar periodicity and, on certain nights, swarm near the surface of the water when attracted by a light. Males are red anteriorly with white posterior segments, the females light green.

Details of fertilization of the Nereis egg will be studied at another time. The first cleavage (90-100 min.) is unequal and forms a smaller AB and a larger CD blastomere. These are divided unequally at the second cleavage forming blastomeres A, B, C, and D in order of increasing size. At the third cleavage four smaller cells (1st quartet of micromeres, la-ld) at the animal pole are separated from four larger vegetal cells (1st generation of macromeres, la-ld).

Observe the cytoplasmic segregation which begins at the time of fertilization (Costell, 1945). This is a segregation of cytoplasmic inclusions along the pole so that by the time of the 3rd cleavage the micromere region is oil free and nearly yolk free. In the unfertilized egg the large oil droplets form a ring around the germinal vesicle and the other inclusions (yolk spheres, mitochondria, granules, etc.) are uniformly distributed. After the germinal vesicle breaks down there is a gradual redistribution of inclusions, the most obvious being a movement of oil droplets and yolk to the vegetal hemisphere. By the four cell stage this segregation is completed and at the 3rd cleavage the micromeres are free of yolk and oil droplets.

Subsequent divisions of micromeres and macromeres lead to the formation of a spherical embryo (20 hours). The anterior is marked by spherical frontal bodies and the posterior end by greenish black pigment. The central part of the protocelar hemisphere bears the apical tuft of cilia.

A. 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. Study larvae from all sides. Consult the figures in E. B. Wilson, 192.

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 stomadaemae; the latter is a short ectodermal invagination.
5. The large macromeres have not yet differentiated into the endodermal 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 post-trochal hemispher. The setae (= chetae) will differ from inside of them. These sacs are the first indication of the first two segments of the worm.

8. Observe the trophophore in locomotion

B. Advanced trophophore, 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: 2-3 days (Wilson, figs. 90-91)

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

1. Sense hairs at the apical pole
2. Additional eye spots; number variable
3. Mesothelial ciliary bands at the boundary of the segments.
4. Parapodia with paraxial cirri appearing on segments 2 and 3
5. Tentacular cirri on the head.

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

REFERENCES


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


Hydrides hexagonus

Hydroides, both male and female, will spawn immediately after being removed from their calcareous tubes. Remove several and place them in finger bowls (one worm per dish to keep sexes separate.) Remove the animals after they have spawned. Let sperm stand for about 3 hours. Add a few drops of sperm suspension to a dish of eggs. Extrusion of polar bodies and cleavage may be studied readily. 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.
Trochophore of Hydroides: The trochophore is a typical Amelius trochophore. Consult the excellent figures in Hatscheck ('86) and Shearer ('11). The larvae show positive phototaxis and gather at the window side of the dish. They are transparent, and proper adjustment of the illumination by moving the mirror and 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 vesical 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 trochophore**
2. **Apical tuft**, (several long cilia, probably function as sense organ)
3. **Apical organ**, a thickening of the ectoderm at the animal pole; a nerve center and probably the primordium of the cerebral ganglion.
4. The **prototroch**, an equatorial band of large cilia. In older trochophores 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. It consists of a few large prototroch cells which become pigmented in older stages.
5. The **metatroch** (paratroch), a circular band of cilia in the middle of the prototrochal 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, stomadaeum (oesophagus-ectodermal), enlarged stomach (endodermal), narrow intestine (endodermal) except for the end portion which is invaginated ectoderm - prototrochaeum, and anus, an opening behind the vegetal pole. All parts are ciliated. Feed india ink and study the mechanism of food intake.
10. The **anal vesicle**, a large vacuolated cell at the posterior end, not found in other trochophores.
11. The cavity between intestine and outer body wall is not a true coelom but a primary body cavity, the persisting blastocoele.
12. The **larval kidneys** (paires) are typical protonephridia with flame cells; they open near the anus. They appear as slender cords near the statocysts, extending between oesophagus and anus. They are best identified in animals with vegetal pole up (consult figures in Hatscheck 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 mesodermal 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 **mesodermal cells** (derivatives of 4a 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.
References:
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Chaetopterus pergamentaceus

This is an exotic species of annelid found in U shaped tubes on mud or sand flats. The sexes are easily distinguished by the color of the parapodia which contain the gonads - the females are orange colored and the males white. Eggs and sperms may be obtained by cutting the posterior parapodia. The eggs, after extrusion from the parapodia, should be washed several times with sea water or filtered through cheesecloth to remove mucous.

The egg (about 100 micra in diameter) contains a large germinal vesicle which breaks down and proceeds to the metaphase of the first maturation division when placed in sea water. Details of fertilization will be studied at another time, however, observe the change in shape of the egg between fertilization and first cleavage.

A polar lobe is formed in the vegetal hemisphere prior to the first cleavage and at the close of cleavage fuses with the OD cell. A smaller lobe appears prior to the second cleavage and passes into the D. Cell which thus becomes the largest blastomere of the 4 cell stage. Subsequent cleavages are of the spiral type (see Plate XL, Mead, 1897). The third cleavage is dextrorotopic, giving rise to the first quartet of micromeres, which are nearly the same size as the macromeres. The embryo becomes ciliated and hatches from the membrane before the end of the first day.

Schedule of development:

1st and 2nd polar bodies 15-27 min.
1st cleavage 62 min.
2nd cleavage 82 min.
3rd cleavage 95 min.
ciliated blastula 8 hrs.
young trochophore 24 hrs.

Trochophore: 24 hour stage

Elongation of the body occurs during the first day. Observe the anterior apical tuft, mouth on ventral surface, eye spots on dorsal side, and mesotrichial band of cilia.

3-6 day trochophore: Elongation of the trochophore continues and it becomes spinule shaped. The mesotrichial band of cilia disappears and is replaced by two lateral flagella. A ciliary band develops in the region of the posterior part of the intestine. The mouth becomes enlarged, forming a triangular slit and the digestive tract becomes more distinct. Observe eye spots, lateral flagella, posterior ciliated esophagus, spherical stomach, thin walled intestine.
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Chapter of available conjugates

Diploblastica and radially or biradially asymmetrical.

Types of individuals: polyp and medusa.

Extensive gastrovascular cavity with mouth surrounded by tentacles.

Intrinsic monoecy.

Clade Hydrozoa

Small polyp and medusa with well-defined metagenesis.

Polyp generation usually colonial.

Reduce with velum.

Clade Hydroida

Polyplid generation well developed

Solitary or colonial.

Building off free or variously reduced medusa.

Subclass Gymnoblastea (Anthozoa)

Hydrothoe without hydrothoe; gonophores without gonothoe.

Gonad borne on medusa.

Free medusa tall; body-like.

Genus: Bougainvillia, Bougainvillia, By-astenia, Penardia, Penardia, Penardia, Tassoria.

Subclass Calyptrataea (Thecophora)

Hydrothoe with hydrothoe; gonophores with gonothoe.

Free medusa bush or medusa-shaped.

Gonads borne on radial clusters or medusa.

Genera: Gomphurania, Gomphurania, Chelia.
Coeleterates

Laboratory Guide

There is a greater abundance of coeletterate material available than you can possibly study in detail. Certain forms, because of their abundance or maturity at this time of the year and because of the relative ease with which developing embryos may be obtained and studied, are dealt with here in greater detail than others. The greater part of your observations will probably be made on hydroid material. Hydroids are very sensitive to environmental conditions and do not survive well in the laboratory, even in aquaria of running water. While adults are difficult to keep for more than a couple of days, the embryos are in general considerably more hardy. Nevertheless neither the embryos nor the adults should be crowded, and both should be kept on the sea water table except during manipulation and observation.

Taxonomy of Available Coeletterates

Phylum Cnidaria
Diploblastic and radially or biradially symmetrical.
Two types of individuals: polyps and medusae.
Extensive gastrovascular cavity with mouth surrounded by tentacles.
Intrinsic nematocysts.

Class Hydrozoa
Small polyps and medusae with well-defined metagenesis.
Polyp generation usually colonial.
Medusae with velum.

Order Hydraida
Polyzooid generation well developed
Solitary or colonial.
Budding off free or variously reduced medusae.

Suborder Gymnothetea (Atheeata)
hydrants without hydrothecae; gonophores without gonothecae.
Gonads borne on manubrium.
Free medusae tall, bell-like
Examples: Bougainvillia, Eudendrium, Hydractinia, Pennaria, Podocoryne, Tubularia.

Suborder Calyptothetea (Thecaphora)
hydrants with hydrothecae; gonophores with gonothecae.
Free medusae bulb or saucer-shaped.
Gonads borne on radial canals of medusae.
Examples: Campanularia, Gonothryae, Obelia.
Order Siphonophora

Free swimming or floating colonies of several types of polypoid and medusa individuals attached to a stem or disk. Medusae never complete and rarely freed.

Suborder Physophorida

Upper end of colony composed of a float (pneumatophore).

Example: Physalia (Portuguese Man of War).

Class Scyphozoa

Acraspedote medusae; polyp generation lacking or represented by a polypoid type (scyphistoma) which develops directly into the adult or gives off medusae by transverse fission.

Order Semaeostomae

Corners of mouth prolonged into four long, frilly lobes. Without septa dividing gastrovascular system. Margin scalloped.

Examples: Aurelia, Cyanea.

Class Anthozoa

Exclusively polypoid.

Not considered here. A common representative is Metridium, a sea anemone.

Family of Turbs with perfect median
Introduction to Hydrozoa

Examine a colony under the lowest power of your dissecting microscope in the case of the smaller forms or with the naked eye in the case of large form such as Tubularia. Clip off a few pieces containing the best embryological material and segregate them in plenty of sea water in a dish for further study.

(a) 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 occurring usually in response to environmental conditions prevailing at certain times of the year. The alternation of sexual and asexual generations (metagenesis) is closely paralleled by an alternation in structure, since zygote production is typically accomplished by medusae and the production of buds by polyps. Exceptions exist, however, in which the polyp produces sex cells (e.g., Hydra) or in which the medusa reproduces asexually by budding (e.g., Rathkea, Bougainvillia, Corymorpha).

In some Hydrozoa no medusa-form exists, while in others there is no polyp-form. All of the intermediate stages, in which the medusa or the polyp forms are variously reduced, exist. 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 jellyfish Campionemus, but unfortunately it is now nearly extinct at Woods Hole. The genera picked for study are arranged in the order of decreasing completeness of the medusa-form, the first having free-swimming medusae and the last mere sporasacs. The utter degeneration (namely the absence) of the medusa form is illustrated by the familiar Hydra.

Laboratory Procedure

1. Study of forms with perfect medusae

Examples: Bougainvillia, Obelia, Pomocorina

Characteristic life history. Zygote shed from medusa; Development to planula larva; Metamorphosis to polyp; Asexual budding of polyps to form a colony; Medusae formed by special buds, in a gonangium (Obelia) or separately (Bougainvillia); Sheding of medusae, which slowly mature 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. The structure of the medusa buds may be made more apparent by the removal of water from beneath the cover slip with absorbent paper applied to the edge of the slip, thus compressing and spreading the buds slightly.

When all of its parts except the gonads are fully formed, the medusa breaks loose and swims away. It lives independently for one or two months, the gonads gradually maturing. Find a well-developed specimen that is swimming actively and sketch its structure roughly. Note the velum, the presence of which is a key
feature in distinguishing hydrozoan from scyphozoan medusae, and the other structures indicated in the accompanying schematic drawing of a medusa.

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 gonangia are several times as large as the hydranths. Examine specimens and determine whether they are located at random along the stems or in regular places.

An *Obelia* gonangium has an enlarged, chitinous, transparent covering, the gonotheca, with a blastostyle extending through it from base to tip. The tip of the blastostyle expands to make a loose plug for the gonotheca when mature.

A cluster of gonophores (medusa buds) is borne on the blastostyle inside the gonotheca. The gonophores mature as medusae, break loose and escape to the outside past the blastostyle plug. Their free-swimming life lasts two months or so, the gonads maturing slowly.

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

With needles, press on the gonotheca of a mature gonangium, and examine under high magnification the gonophores which are released. The oldest ones may show swimming movements. Younger stages may be teased out from the gonangium 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 found with the naked eye in good light. Examine some when they are available and note their slightly reduced condition 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.

The newly shed medusa of *Obelia maniculata* has 24 tentacles, while that of *O. commissurabilis* has 16. Both 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. Found primarily on *Mysa* shells)

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 (*trophoecoids*), gonozoids and actinolozoids (*protective polyps*). *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 indulging in gamete production. See Goette,
16. (The sporosacs of *Hydraactinia* bear very little resemblance to medusae, being greatly reduced.)

Sketch the three main types of polyps and show several stages in medusa development. Cuvads can be distinguished 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: *Pennaaria, Tubularia*.

*Life Histories: Zygote shed from short-lived imperfect medusa (Pennaaria) or retained in reduced sessile meadsa form (Tubularia); or development to actinula larvaand growth to polyp (Tubularia); Asexual budding of polyps 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 (Pennaaria).*

*Pennaaria* (July, August, September; begin 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 cut 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 asexually reproducing colonies bearing male and female gonophores respectively.

Mature *Pennaaria* colonies festooned with shedding medusae provide an astonishing and beautiful spectacle that every student should certainly see. The material ripens in the season of warmest water. 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 on 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, at the expense of great effort, the three to six pink eggs. In the south, *Pennaaria* 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. Do not crowd! Next day, remove the stems and look with naked eye for free medusa as evidence of shedding. If they are found, look for developing eggs.

The eggs are very simple and slightly amoeboïd, 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 hrs., and stages in the very simple metamorphosis to the polyp stage may be observed. (Hargitt, G.T., '00, '09)

Tease apart male and female gonophores and examine the eggs and sperm. Sketch several stages in the development of the medusa, male and female medusae, and
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 embryonic development of Tubularia is figured in an accompanying plate. The gonophores of a mature specimen form long racemes or clusters, crowning and drooping from the region between the two circles of tentacles on a hydranth. The gonophores are quite severely reduced medusae which never become free-swimming, usually have no evident radial or circular canals, and develop nothing but buds for tentacles. Several different species occur in the Woods Hole region, two of which have apparently never been classified. There is a possibility that they are introduced via the hulls of ships, however, and are indigenous to other parts of the world. These species differ in their color, the presence or absence of lateral branches on the main body of the stem, the height which individual polyps attain, and the morphology and number of the tentacle buds or "apical processes" of the gonophores. The unbranched forms are larger and withstand laboratory conditions better, but these are the apparently unclassified species. They possess four or eight laterally compressed apical processes per gonophore.

T. crocea, the form which is commonest in early summer, is branched and possesses four nipple-shaped apical processes per gonophore. Another species, (probably T. tenella), is very short, possesses hydranths of a vivid, deep orange color, and has, like T. crocea, four nipple shaped apical processes on each gonophore. This is the least satisfactory species for your observations because of its extremely low temperature tolerance.

Male and female gonophores occur in separate colonies. Occasionally a colony may be found possessing gonophores of both sexes (on different individuals), but this is not a true "colony". Rather it has been formed by the settling of more than one actinula, of different sex, near one another. Diagnosis of sex by sight is impossible in the immature specimens, but gonophores that contain embryos are easy to distinguish from those filled with a cloudy mass of sperm. Early stages of developing embryos are found by teasing with needles; those near the hatching stage (actinulae) 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 examine the sperm under high power of your compound microscope.

Examine and sketch a ripe female gonophore. Determine the shape and number of the tentacles (apical processes) on its distal end. The eggs come from favored oocytes that progressively swallow up their neighbors. They lie 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 (hollow) or a morula (solid) may be formed (Lowe, '26), and gastrulation of the former has been described as a mixture of delamination and multi-polar proliferation. The embryos develop to the actinula stage within the gonophore. The actinula larva is to be considered as a precociously metamorphosing form, part planula and part polyp.

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

Study and sketch the structure of a fully formed actinula larva. Look over some ripe "female" colonies for actinulae beginning to escape from the gonophores. Actinulae will not develop further without feeding. They may be few brine shrimp, the eggs of which may be obtained from the assistant.

Besides the accompanying illustrations, you may find others in the text books of Korschelt, '36, and Hyman, '40. Cleavage shown in Allen, '00; Hargitt, G. T., '09. For gonophore development see G. ette, '07.

C. Study of forms with degenerate medusae

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

Life histories.

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

(b) Hydractinia, Eudendrium: Zygote develops into planula larva either inside gonozoid (Eudendrium) or after being shed (Hydractinia); Planula metamorphoses into polyp; Asexual multiplication by budding; Colony formation; Gonozoids formed from hydorhiza (Hydractinia) or by transformation of hydranths (Eudendrium); Gonophores (highly reduced medusae or "sporosacs") borne on gonozoids; Eggs and sperm formed in the sporosacs; Eggs fertilized in situ (Eudendrium) or during shedding (Hydractinia).

Campanularia (June, July)

It is not safe to try to distinguish this genus from Obelia by the anatomy of the feeding individuals. Even the gonangia 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, gastrulated by delamination and reaches the free-swimming planula stage, still in situ. Campanularia therefore releases from its gonotheca not medusae, but planulas.

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 themselves reproduce asexually by budding.
Select from a "female" colony a gonangium showing eggs in the basal gonophores. Mount it on a slide, study under various magnifications of the compound microscope, and sketch it.

Select and sketch another gonangium 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 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 gonangia 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 slip while watching it under the microscope.

For illustrations of Campanularia development (gonophores) see Guette, '07.

Gonothyraeae (July, August)

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

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

Gonothyraeae cleavage illustrated in Wulfert, '02; medusa development in Guette, '07. See also texts of Hyman, '40 and Korschelt, '36.

Hydractinia (June, July, August. Found on Littorina shells)

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 (trophozoids), threadlike coiling forms with no mouth and an apical knob of nematocysts (actinyzoids, commonest around the tip of the shell), and gonozoids. The three types all arise from a hydorhiza network covering a rust-red, spine-studded crust.

a) Gonozoids and Gonophores:

The gonozoids or reproductive individuals are usually without tentacles and have a large knob of nematocysts on the proboscis (manubrium); each bears a number of gonophores, which are medusa buds reduced to the state of sporozoa. Ripe "male" and "female" colonies can be distinguished with the naked eye, since the eggs
within the sporo sacs are a dull green against the red hydrorhiza, and the sperm when mature are a whitish mass.

Remove several "male" gonozoids showing ripe sporo sacs, 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 sporo sacs fall far short of being perfect medusae. Note stages in the reproduction of sperm within them. Burst a mature sporo sac and study the sperm under high power.

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

b) Cleavage and Development:

The embryonic development of Hydractinia is illustrated in an accompanying plate. If a number of "male" and "female" colonies are put together in a large dish of sea water (or a pair of prime colonies in a fingerbowl) and left overnight, eggs should be shed and fertilized between 7 and 9 A.M. The shedding can be controlled by light. If fertilization and cleavage stages are needed later in the day or in the evening, the colonies may be kept illuminated (that doesn't mean heated) during the preceding night, put in the dark for a couple of hours and reilluminated one hour before the time when shedding is desired. Eggs are shed in 55 minutes, sperm in 50 minutes. See 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. Do you see protoplasmic processes connecting the just-cleaved blastomeres? Sketch an 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 body formation occurs soon after, a loose jelly being the only covering of the egg.

Cleavage may be irregular or even chaotic, but usually the slightly amoeboid egg undergoes three equal total cleavages, each at right angles to the preceding. The separating pairs of blastomeres tend to retain broad protoplasmic processes connecting each to the other on the side opposite the cleavage furrow until the next cleavage is about to 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 the sea water, or other unfavorable circumstances.

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

At the end of 24 hours the embryo is a "preplanula" (Teissier, '27) with an elongated ovoid form, recognizable polarity and ciliation which enables it to swim heavily. During several days it lengthens, one end becoming slimmer and slimmer,
while it rolls and crawls along the bottom like a planarian. The big end which goes first in this movement (anterior) 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 substratum, where it shortly produces a mouth and a succession of tentacles. The new polyp elongates, its attached end meanwhile sending out a number of anastomosing and encrusting hydrothecae from which sprout new polyps. Is the first polyp to form in a colony (that which metamorphoses from a planula) always one type of zooid or may it be any one of three types?

Illustrations other than those accompanying may be found in Bunting, 1924 and Beckwith, 1914 (cleavage); Teissier, 1927 (later planula development); and Guette, 1907 & 1916 (developing gonozooids).

Euphorium (July, August)

The gonozooids 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 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. T., 1904) Tease out embryos from different colonies and study all stages found. The eggs are so rich in yolk that they cleave like insect eggs. The gastrulation is by an extraordinary syncytial delamination. Metamorphosis is simple.

Eudendrium cleavage stages illustrated in Hargitt, 1904 (Zoöl. Jahrb.);
Gonozooi development in Guette, 1907. See also texts by Korschelt, 1936 and Ryman, 1940.

Development of Scyphozoa

Aurelia and Cranae (April, May, 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 brown sacs in the lobes. Tease off some of this material into a drop of sea water on a slide and examine under the microscope. Embryos of different stages can be found, from spherical cleaving eggs to oval gastrulating forms and fully formed, stocky, active planulae. (Hargitt, C. T., 1909; Heim, 1900). Illustrations of Aurelia development are included in this outline. 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
scyphistoma stage can be easily followed. The resemblance of the scyphistoma to a simple polyp is obvious. Attachment plus the formation of 2 to 4 tentacles occurs on the second day, as does the development of an open mouth. There are 8 tentacles at 4 days, 16 at 2 weeks, 24 at 1 month. Sketch the scyphistoma in side view and in top view.

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

Aside from the accompanying illustrations you may find Aurelia life history illustrated in the texts of MacBride, '14; Korschelt, '36; and Hyman, '40.

GLOSSARY

Acraspedote - lacking a velum.
Actinula - A tentaculate larva resembling a short, stalkless polyp.
Blastostyle - A reduced gonozoid; a club-shaped projection bearing the gonophores on its sides.
Circular canal - Same as ring canal. Part of medusoid gastrovascular system encircling the margin of the umbrella and connecting with the radial canals.
Coeleenteron - Gastrovascular cavity of a polyp.
Coebloblastula - A hollow blastula. Synonymous with conventional use of "blastula".
Coenosarc - The living tissue of the hydrocaulus or stolon.
Dactylozooid - Polyp specialized for protection and bearing a battery of cnidoblasts (namatocyst-containing cells).
Diploblastic - Having two body layers, ectoderm and endoderm.
Ephyra - Free-swimming, immature medusa stage of scyphozoans, budded from scyphistoma.
Gonangium - The gonotheca plus its enclosed blastostyle with gonophores if present.
Gonophore - A medusa bud. (Pertains to buds of both complete and reduced medusae.)
Gonosome - Collective term referring to the total assemblage of sexual parts of a polyp.
Gonotheca - The perisarc structure surrounding the gonangia in Calyptoblasts.
Gonozoid - Polyp specialized for reproduction.
Hydranth - Elongated polyp structure with terminal mouth and bearing tentacles.
Hydrocaulus - The "stem" of a polyp.
Hyurorhiza - The stolon or attachment region of a polyp.
Hydrotheca - The perisarc surrounding the hydranth in Calyptoblasts.
Manubrium - Elongated distal part of body of hydranth, or of corresponding medusa region.
Metagenesis - Alternation between polyp and medusoid generations.
Perisarc - Chitinous, secreted exoskeleton of certain polyps.
Planula - Ciliated, elongated, tentaculate larva of Cnidarians.
Proboscis - The manubrium.
Radial canal - Part of medusoid gastrovascular system connecting gastrocoel of medusa with circular canal.
Scyphistoma - Polypoid larva of Scyphozoans.
Spadix - Central core of gonophore or sporosac on which sex cells develop.
Sporosac - Highly reduced gonophore.
Trophozoid - Polyp specialized for feeding.
Velum - A "shelf" extending centripetally from just above margin of medusa bell.
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PAPERS ON SCYPHOZOA


Embryonic Development of hydractinia

A  2nd maturation division
B-F Cleavage stages
G Gastrula (surface view)
H Gastrula (section)
I Early planula
J-X Planula elongation
N-U Planula truncation
P-Q Tentacle bud formation
R Stolon formation
S Adult with stolon network and bud
1. ectoderm
2. endoderm
3. attachment region (anterior)
4. tentacle bud
5. manubrium
6. stolon
7. newly budded individual

A-I adapted from Bunting, 1894; J-S adapted from Teissier, '27.
Plate 3
A  Undivided egg
B-C  Cleavage stages
D  Section through late "blastula"
E  Establishment of germ layers (section)
F  Establishment of coelenteron (section)
G  Flattened planula
H-M  Tentacle formation and establishment of definitive form

1. ectoderm
2. endoderm
3. coelenteron
4. tentacle bud
5. manubrium
6. attachment region

A-F adapted from Lowe, '26; G-M adapted from Berrill, '52.

Plate 2
Embryonic Development of Cyanea
(holds also for Aurelia)

B-D Cleavage stages. Somewhat irregular.
E Blastula. (surface view)
F Blastula. (section)
G Gastrula. (section)
H Late gastrula. Endoderm discreet and blastopore closing. (section)
I Planula. (section)
J Encysted stage (may occur under poor conditions)
K Attached planula
L-N Tentacle and manubrium formation
O Scyphistoma with beginning strobilization
P Organization of "metameres" into ephyrae
Q Tentacle resorption by terminal ephyra
R Terminal ephyra ready to be liberated as d medusa

Adapted from J.W. & G.T. Hargitt, '10.

Plate 4
FERTILIZATION

The study of fertilization and artificial activation has long been a meeting point for embryologists interested in the initiation of development and for physiologists attracted by more general aspects of cellular stimulation or interaction. The selected bibliography for this section of the course will illustrate the scope of studies carried out in this field. The laboratory exercises are designed for orientation in regard to the handling of marine eggs and the appreciation of the myriad problems in fertilization, activation and early development which await solution.

Classification

It is well to remember that the differences among eggs can in part be explained by taxonomic position. Wider differences among fertilization mechanisms would be expected, for example, between eggs of an annelid and an echinoderm than between a chordate and an echinoderm. To a large extent this expectation is realized. In the area of comparatively small differences, the eggs of Arbacia, for example, differ more widely from the eggs of the camardont sea urchins than do two members of the latter group (e.g., Psammochinus and Paracentrotus). Failure to take into account taxonomic relatedness can lead to errors of judgement in generalizing. The following classification is taken from Hyman's "The Invertebrates: Echinoderms" and from "The Invertebrata" (2nd Ed.) by Borradaile and Potts.

PHYLUM ANNElIDA

Class Polychaeta

Order Errantia

Nereis virens, N. Limbata (Woods Hole)
Platynereis (Woods Hole)

Order Sagartia

Arenicola (Woods Hole)
Amphitrite (Woods Hole)
Chaetopterus pergamentaceus (Woods Hole, Europe)
Pomatoceros triqueter (Scandinavia, Naples)

PHYLUM MOLLUSCA

Class Pelecypoda

Order Filibranchiata

Mytilus (Woods Hole, California)
Pecten (Woods Hole)

Order Bulimulinbranchiata

Spisula solidissima (Macra) (Woods Hole, New Jersey)
Macra (Naples, Florida, Australia)
Ostrea (universal)
Pholas (Europe, New Jersey)

PHYLUM ECHINODERMA

Subphylum Eleutherouzoa

Class Asteroidea - the starfish

Order Fiercifera

Asterias forbesii (Woods Hole)
Asterias vulgaris (Woods Hole)
Asterias rubens (European)
Asterias glacialis (arctic)
Henricia (Woods Hole)
Pisaster (California)
Class Ophiuroidea
Order Ophiurae
Ophioderma brevispinula (Woods Hole)

Class Echinoidea
Order Asteroidea
Diadema (Bermuda)

Order Stichasteridea
Arbacia punctulata (Cape Cod, Florida, West Indies)
Arbacia lixula (Naples)

Order Echinoidea
Lytechinus (St. Atlantic, California)
Toxopneustes (Bermuda)
Tripneustes (Bermuda)
Echinus esculentus (Britain, Scandinavia)
Psammochinus miliaris (Britain, Scandinavia)
Psammochinus microtuberculeatus (Naples)
Parametra livida (Naples, Roscoff)
Strongylocentrotus depressus (Hain, Scandinavia)
Strongylocentrotus purpuratus (California)
Strongylocentrotus franciscanus (California)
Strongylocentrotus pulcherrimus (Japan)
Sphaerechinus granularis (Naples)

Order Clypeasteroida - the sand dollars
Echinocardium cordatum (Scandinavia, Mediterra, elsewhere)
Briareus lyrifera (Scandinavia)

Class Holothuroidea
Order Dendrochirotida
Thyone biaureus (Woods Hole)

PHYLUM CHORDATA
Subphylum Hemichordata
Class Enteropneusta
Saccoglossus kowalevskii (Woods Hole)
Discoglossus

Subphylum Turicata
Glycera (Woods Hole)
Holguina (Woods Hole)

Subphylum Cephalochordata
Amphioxus (Japan, Scandinavia, Bermuda)

Subphylum Vertebrata
Class Osteichthyes
Fundulus heteroclitus (Woods Hole)
GENERAL PRECAUTIONS IN THE HANDLING OF THE GAMETES AND LARVAE OF MARINE ANIMALS.

1. **Cleanliness**: Use only dishes that are biologically clean. Avoid using bichromate cleaning solution unless followed by prolonged rinsing. Detergents are good fertilization inhibitors; rinse dishes carefully and lay them upside down on towels. Pipettes used for eggs and sperm should be rinsed immediately in sea water to avoid encrustation of dried gametes.

2. **Avoid overinsemination**: Remember that very few spermatozoa are required to bring about fertilization. If the egg suspension becomes noticeably more milky after insemination, you have added too much sperm. Either use a toothpick to introduce sperm, or measure amount as described under section on echinoderms. Label sperm pipette and use only for that purpose.

3. **Keep temperature low**: Remember that marine animals are accustomed to temperatures 5 to 15 degrees C. below room temperature. Never leave stock cultures at room temperature; they may not develop normally. Remember that eggs which normally develop, for example, at 30°C. may be heat-killed at 15°C!

4. **Overcrowding**: Remember again that eggs spawned under natural conditions probably never have to face overcrowded conditions. Avoid this very common source of abnormal development by placing so few eggs in a single culture that they can hardly be seen. If they form anything like a continuous layer on the bottom of the dish, they are too numerous. It is far better to discard half a culture than lose the whole culture through overcrowding.

5. **Equipment**: The climate at Woods Hole is especially hard on equipment and optics. Be sure to rinse off objectives or any other parts of microscopes that may come into contact with sea water or the sea fog that carries salt. Use stop-clock grease on the mechanical parts of your microscope.

FERTILIZATION IN NEREIS

**Breeding habits of Nereis limbata**

Nereis can be made to swarm in the Eel Pond about an hour after dark at certain phases of the lunar cycle (see Lillie and Just, 1913) by shining an artificial light on the water from the dock. This procedure will yield a few animals almost any evening. The females (fat and greenish bodies) should be isolated in separate finger bowls. Separation of the males is not so crucial.

Females may burst while in captivity and shed their eggs; if not, they can be cut open and the eggs will then be shed into the dish. Place a few eggs in a Syracuse dish and observe with a low power of the microscope (100X: 10X ocular and objective). Nereis eggs are anisodiametric: 140 microns as seen from above, and 100 microns high seen from the side. (How would you go about seeing an egg from the side?) Note the large germinal vesicle (typical of eggs before meiotic divisions), the oil droplets (note what happens to these in the first few minutes after fertilization), the thick, finely granular cortex, and the vitelline membrane. Is there an external jelly layer? How can you tell?

In your observations of fertilization and early development, never rely on
preparations that have been kept on the stage of the microscope, as temperature and possibly other factors may be operating to make these cultures abnormal. A convenient way to keep constant observations on normal material is to remove a drop of eggs, place it on a slice under a cover glass supported at the four corners by scarring each corner over soft plastigene. Observations should be made on the eggs in the center of the preparation; an occasional look at eggs near the edge of the cover-glass will tell when the preparation should be renewed. All powers of the microscope can be used with such a preparation, and manipulations of various kinds (sorting of eggs, isolation of blastomeres, etc.) can be carried out under such plastigene-supported cover glasses.

After you have become familiar with the morphology of the unfertilized egg, look closely at sperm under the oil immersion objective. You will see the most detail if you partially dark-adapt your eyes and use low light intensities. Note the head, middle-piece and tail regions of the sperm and their characteristic movements. Draw a sperm cell to scale, noting especially the acrosome in front. Next observe some of the "rejected sperm" attached to the extruded jelly of fertilized eggs. Is their shape the same? Note especially the front.

Find two eggs that are touching one another and inseminate them. Note how they are pushed apart by jelly extruded from their cortices. The margin of the jelly layer can be seen by the ring of rejected sperm attached to it. By 5 or 6 minutes after fertilization, the "Fertilization membrane" has raised slightly from the surface. Find an egg showing a tangential view of sperm attachment; by 8 to 10 minutes an entrance cone begins to form. The sperm head becomes hidden from view within the next ten minutes, but does not enter for some time (how long?). When it finally penetrates (or is drawn in), the middle piece and tail are left outside.

The first developmental changes to follow insemination are those involved in the meiotic divisions. The circulation of the germinal vesicle is followed by the extrusion of the first and second polar bodies. These establish the animal pole of the egg. What relation has the polar axis to the plane of cleavage? At 21°C, half of the eggs in a developing culture will form the first polar body at 45 min., the second polar body at 60 minutes, and will cleave at 95 minutes. If time permits observe the second and third cleavages. The third division, from 4 to 8 cells, produces 4 micromeres by spiral cleavage.

Centrifuged Nereis eggs

Fill 2 centrifuge tubes half way with 0.95 M sucrose (made with glass-distilled water) and layer eggs in sea water carefully over them. Shake one of the tubes laterally so that a sea water-sucrose gradient is established and mark the two tubes suitably. Spin the eggs preferably in an air-turbine centrifuge (ask for instructor's help in this) or in the Emerson electric centrifuge. The former will give forces up to 200,000 X gravity, whereas the latter will apply only about 10,000 X g. About thirty minutes with the air-turbine centrifuge at 65 lbs. pressure should give good stratification. An hour with the Emerson centrifuge does almost as well. Is there a difference between the eggs in the two tubes? Note the position of the oil drops, yolk; where has the cortical material gone? What new structures can be seen inside the germinal vesicle? Note the "vertex" near the centrifugal pole; why did this form? Wash away the sucrose and inseminate to observe jelly extrusion. Does all the cortical material disappear in this process? (To observe jelly extrusion, rub some Chinese ink in the sea water, but remove it again as it will disintegrate completely!)
Relation of sperm entrance to plane of cleavage

Insemination in the presence of Rhode ink will reveal an arrow the point of sperm entrance. Observe these eggs during cleavage to determine the relationship of the entrance point to cleavage plane.

Exaggerated entrance cones

Place some Neris eggs inseminated 5 to 8 minutes earlier in a Syracuse dish containing alkaline NaCl (pH 10.3 to 10.5). Observe immediately. The vitelline membrane will elevate due to a sudden inhibition of jelly release through the membrane and a subsequent accumulation of jelly in the pravitelline space. The vitelline membrane remains permeable to water; swelling of the pv. space is due to colloid osmotic pressure. Elevation of the membrane stretches out the sperm entrance cone between the membrane and the egg surface, forming a long filament which frequently causes marked indentation of the membrane. Eggs kept in the refrigerator may become polyspermic upon insemination and show several of these exaggerated cones upon treatment with alkaline NaCl. Watch the sperm moving across the pv. space to the egg surface. Repeated alkaline NaCl treatments may free eggs of their membranes. This method has been employed in isolating blastomeres.

Later development

Keep a sparse culture of Neris eggs going as long as possible to have an occasional look at the development of the trochophore larvae. These will be studied in detail later.

Chaetopterus

The polychaete Chaetopterus neogentilus is collected in the muddy bottom coves of nearby islands. Each specimen lives in a parchment tube with two commensal crabs. Eggs are obtained simply by shaking an excised parapod in a dish of sea water. Sperm should be kept "dry" by squeezing one or two male parapods into a dry dish and keeping the seminal fluid covered in a refrigerator. To inseminate, add one drop of "dry" seminal fluid to 10 ml of sea water; with another pipette, add 2 or 3 drops of this "stock sperm" to 10 ml of sea water containing eggs.

Freshly shed eggs are in the germinal vesicle stage. Unlike Neris, the eggs of Chaetopterus begin to mature spontaneously when shed into sea water. Calcium ion is necessary for this process, which has been studied extensively recently by Goldstein. The maturation process which begins in sea water becomes arrested in metaphase of the first meiotic division. The spindle can be seen at this stage as a clear area near the animal pole. The eggs of Chaetopterus are highly pigmented and granular, so that little structural detail can be observed unless these eggs are stratified by centrifugation. Stratified eggs in the germinal vesicle stage can be obtained by spinning whole parapods; later on, at metaphase of the first maturation division, stratification is more easily accomplished. For information on centrifugation of these eggs, see papers by Heilbrunn and Wilson. In centrifuged eggs, note the cortex and its granular complement, the spindle (or the germinal vesicle in earlier stages), and the various granular layers.

In observing the process of fertilization, note changes in the egg surface and in the germ head. Can you see any? Is a membrane elevated from the surface? How far?
In the early development of Chaetopterus eggs, the first discernible event is the formation of the first polar body, which appears at 14-15 minutes in 50% of the eggs at 21°C. This is followed by the second polar body at 27 minutes, the "pear shaped stage" at 46 minutes, the polar lobe bulge, 52 minutes; cleavage with polar lobe attached, 58 minutes; cleavage completed with polar lobe reserved into one blastomere, 62 minutes; and the 4-cell stage 62 minutes.

Note during development to the 2-cell stage the changes in shape that the egg undergoes. What could these mean? Keep a few Chaetopterus larvae to observe trochosphere stages.

**SPISULA SOLIDISSIMA**

Small specimens of the surf-clam Spisula solidissima can be found locally and may be mature from late in May through August. The larger specimens more generally used come from the colker water on the north side of the Cape. For this reason, there is some difficulty in keeping these animals in good condition in the laboratory. When placing these animals in the tanks, it is necessary to hold them each under water for a few seconds with siphons up to expel gas trapped in their gill chambers; without this ritual, the animals suffocate.

Large numbers of gametes can be obtained by breaking the shell near the umbo, cutting the adductor muscles and removing the visceral mass and foot intact. The gonad is exposed by trimming the gills and cutting directly into the visceral mass. The ovary or pieces of the ovary are placed in an egg strainer made by cutting off the bottom of a plastic centrifuge tube (diameter 1 inch) and fastening a circle of bolting cloth to the inverted lip by means of an elastic band. Filtered sea water is then poured over the ovary in the egg strainer and eggs pass through the strainer into a beaker, leaving the ovarian tissue and other debris behind. Eggs should be allowed to settle in the beaker, and the supernatant should be decanted. This procedure should be repeated at least three times. The use of a siphon for removing the supernatant results in less loss of eggs, although it is not a good idea to lose some of the lightest eggs, as these are probably the least mature. Excised testes should be cut up and placed on a 3 inch square double layer of cheesecloth which has previously been wet with pyrex distilled water and wrung dry. The corners of the cheesecloth are then gathered and twisted to press the seminal fluid out of the bag so formed and into a dry dish. This can be covered and kept refrigerated for at least 24 hours and possibly longer. This "dry" sperm is diluted 1 drop to 10 ml. for the stock suspension, and 1 to 3 drops of this is used to inseminate eggs in 10 ml. of sea water, the quantity of sperm varying with the number of eggs and the purpose of the experiment. Spisula eggs are very susceptible to polyspermy; the presence of more than two pronuclei at about 50 minutes after insemination is a good criterion of polyspermy. Spisula eggs are also very susceptible to crowded conditions; these can lead to delayed or inhibited mitosis (see Allen, 1952).

The eggs of Spisula solidissima are in some respects similar to eggs of Macrta stultorum, which were described early in the century by Kostanecki at Naples. Early workers at Woods Hole called the local clam "Macrta", even though the name Spisula had been given to it in 1833 by Dillwyn. A true "Macrta" egg is available in Florida. The Woods Hole Spisula egg has been used in only a few major studies; Schechtman studied the aging responses in this egg, and more recently a study of fertilization and artificial activation was done by R. D. Allen.

The Spisula egg is about 56 microns in diameter and quite transparent. Slightly
eccentrically located is a large (31 microns) germinal vesicle nucleus containing the large amphinucleolus (true nucleolus and nucleolus) and the chromosomes, which are attached to the inside of the nuclear membrane except for the nucleolus chromosome. The cytoplasm is only moderately granular and contains about 11% centrifugeable fat particles. Stratification by centrifugal force takes several minutes at 100,000 x gravity in the air-turbine centrifuge. The cortex of the egg is thicker than that of the sea urchin and contains large cortical granules which do not break down at fertilization except for a few at the point of sperm entrance and which cannot be torn from the cortex when forces of 200,000 x gravity is applied. The egg is surrounded by a jelly layer about 2 microns thick, which is best seen when eggs are crowded together as an invisible barrier between them, but which is also stainable with Janus Green. Recently shed eggs are invariably irregular in outline. They soon become spherical on standing in sea water.

Fertilization details are difficult to see in this species. Look for changes in the sperm's acrosomal region on contact with eggs. Sperm penetration is sometimes seen on the top or side of an egg. The sperm enters much more readily than in Nereis; it probably is inside within 2-3 minutes. Watch the egg surface for an indentation of the cytoplasm which sometimes occurs on fertilization.

The ease with which details of early development are seen in this species makes up for the difficulties in observing details of fertilization. Within 5-7 minutes after insemination, the nucleus begins to change; the transparent true nucleolus disappears, leaving the nucleolus alone in the nucleus. Then this disappears in the living egg, although in fixed preparations it can be seen that it remains intact until after the nucleus breaks down, the nucleolus contributes some staining substance, probably a polysaccharide to the spindle of the first meiotic division (Allen 1952). The first meiotic metaphase lasts from 13-26 minutes after insemination. The first polar body appears at 29 minutes, and its position establishes the animal pole of the egg. The animal pole flattens, then rounds up at 35 minutes. The second polar body appears at 39 minutes after fertilization. There then follows a brief flattening period before the male pronucleus becomes visible at 50 minutes and the appearance of the female pronucleus from the animal pole follows within 2-3 minutes. The two nuclei swell almost simultaneously to a diameter of 11-12 microns before they merge and break down, giving rise to a clear area in the center of the cell. Cleavage is unequal, with the polar bodies lying at the animal pole in the furrow. (Times are for development at 21°C.

Artificial activation

If time allows, artificially activate some Spicula eggs with 4% isotonic KCl in sea water, potassium free sea water (sodium stimulation) or with an ultraviolet lamp. Watch the immediate reaction of the eggs to the stimulation. What does their change in shape indicate? How far do these eggs develop?

ARBACIA PUNCTULATA

The eggs of various sea urchins have been possibly the most-used classical egg material for embryological and physiological studies. The egg of Arbacia is rather different from the eggs of most other sea urchins in being smaller and containing red echinochrom granules. It is interesting to note (see classification earlier) that Lyman has classified Arbacia in a different order from the other sea urchins so often studied.

In the early years at Woods Hole, investigators placed a heavy drain on the supply of urchins; whether this was the reason for a depletion in their supply is not known.
However, for the last five or ten years, the general supply of urchins has been jealously guarded. Recently there have been signs that urchins were becoming more common locally; it is not known how much of this is due to "stocking" operations carried out by the supply staff in the area of former urchin beds, and how much is due to other factors. Sea urchins normally shed several times during a season, often at rough weather.

To preserve the supply of urchins, it is highly desirable to sex them to avoid opening several males in a row, for example, while searching for a female. Mrs. E. B. Harvey has described a simple electrical method for sexing Arbacia; this method is preferable to the KCl injection method, which can also be used. The apparatus department provides a simple electrical set-up with which to stimulate the surface of the sea urchin (usually with a lead electrode). If the animal is ripe, gametes will ooze out of the gonopores (aboral surface) in sufficient quantity to determine the sex of the specimen. Eggs are blood-red; sperm are white. Sexing usually stops if the animals are rinsed in sea water and replaced in the aquarium. For experiments, eggs can be sexed electrically to give the desired number for an experiment, or the animal can be cut open around the oral surface and the gonads (5) removed with a stainless steel spoon. The gonads are then transferred to an egg strainer (see section on Spisula) made of coarse balting silk. If the eggs are strained too rapidly, their jelly layers may be torn off. Sperm should be stored "dry" as described with the other species. Excess testes can be wrung in moist double-layer cheesecloth, or sperm can be shed dry into a clean dry dish by the electrical method.

**Spermatozoa**

Undiluted Arbacia seminal fluid contains approx. $2 \times 10^{10}$ spermatozoa/ml. One drop of this "dry sperm" in 10 ml. of sea water brings the concentration down by a factor of 200 to 10 ml. Two drops of this stock sperm suspension in 10 ml. of sea water containing eggs dilutes the sperm concentration by another factor of 100 to $10^8$ sperm/ml. This sperm density is adequate for fertilizing small numbers of eggs. Three times this much gives about the maximum rate of fertilization without polyspermy. Densities between $10^7$ and $10^8$ sperm/ml. result in varying percentages of polyspermy. Undiluted sperm will keep in a refrigerator for at least two days; dilution, however, rapidly destroys their fertilizing power. Consequently, a new stock sperm suspension should be made up for each insemination.

Observe some living spermatozoa under oil immersion. The acrosome, nucleus and midpiece are roughly 0.3, 1.0 and 1.0 microns in length respectively. The tail is about 45 microns long and 0.1 micron wide; its axial filament protrudes a short distance beyond the end of the sheath. Sketch a spermatozoon and describe its movement. Place side by side on a slide a drop of stock sperm suspension and a drop of "egg water" (supernatant from eggs which have stood for an hour or more). Place a cover-glass over the two drops so that they mix at the middle of the cover glass. Note the agglutination; do the spermatozoa stick together by their heads or tails? Can you detect any changes in the sperm acrosome?

Add one ml. of $10^8$/ml stock sperm suspension to A. 9 ml. sea water, B. 9 ml. of egg water. Observe at 10 minute intervals. What effect does egg water have on the sperm besides agglutination? Will these sperm activate eggs?
Unfertilized Ova

Arbacia punctulata eggs complete both meiotic divisions while in the ovary; presumably the divisions themselves must be rapid, for very seldom are eggs observed with maturation spindles or polar bodies. Unripe gonads often do exhibit large numbers of eggs in the germinal vesicle stage. These will often respond to insemination by forming hyaline blisters at the points of sperm entrance. At this stage, the mechanisms for membrane elevation and for preventing polyspermy have not developed. No development results from insemination at this stage.

The mature Arbacia egg has a transparent jelly layer that swells to about 30 microns in diameter. Most of the jelly can be removed if desired by swirling the eggs for 20-40 seconds in sea water pH 4.5. Longer treatment removes the jelly entirely, but may injure the eggs. Beneath the jelly layer is the vitelline membrane. It is too thin to see on the egg surface, but is visible as it elevates from the surface at fertilization. Beneath the vitelline membrane is the cortex, containing characteristic granules from 0.5 to 0.8 microns in diameter. The cortex is apparently an extracellular layer (Farr, and Larsen), and in the sea urchins Psammochirus and Arbacia can be removed in strips (Allen). The cytoplasm of Arbacia contains oil droplets, mitochondria, yolk granules and the slightly larger red echinochrome granules. (As Hartmann (1939), and Tyler (1939) showed, the red echinochrome is a substituted naphthoquinone related to the K vitamins.) The formed cytoplasmic components and the cortical granules are best seen in centrifuged eggs. The polarity of the egg can be determined only by marking the position of the micropyle or funnel with Janus green or Chinese ink. The polar bodies, which were extruded at the animal pole have by this time been lost in the medium. The position of the nucleus probably marks the animal pole only in very unripe eggs (see Hadley, 1934).

Centrifuge a sample of unfertilized eggs at 10,000 g for 1/2 hour and sketch one in "side view", noting the five layers of stratified material: oil cap, hyaline zone, mitochondria, yolk zone and pigment zone. Where is the nucleus? Have the cortical granules been displaced? Are they thicker in the centripetal hemisphere? If time allows, centrifuge some eggs at higher speeds in the air-turbine centrifuge (consult manual of E. B. Harvey) to separate them into halves and quarters. Inseminate these and watch development. The mitochondrial layer is stained brilliantly by either Janus B Green or by Cresyl violet.

Cortical reaction

Place a drop of dilute eggs on a slide and cover with a coverslip. Support the coverslip with plasticine "feet". Withdraw a little water so that the water interphase shrinks away from the outline of the coverslip. Add a drop of stock sperm suspension to one corner of the preparation and select the sperm density that is most convenient for the subject under study. (For the details of sperm penetration, select an egg from the point of insemination to get a low sperm to egg ratio; for observing the time of membrane elevation, select an egg with plenty of sperm around it.) The penetrating sperm is usually one of the first to arrive at the egg surface (see Walton's "first orbit theory". Exptl. Cell Res., in press 1956), and it is safe to assume that the sperm has entered by the time the membrane begins to elevate. Watch for a characteristic wrinkling on the egg surface; this is caused by expulsion of the cortical granules. Watch also for the appearance of the hyaline layer. Where did the substances occupying
the vitelline space come from? What is the mechanism of membrane elevation? 
Compare the appearance of an unfertilized egg with one 10 minutes after fertilization; note the distribution of the red pigment granules. What happens to the pigment granules in fertilized centrifuged eggs?

If a Wochsel-Prasensor nach Siedentopf (Zeiss) is available (or a standard dark-field condenser will suffice), observe unfertilized eggs under dark-field with the most brilliant light source possible (direct sunlight is best). Note the orange ring around the egg surface; this is the unfertilized cortex and "luminous" hyaline layer. Beneath it are the cortical granules and the plasma membrane. 
Incubate and watch the orange ring fade, either gradually and regularly if the sperm enters directly above or below the egg, or as a wave if the sperm enters anywhere near the equator. For the kinetics of the cortical change, see papers by Lord Rothschild, and by Allen and Hagstrom.

Early development

With high dry or oil immersion, watch the growth of the sperm aster and approach of the two pronuclei. If time allows, plot the paths of these nuclei prior to fusion. Note the clear streak in the egg both before and after the nucleus breaks down. Later on, watch the spindle form, and try to trace the astral rays out to the cortex. Observe the cleavage furrow; how does this furrow differ if the hyaline layer is dissolved away by calcium-free sea water or digested by trypsin? Set aside a sparse culture of Arbacia eggs and observe through the pluteus stage.

Special optics

If phase optics are available for demonstration, look at the granular components of the cell, the nucleus, the spindle, the skeleton of the pluteus. Repeat these observations with an interference microscope, if available. Using white light, this instrument gives variable color contrast depending on the thickness of the specimen and its index of refraction.

ASTERIAS FORBESII (or A. VULGARIS)

Specimens of the common starfish, Asterias, are dredged in the neighborhood of Woods Hole. Fresh material seems to yield the best eggs. The largest and fattest specimens are really only worth keeping. Sexes are separate in Asterias, but it is not possible to distinguish them by external characteristics. To obtain gametes, make a small puncture in one arm close to the disc and pipette a few gametes onto a slide to determine the sex of the specimen. If it is a female, remove the arm completely, and slit it along the mid-dorsal line to expose the pair of bulging ovaries, which are a typical pale salmon color. Then, with a pair of clean forceps, carefully extract each plume-like ovary by grasping it near its point of attachment at the disc end, closing the gonoduct, and rinse it with as little injury as possible in one 1750 ml. finger bowl and transfer it to another container of the same size. Do not cut up the ovary, but rather let it shed normally for about 5 minutes; these first shed eggs are the best. Discard the rest of the ovary and save the specimen for use later in the day. Injured animals should be kept in a separate aquarium with rapidly running sea water and not kept for more than two days. The eggs should be washed by siphoning off the supernatent fluid and replacing it with sea water at least twice. During this time, the eggs should be examined occasionally to observe meiotic stages. If the eggs have been left relatively undisturbed for about 30 minutes, it is time to
decide whether they are suitable for use in an experiment. About the best that can be expected of Asterias eggs is 85-90% germinal vesicle breakdown; of the eggs that spontaneously initiate maturation, nearly all should elevate fertilization membrane.

**Spermatozoa.**

One way to obtain sperm rapidly is to keep a "dry" testis in the refrigerator; when sperm are needed, a small piece of the male gonad is shaken in sea water to make up the stock sperm suspension, and this is added in small quantities to the egg. A more quantitatively reproducible method of insemination is that already suggested for the sea urchin and the clam above. Testes can be placed on a square of moist cheesecloth which is then gathered by the four corners into a bag and pressed. Seminal fluid so obtained can be kept in the refrigerator for at least two days and can be measured out dropwise as needed.

The sperm of the starfish are perhaps the best material in which to detect acrosome filaments. These can be seen with phase contrast, and perhaps even with bright field. It used to be thought that the filament which Hol (1879) and later Chambers (1930) saw joining the sperm and egg was a product of the egg; now, however, this has been shown to be the acrosomal filament of the sperm. As the Colwins have shown in Japan and Woods Hole, the egg reception cone climbs up the acrosome filament after the latter attaches to the egg surface.

**Eggs.**

Observe some freshly shed Asterias eggs. Note the large clear germinal vesicle and the yolky, yet surprisingly transparent and unpigmented cytoplasm. These eggs contain so much yolk that it is difficult to stratify them by centrifugation. Note the vesicular nucleolus. If the oocyte is placed on the stage of a horizontal microscope, the nucleolus will fall through the nucleus (Gray, Harding). Note the cortical layer of the immature Asterias egg. The number of cortical granules in this layer seems to increase during maturation. (The origin of these granules has been studied in the eggs of the sea urchin by Manne and Harvey and by McCulloch).

**Fertilization.**

Watch the equator of an egg being attacked by only a few sperm. Remember that the chances of sperm penetration in the equatorial band that you can see are about one in three. Accordingly, make several observations. Can you see a reception cone? A filament between the egg and sperm? It must be stressed that for extremely fine observations of this sort, clean optics and slides are required; also good illumination and patience! Watch for details of the cortical reaction (cortical granule breakdown, membrane elevation, dark-field color change as in the sea urchin).

**Early development.**

Development in Asterias begins at the same stage as in Chaetopterus. The extrusion of polar bodies must precede egg and sperm nuclear fusion and cleavage. The type of cleavage and gastrulation is similar to the sea urchin. Again, save a few larvae to have a preliminary look at the bipinnaria stage.
Precautions

Remember especially that the eggs of Asterias are more fragile than those of many other marine animals. If treated carefully, they will yield good results in fertilization and development experiments. Be careful of the following: 1. avoid contamination by perivisceral fluid, 2. do not overinseminate, 3. do not overcrowd; a solid single layer of eggs on the bottom is too many! and 4. use only fresh motile sperm.

ECHINARACHNIUS PARVA

The supply of Echinarachnius comes from the colo water on the north coast of the Cape; consequently even the temperature of the sea water table is likely to put quite a strain on eggs of this species. They are best used on cold days or in an air-condition room. If a specimen turns green, either all over or in spots, it is probably ready to be discarded.

Gametes can be obtained either by cutting the oral surface completely around, about 1/4 inch inside the margin. The oral part of the test and the Aristotle's lantern can be removed. Specimens cut open in this way will usually shed if placed aboral surface down over a stemmer dish. If desired, the gonads can be removed from the test and placed in an egg strainer (see section on sea urchin or clam) and washed through. A third method to obtain gametes is to inject isotonic (0.53M) KCl into the visceral cavity. Sperm should be shed dry by the first or third method, or the testes can be kept dry in small dishes in the refrigerator. It is important that the eggs should be washed a few times before insemination. Sperm should not be diluted until ready to use.

Fertilization

Sand dollar eggs are larger than those of Arbacia (135 microns instead of 74) and are surrounded by a much thicker jelly layer in which very beautiful red pigment granules are suspended. The egg itself, freed from jelly, is pale yellow and resembles very closely the eggs of the Camerata urchins (Psammochinus, Paracentrotus, etc.) Echinarachnius eggs are fragile compared to those of Arbacia, but they can be stratified by centrifugation in much the same way. The cortex can easily be seen without stratification - especially with a 50X water immersion lens, if available. The cortical granules of Echinarachnius are larger than those of Arbacia and other sea urchins, and, consequently, this form is the best one at Woods Hole in which to observe cortical reaction phenomena. If you can locate the cortical granules at the top of an egg under high power, water or oil immersion, inseminate and watch these granules break down in a wave which sweeps the cell from end to end. Watch the membrane elevate and try to determine the time relationships between dissolution of the cortical granules and membrane elevation. Could there be a causal relationship between these two events? Do you see a hyaline layer?

The early development through cleavage stages of Echinarachnius eggs is not sufficiently different from that of Arbacia to warrant more than a review. However, save a sparse culture of larvae for comparison with those of Arbacia.

FUNDULUS HETEROCOLITUS

Fertilization in the eggs of the fish, Fundulus, is worth looking at because of the striking wave-like breakdown of its large cortical alveoli. The cortical reaction in another fish, Orzyzies latipes, had been much studied recently by Yamamoto in Japan.
SUGGESTIONS REGARDING EXPERIMENTAL PROJECTS ON FERTILIZATION AND ACTIVATION

1. Research projects should not be undertaken until the student has familiarized himself with the normal course of fertilization and early development in most if not all of the foregoing eggs.

2. It is suggested that the class split up into teams of two or three, several groups of which would attempt experiments in the same area if their interests coincide. Where specialized equipment is required, duplication of effort must be kept at a minimum.

3. It is preferable for members of a team to persevere until they have succeeded with one type of work, rather than switch to another type of experiment at the first disappointment.

4. Experiments that often prove to be the most interesting to follow sometimes suggest themselves during routine observations of early development. Don't hesitate to discuss possible research tasks with the staff. They will perhaps know whether any work has been done and can direct you to the proper source.

5. Careful and accurate data should be kept on all experiments, regardless of their outcome. Record temperatures, times, concentrations; make sketches of abnormalities etc. For all experiments, keep adequate controls; ask yourself if you have isolated a single factor for experimentation.

SAMPLE PROJECTS

The following is a very incomplete list of areas in which profitable experimentation can be carried out.

1. Artificial activation (both from the standpoint of cellular stimulation problem and of induction of development)
2. Interaction of gametes (an opportunity to repeat work on the interaction of egg and sperm substances)
3. Sperm aggregation rate (utilization of a new technique for determining the rate at which sperm impinge on the egg surface and adhere there)
4. Fertilization rate (separately or in combination with 3), a study of factors which retard or accelerate activation of the egg by the sperm
5. Centrifugation (experimental regulation of half and quarter production in sea urchin eggs, so that different gamete fractions go into different fragments; experiments with little-studied eggs)
6. Experimental hybridization (modification of egg surface so that heterologous sperm can enter and perhaps initiate development)
7. Partial fertilization (inhibition of the cortical reaction, interactions of nuclei with fertilized and unfertilized cytoplasm)
8. Experimental polyspermy (nuclear interactions, developmental abnormalities mechanism of block against polyspermy, role of hyaline layer, membrane)
9. Isolation of mitotic apparatus (repetition of D. Karrer's experiments)
10. Action of agents on eggs (effect of proteolytic enzymes on the surface, action of versene, peroxide, Ca-free medium, etc. see references)
TUNICATES

The subphylum Tunicata includes the sessile ascidians, the pelagic thaliaceans and the appendicularians with a permanent chordate tail. Each group has a unique organization and their interrelations are obscure. An excellent survey is available in Berrill's ('50) monograph.

Only forms representative of the sessile tunicates, i.e. the Ascidiaeae, will be studied here. Examples of both solitary and colonial forms will be available; review their adult structure and life history using some standard text or Berrill's ('50) account.

A. SOLITARY ASCIDIANS
DEVELOPMENT TO THE TADPOLE STAGE

Styela partita
Molgula manhattensis
Ciona intestinalis

1. Methods for obtaining eggs. All ascidians are hermaphroditic; some are self-fertile, others self-sterile. Styela, Molgula and Ciona are mostly self-sterile. The best material for study is obtained from naturally shed gametes.

Styela: normally sheds gametes between 4 and 7 p.m. Rose ('39) devised the following procedure for inducing natural spawning at a more convenient hour. Animals are kept in the dark in running sea water until 10 and 12 hours before eggs are needed. An artificial day is then started by illuminating the tank with a 40 watt bulb. Eggs and sperm are shed in clouds at the desired time and may be collected with a long pipette.

Molgula and Ciona: spawning usually occurs at daybreak. Rose's method for controlled spawning is to put a few animals in the dark in a large finger bowl of sea water. Molgula will shed within about 15 minutes after being brought out into the light, Ciona immediately.

"Minced Cultures". A more rapid, but less satisfactory, method of obtaining eggs is to mince the gonads from several animals (Molgula or Ciona) or several whole animals (Styela) in a dish of sea water. If the debris is carefully washed and the eggs removed, several of the latter will be fertilized and start normal development.

2. Normal development. The egg of Styela was made famous by Conklin ('05) whose superb plates should be studied with care. The following description holds for the eggs of Styela and, by and large, for Molgula and Ciona as well. There are certain important differences - such as the occurrence of yellow pigment only in Styela and the excessively large and beautiful follicle cells in Ciona - which the student should work out for himself.

a. Mature unfertilized egg. Eggs are of moderate size (Molgula: 110 u; Styela: 150 u). Note: choriand with outer follicle cells, inner follicle cells (pigmented?) in perivitelline space, clear egg cortex (pigment granules present?), endoplasm with gray yolk platelets, germinal vesicle.

b. Post-fertilization rearrangements. These changes can be observed only in Styela, and then only in eggs with sufficient yellow pigment. The latter varies in amount from season to season. Critical microscopy is essential. Use only
daylight for illumination and have the condenser diaphragm wide open. If your microscope has a dark-field attachment, this may be used to advantage.

Within 2-3 minutes after fertilization, the peripheral pigmented ooplasm begins to stream toward the vegetal pole (near which the sperm has entered). The inner follicle cells also move similarly through the perivitelline space. While the cortical pigment becomes concentrated in a vegetal cap, the gray yolk rises toward the animal pole. The sperm nucleus migrates up the periphery of the egg to a point just below the equator, then moves inward toward the center of the egg where it joins the female pronucleus. The yellow pigment tends to follow the sperm toward the equator and soon assumes the form of a broad crescent. As soon as this stage is reached, the three major axes (antero-posterior, dorso-ventral, and right-left) of the future larva can be determined. The center of the yellow crescent marks the posterior pole, and the arms of the crescent wrap around the right and left sides. The vegetal pole is directed dorso-posteriorly, the animal pole ventro-anteriorly.

Another remarkable feature of the egg at this stage is the fact that several areas are already visibly different from one another. Each of these areas, is destined to form specific larval organs. For example, the yellow crescent goes exclusively into muscle and mesenchyme cells, the gray cytoplasmic crescent on the opposite side of the egg constitutes neural and chordal materials, etc.; c.f. Conklin's figures.

c. **Cleavage.** The following approximate time schedule is for normally shed eggs; eggs from "minced" cultures are delayed apparently because they ripen at variable intervals after striking sea water.

<table>
<thead>
<tr>
<th>From</th>
<th>To</th>
<th>Time required</th>
<th>Total time since fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilization</td>
<td>2 cells</td>
<td>10 min.</td>
<td>40 min.</td>
</tr>
<tr>
<td>2 cells</td>
<td>4 cells</td>
<td>30 min.</td>
<td>1 hr. 10 min.</td>
</tr>
<tr>
<td>4 cells</td>
<td>8 cells</td>
<td>30 min.</td>
<td>1 hr. 40 min.</td>
</tr>
<tr>
<td>8 cells</td>
<td>16 cells</td>
<td>20 min.</td>
<td>2 hrs.</td>
</tr>
<tr>
<td>16 cells</td>
<td>32 cells</td>
<td>20 min.</td>
<td>2 hrs. 40 min.</td>
</tr>
<tr>
<td>32 cells</td>
<td>64 cells</td>
<td>20 min.</td>
<td>3 hrs.</td>
</tr>
<tr>
<td>64 cells</td>
<td>112 cells</td>
<td>20 min.</td>
<td>3 hrs. 20 min.</td>
</tr>
<tr>
<td>112 cells</td>
<td>218 cells</td>
<td>20 min.</td>
<td>5 hrs. 20 min.</td>
</tr>
<tr>
<td>218 cells</td>
<td>Neural plate</td>
<td>2 hrs.</td>
<td>12 hrs.</td>
</tr>
<tr>
<td>Neural plate</td>
<td>Tadpole larva</td>
<td>6 hrs. 40 min.</td>
<td></td>
</tr>
</tbody>
</table>

(i) **First Cleavage.** Meridional and equal; cuts yellow crescent in half, hence separates future right and left halves. Is the plane of reference for all cleavages after the third; hence cleavage is bilateral. According to the system of nomenclature proposed by Conklin ('05), the 2 cells are designated A02 (right) and A02 (left).

(ii) **Second Cleavage.** Meridional and nearly equal, at right angles to the first. Separates 2 anterior blastomeres (A3 on right, A3 on left) from 2 posterior blastomeres (B3 and B3). The two B cells contain little yolk and practically all of the yellow crescent. The clear cytoplasm goes equally to all four cells.

(iii) **Third Cleavage.** Equatorial and unequal, producing 4 animal cells (A4.2, A4.2, B4.2 and b4.2) somewhat smaller than the 4 vegetal cells (A4.1, A4.1, B4.1 and B4.1). Not strictly in the frontal plane but roughly separates future
(iv) Fourth Cleavage. Oblique; varies in different blastomeres. In the
two vegetal A4.1 and the two animal b4.2 cells, the furrows cut in an antero-
posterior direction to meet the second cleavage plane. In the two vegetal B4.1
and the two animal a4.2 cells, the furrows run from the lateral border of the egg
obliquely inward to reach the first cleavage plane (future mid-sagittal plane).
Thus, the animal and vegetal hemispheres (as well as the right and left halves)
become mirror images. Sixteen cells are produced and they and their subsequent
derivatives are labelled as follows:

<table>
<thead>
<tr>
<th>4 cells</th>
<th>8 cells</th>
<th>16 cells</th>
<th>32 cells</th>
<th>64 cells</th>
<th>Gives rise to</th>
</tr>
</thead>
<tbody>
<tr>
<td>A5.1</td>
<td>A6.1</td>
<td></td>
<td>A7.1</td>
<td></td>
<td>Entoderm</td>
</tr>
<tr>
<td>A5.2</td>
<td>A6.2</td>
<td></td>
<td></td>
<td>A7.2</td>
<td>Chorda</td>
</tr>
<tr>
<td>A4.1</td>
<td>A6.3</td>
<td></td>
<td></td>
<td>A7.3</td>
<td>Sp. cord</td>
</tr>
<tr>
<td></td>
<td>A6.4</td>
<td></td>
<td></td>
<td>A7.5</td>
<td>Entoderm</td>
</tr>
<tr>
<td>A3</td>
<td>a5.3</td>
<td>a6.5</td>
<td>a7.9</td>
<td>a7.6</td>
<td>Mesenchyme</td>
</tr>
<tr>
<td></td>
<td>a5.4</td>
<td>a6.6</td>
<td></td>
<td>a7.10</td>
<td>Notochord</td>
</tr>
<tr>
<td>a4.2</td>
<td>a6.7</td>
<td></td>
<td></td>
<td>a7.11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>a6.8</td>
<td></td>
<td></td>
<td>a7.12</td>
<td>Cerebral vesicle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a7.13</td>
<td>Epidermis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a7.14</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>a7.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a7.16</td>
<td></td>
</tr>
<tr>
<td>B5.1</td>
<td>B6.1</td>
<td></td>
<td>B7.1</td>
<td></td>
<td>Entoderm</td>
</tr>
<tr>
<td>B5.2</td>
<td>B6.2</td>
<td></td>
<td>B7.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B4.1</td>
<td>B6.3</td>
<td></td>
<td>B7.3</td>
<td>B7.4</td>
<td>Mesenchyme</td>
</tr>
<tr>
<td></td>
<td>B6.4</td>
<td></td>
<td>B7.5</td>
<td>B7.6</td>
<td>Mesoderm</td>
</tr>
<tr>
<td>B3</td>
<td></td>
<td>B7.7</td>
<td>B7.8</td>
<td></td>
<td>Mesoderm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>B7.9</td>
<td></td>
<td></td>
<td>Epidermis</td>
</tr>
<tr>
<td>b5.3</td>
<td>b6.5</td>
<td></td>
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<td></td>
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</tr>
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<td>b7.13</td>
<td></td>
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</tr>
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<td>b5.4</td>
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<td>b7.14</td>
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<td></td>
<td></td>
<td></td>
<td>b7.15</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>b7.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In using the above plan of cell lineage, note that: (1) after 3rd cleavage,
animal cells receive small letters (a and b), vegetal cells, capital letters (A and
B); (2) cells of right half have underlined letters (a, b, A and B); (3) anterior
cells are A or a, posterior cells are B or b; (4) first exponent after a letter
gives the generation to which a cell belongs; (5) second exponent indicates position
of cell relative to vegetal pole. To determine the mother cell of a given blasto-
mere: (1) leave letter unchanged; (2) subtract 1 from first exponent; (3) if second exponent is even, divide it by 2; if second exponent is odd, add 1 to it and then divide it by 2. To determine daughter cells, reverse the process.

(v) Fifth Cleavage. The fifth and subsequent cleavages are more difficult to follow. Some details may be observed with the help of Conklin's figures. The scheme of cell lineage should be mastered. In favorable material, the student may confirm Conklin's observation that different cells can be recognized by their natural color, as follows: entoderm-slate gray; chorda - lighter gray, less yolk; ectoderm and neural plate - clear and free of yolk; mesoderm - yellow.

d. Gastrulation and Neurulation. Gastrulation begins during the 7th cleavage and is substantially over by the close of the 8th; some 40 minutes are required. The gastrula passes successively through disc-shaped, cup-shaped and egg-shaped stages. Observe the gradual transition to the neurula stage and, finally, the appearance of the definitive tadpole.

e. Metamorphosis and post-metamorphic development. Prepare cultures of developing Styela, Molgula and Ciona and study them at 1 day intervals. The small size of the larvae makes observation of their internal organization difficult, but many external changes can be followed. Older cultures which have been developing for a week or more will also be available for study.

B. COLONIAL ASCIDIANS
METAMORPHOSIS, ASEXUAL REPRODUCTION

Amarocucium constellatum
Botryllus schlosseri
Perophora viridis

1. Amarocucium. This colonial ascidian is viviparous. The large tadpoles (ca.1.5 to 2 mm. in length) are easily obtained from ripe, freshly collected colonies.

Tadpoles usually leave the parent colony shortly after sunrise. These undamaged and fully developed individuals 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 a half an hour. Nearly all of them will commence metamorphosis within an hour of being shed.

a) Tadpole structure and behavior. Watch Amarocucium 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; Scott, '46)

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), and the oral siphon (usually larger) and the sensory vesicle between the two siphons.
The small pigment cup with lens is the light-perceiving organ, and there is a small round black static organ also within the sensory vesicle. In the tail, not the muscle cells and notochord.

b) Metamorphosis. Place a dozen or so tadpoles in a drop of water on a dry watch glass for study of 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 tissues of the tail withdraw into the body, the test swells and metamorphosis is under way. 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 to a week later (Grave, '35). Make several timed sketches of the external aspects of metamorphosis.

c) Later Stages. Observe and sketch metamorphosed Amorouclium individuals which have been growing for four days or so after attachment. They are fastened to watch glasses which have been stored in frames under water. Gently flush debris from them in the sea-water tank, and avoid tipping off their cover of water. After making your records, return the specimens, still living in their watch glasses, to the frames from which they were taken.

Examine the specimens under low power first, for orientation. If they are growing upright they may be flattened out by gently lowering a cover glass on them. At one end of the animal are the atrial and oral siphons; at the other is the post-abdomen, with the heart at its tip. Watch the heart for periodic reversal of beat. Identify the epicardium, a usually pigmented strand of tissue running throughout the post-abdomen from pharynx to heart region. It is the agent in asexual reproduction and colony formation.

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

d) 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 finger-like lobes of a large healthy colony.

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

e) Permanent preparations of larvae and metamorphosed individuals can be made by the following method, kindly supplied by Sister Florence Scott.

Fix about a dozen specimens of desired age in a small Stender dish in Schaudinn's fluid (sat. soln. HgCl₂: 80 pts; abs. alc.: 20 pts; HAc, gl.:5 pts.)
added before using). Heat fixative (60°C) and pour over animals in a small amount of sea water. After 10 minutes, change to pure fixative for 1½ to 2 hrs. In this and subsequent changes, use pipette to remove and replace fluids, keeping specimens always in the same dish. From fixative, change to 50% alc. (15 minutes); 70% alc. plus iodine (overnight); 70% alc. (rinse out iodine); 50% alc. (15 minutes); 35% alc. (15 minutes); dist. water.

Change to IN HCl (60°C) for 7 minutes; rinse in water; Teulgen stain for 6 minutes; 50% alc. for 5 minutes (1 change). Up grade slowly through 35% alc. (10 minutes), 70% alc. (15 minutes), 80% alc. (20 minutes), 95% alc. (½ hour), abs. alc. (1 hour), abs. alc. plus toluene (½ hour), toluene (1 hour). Change to small quantity of fresh toluene and add balsam a drop at a time over a period of 30 minutes. Permit toluene to evaporate. Mount in balsam, supporting cover slip with paper or chips of cover slip.

2. Botryllus. 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 normallyshed tadpoles are placed in a little sea water in a watch glass, they soon attach to the dish and commence their rapid metamorphosis.

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

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

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 attach a day or so later.

b) Two-day-old Form Atrial Budding. Allow tadpoles to attach to watch glasses and grow for two days. They are now larger and clearer, and usually so oriented that the observer looks directly down into the atrial and oral siphons. The large pharynx is in the shape of a truncated cone and bears the three rows of stigmata 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 oozoid) as an evagination of the atrium at one side. It is furnished with a blood supply, and presently the differentiating parts can be distinguished. Coming from the atrium, the whole bud, and all parts of the new individual, are derived from ectoderm. This is the atrial type of budding. (Berrill, '41).
c) **Week-old form** Will be provided. In these animals, general structure already studied may be easily seen under low power. The pharynx of the oozooiid has developed 4 rows of stimata, and the first blastozooiid may also have three or four rows. Blastozooiid buds of the second and third order may be present.

The first blastozooiid 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 oozooiid does not develop gonads. Through rearrangement of the individuals, the completed colony shows a common atrial pit in the center, with separate pharyngeal openings at the periphery (see wall chart).

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 atrial chambers, the middle one the pharynx. (Later stages show differentiation of the rest of the organs from the pharynx-vesicle (Berrill, '41).

3. **Perophora**. Perophora is a little green-colored ascidian, which by means of stolons forms loose colonies on wharf pilings, etc. Pieces of the colony may be gently stuck to watch glasses with vaseline, and stored in running sea water. After a day or so, stolons will be sent out over the surface of the glass and new blastozooids will be formed at intervals. Examine the 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 epidermis of the colony and an inner vesicle formed by the splitting of the mesenchymatous stolon septum. (See Barth, '49; Goldin, '48)

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

4. Excellent accounts of asexual reproduction in Tunicates, both descriptive and theoretical, can be found in Berrill (1945, 1951) and O'Brien (1948).
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* Indicates especially rewarding papers.

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* 1941c Spatial and temporal growth patterns in colonial organisms. Growth, Third Growth Symposium, 89-111.


**TUNICATE REGENERATION AND REDUCTION**


Sponges

The laboratory outline is prepared to include sporadic observation on the weekend. In order that the material may be observed at intervals throughout the day, the regeneration experiments should be started first, since the most interesting observations occur later.

Regeneration: Read all instructions before starting.

Each pair of students will need the following equipment: two 50-100 ml. beakers, 6 large styler dishes, 2 fingerbowls, clean slides and cover slips, pipettes, fine (clean) glass needles, and a square of the fine bolting cloth. The reaggregation studies will be performed on three species: Microciona, Cliona and Halichondria (if available). Of these, Microciona shows the fastest reaggregation so that greatest attention will be paid to it during the observations. The expressate from each sponge should be made in the following way and attention should be paid to the quantities of sea water and sponge used. Work quickly.

One gram of sponge (estimated from 1 gram samples demonstrated up front) (blotted dry with towelling) is placed in the center of a square of bolting cloth which is then folded into a small bag around it. This bag is squeezed either with clean fingers or a heavy forceps into 40 ml. of sea water in a beaker. Don't spare the muscle power. Quickly wash the cloth (or get a clean one) with sea water and use it to filter the expressate into a second beaker attempting to remove all macroscopic pieces of sponge. Ten to 20 cc. (depending on the size of the styler dish - be consistent in all experiments) should be transferred to a styler dish and this placed on the sea table. Save the excess suspension.

Experiments will be performed with mono- and bispecific expressates of sponges. Specifically prepare the following dishes (and number them) by the above method (except for indicated modifications) and carefully record the time at which each is made.

1. A styler dish containing an expressate of Halichondria, one of Cliona and one of Microciona diluted 5:1 with fresh sea water.

2. A styler dish containing a 50:1 dilution of the expressate of Microciona (keep the same volume as the Microciona expressate of no. 1). 

3. A styler dish containing a half and half mixture of Microciona with either Cliona or Halichondria (different groups of students should try different sponges except that Microciona must always be in the mixture)(diluted 5:1 with fresh sea water) made within 15 minutes of the time of preparation of the expressate.

4. A styler dish containing a mixture made in the following way (see DeLaubenfels, 1926-1928): 1/10 gram of Microciona + 1/2 gram of either Cliona or Halichondria are placed in the square of bolting cloth and expressed as described above for monospecific expressates into 40 ml. of sea water.

5. A fingerbowl with two slides in the bottom and containing an expressate made with 1/2 gram of Microciona in 100 cc. sea water (same proportions as already used).

6. A fingerbowl with as many coverslips on the bottom as will go and covered with the same solution as in no. 5.
Monospecific aggregation: a variety of specific processes take place in sequence during aggregation which you are to observe. You will have to watch the cultures at various intervals during the next 24 hours or so and try to answer the following questions. Carefully record the time at which observations are made. Do your observations primarily on Microciona but note differences with the other sponges.

a) Take a small piece of Microciona and express it through bolting cloth on to a slide and cover with a cover slip. Make observations of this at times during the next hour adding fluid at the cover slip edge to avoid evaporation. You may be aided in this by putting vaseline on three edges of the coverslip before putting it on the fluid.

The structure of the reunion mass and the cells involved are discussed in papers by Wilson and by Galitsch, copies of which are available in the laboratory. Many of the cell types, the syncytium which forms the surface layer of the intact sponge, and all skeletal elements are retained by the bolting cloth. Wilson considers three types as represented among the free cells passing through the cloth: (1) the choanocytes, which remain specific (although retracting their collars and flagella in many cases) and produce a new lining for the internal cavities; (2) cells with nucleolate nuclei and a variable degree of granulation in the cytoplasm; and (3) non-nucleolate cells possessing fine cytoplasmic granules of uniform size which give the cells a grayish color. Wilson regards the nucleolate cells as primordial cells, which produce in the reunion mass, as in the intact sponge, skeletal and reproductive cells. The non-nucleolate cells produce the syncytium which covers the body and lines those internal spaces not possessing choanocytes. Some indications of cell types may be learned from your fresh preparations, but fixed and stained material has been found more satisfactory. Do not spend too much time trying to identify the different types of cells.

b) At approximately hourly intervals for 3 or 4 hours use a small pipette to blow water at the reunion masses. When does a change in behavior of the masses occur?

c) After masses appear make some effort to measure the size of some of the masses during the day (say, every 2 hours) by placing a millimeter rule beneath the stender. Do this especially for Microciona expressages of different dilutions and compare.

d) Place a coverslip on a slide from dish no. 5 after 2-3 hours and observe the masses. Are the masses smooth or rough edged? By carefully focusing on the surface of a mass see if you can see granulated cells with a clear circular space in them ("gray cells"). Try again in 4-5 hours, etc. When they appear you will notice the change in surface texture of the masses, seen especially at the edge of a mass. If you are careful, you can return the slide to the fingerbowl and remove the coverslip under water without dislodging too many aggregates.

e) Rough-edge masses may be combined by pushing them together with glass needles or they may be cut in half with crossed needles. Wilson and Penney (1930) say that masses of 1-2 mm. will form the most completely regenerated sponges. You should combine or divide several masses on slides in finger bowls (no. 5 above) for later observations.

f) In from 18-24 hours many masses will be firmly attached by visible extensions of the aggregate. Observe such aggregates on a slide and on a coverslip from dish no. 6 inverted into sea water in a depression slide (or on an ordinary slide). Describe the attachments and the shapes of the masses.
Sponges 3.

g) To observe further differentiation of the masses, they will have to have access to running sea water. They should be quite well attached by now. Invert a Syracuse dish in the sea table (at the back where it is deep) and carefully lay a slide and several coverslips with attached aggregates on it. At intervals in the next day or so, watch the aggregates. Flagellated chambers, canals, escula, spicules, etc. develop in about three days. Rounded, unattached masses may live for weeks, but never develop into sponges (for details of development see Wilson and Penney, 1930; Huxley, 1912 and Galtsoff, 1925b). If the masses are cared for they may be kept for weeks and attain a high degree of organization.

Diplospecific aggregates:

a) Observe dishes no. 3, comparing colors with those of Microciona and Cliona (or Halichondria) aggregates; note the colors of the aggregates in the "mixed" dishes. Are they uniform in color?

b) Taking into account the fact that only half as much Microciona tissue is used compare the sizes of the aggregates here with those in dish no. 1.

c) In stender dish 4 is the same result obtained as in no. 3?

For discussion of these results see papers by de Laubenfels (1926-1928); Wilson and Penney, 1930; Galtsoff, 1923 and Spiegel, 1954. When you are finished with your material clean it up or life will be unbearable for the rest of us.

The observational parts of the laboratory may be done after the aggregates are started.

Details of sponge structure may be found in Myman’s treatise, The Invertebrates. The species found in the Woods Hole region and methods for observing them are described in the monograph of de Laubenfels (1949).

The following species will be available: Cliona celata, Halichondria panicea, Leucosolenia cancellata, Microciona proliferata, and Scypha lingua. Scypha is referred to as Sycon and as Grantia in older literature. Identify these sponges, and determine whether they are the Ascon, Sycon or Leucon type.

Using a razor blade, make thin cross-sections of Leucosolenia and Scypha. Place these sections on a slide, cover with a cover slip and examine under high power. Notice the arrangement of the cells, the spicules and their distribution.

In Leucosolenia and Scypha, look for embryos in the cross-sections. It may also be possible to find embryonic stages in Halichondria by looking down through the osculum.
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PLANKTON TOW

The last morning of the descriptive part of the course will be devoted to a plankton tow. The primary idea is to identify as many larval forms as possible. In most cases consider yourshelf in fine shape if you can put what you see into classes. Make this a class cooperative effort and don't hesitate to call on any available source, human or otherwise. A list of minor phyla not covered in the course is appended as a guide to literature on some forms which you may encounter.

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(Many additional references may be found in Hyman: The Invertebrates volumes I-IV)

CTENOPHORA


PLATYHELMINTHS


SIPUNCULOIDEA


PHORONIDEA


ENDOPROCTA


**BRACHIOPODA**


**CHAETOGNATHA**


**ROTIFERA**


**NEMATODA**


**NEMATOMORPHA**


GASTROTRICHA


PRIAPULIDA


ACANTHOCEPHALA


NEMERTINES


Wilson, E. B. 1903. Experiments on cleavage and localization in the Nemertine egg. Roux' Arch. 16.


ENTOPROCTA


As far as possible, study each major larval type from the following points of view:

1. Polarity, symmetry, and size of larva; relationship of these factors to corresponding conditions in egg and adult.

2. Early embryology - size and position of egg, manner of gastrulation, time and developmental stage of hatching; amount of differentiation and relative growth of germ layers necessary to produce larva.

3. Duration (relative and absolute) of larval period; presence or absence of sequential larval stages.
LARVAE

Aim: The objective will be to identify and to study different marine larvae and, if possible, to observe the development of some of them. A corollary objective, in conjunction with theoretical considerations, is to gain appreciation of the significance of larval forms in the development, the ecology, and the evolution of animal organisms.

In your deliberations, do not forget to take into account three major groups of larvae not dealt with here: those of parasitic flatworms, of insects, and of vertebrates.

Material:

1. Mixed, unsorted plankton collection, with larvae.
2. Staged and timed series of a variety of larvae (e.g., Artemia, Nereis) as available.

Procedure:

1. From a rich fingerbowl-full of the mixed collection pipette out as many different larval types as you can find.

2. Using the descriptions below, distribute the larvae phylum by phylum into separate dishes (as nearly as can be made out by superficial examination). Try here to become acquainted with the general diagnostic features of major larval types.

3. Now study each larval type more carefully, disregarding variations of type below class rank. From morphology, observed behavior, and embryological and ecological considerations, try to assess the significance of the larva in the life cycle of the organism. In this, the approach outlined below will be helpful.

4. Set aside representative specimens of different larval forms for prolonged observation (overnight to several days) for a study of further development and, conceivably, of metamorphosis.

Approach:

As far as possible, study each major larval type from the following points of view:

A. Developmental complexity

1. Polarity, symmetry, and size of larva; relationship of these factors to corresponding conditions in egg and adult.

2. Early embryology—size and yolkiness of egg, manner of gastrulation, time and developmental stage of hatching; amount of differentiation and relative growth of germ layers necessary to produce larva.

3. Duration (relative and absolute) of larval period; presence or absence of sequential larval stages.
4. Amount of differentiation and growth necessary to produce adult; nature of metamorphosis.

5. Transient larval vs. emergent adult structures - relative bulk, rates of growth, degree of neoteny in adult; degree of coexistence and codvelopment of larval and adult tissues.

B. **Structural and functional complexity**

1. Habitat and way of life of larva.

2. Locomotor, sensory-neural, and alimentary equipment, in relation to way of life; other pertinent features.


C. **Adaptive complexity**

1. Can larva function as permanent organism? Is there evidence of neotenic macroevolution?

2. Can larval period be suppressed or dispensed with altogether? Is there evidence of abridged development?

3. Relation between egg size, amount of yolk, duration of free-swimming larval period, and developmental stage at hatching (oviparous or viviparous). Is direct, indirect (larval), or are both types of development typical of the group? Relate to ecology.

4. Does larva recapitulate an ancestral stage, or is it an independent invention of the group? If larva resembles another larval type, is this parallel evolution or ancestral relation?

5. Net adaptive advantage and disadvantage of larva - probable direction of selective pressure.
PORIFERAN LARVAE

AMPHIBLASTULA

This larva is characteristic of all poriferan families. The early larva is a sphere of cells, composed anteriorly of small columnar, flagellated locomotor cells and posteriorly of large, granular cells. In later stages the larva flattens out, the large posterior cells proliferate rapidly and progressively envelop the small anterior cells. The process is reminiscent of epibolic gastrulation. In the final condition, the flagellated cells form an inner lining layer in the gastrula-like larva (which for this reason is often called "pseudogastula").

The free-swimming larva stays in surface waters for about a day, then descends and attaches to a substratum at the anterior pole. The flagellated cells develop collars and become choanocytes. All other adult cell types differentiate from the outer granular cells, some of which migrate inward and settle among the choanocytes. After several days an osculum breaks through opposite the area of attachment, and lateral canals differentiate slowly.

The Amphiblastula is probably not an ancestral forerunner of the true gastrula of higher invertebrates; rather, parallel evolution is indicated.

COELENTERATE Larvae

PLANULA

This is the basic larval type of all coelenterates, even though, in several cases, the planula phase is abridged and does not represent a free-swimming phase (e.g. Hydra, Tubularia).

The free-swimming planula is a 2-layered ovoid, with an ectoderm of ciliated, columnar cells. The endoderm, solid in Hydorzoa, hollow in most Actinoderm and Scyphozoa, develops from the single-layered blastula either by delamination or by multi polar ingestion.
Where the endoderm is solid, a gastral cavity develops at the time of larval attachment. The free-swimming period lasts about a day.

An attached planula may metamorphose either into a permanent polyp phase (Actinzoa), or into a polyp phase which may later alternate with a medusa phase (Hydrozoa), or into a permanent medusa phase via an Ephyra larva (Scyphozoa).

(Actinzoan)  (Hydrozoan)  (Ephyra)

**EPHYRA**

After the scyphozoan planula has become attached, tentacle buds form anteriorly and an "oral disc" begins to constrict off transversely. Many more such discs may subsequently form in posterior succession. This process of strabilization directly transforms a single planula into many medusa-type adults. The strabilized larval condition represents the Ephyra phase.

**ACTINULA**

In Tubularia, Hydra, and other hydrozoans, the planula does not become free-swimming, but is represented by an abbreviated 2-layered embryonic stage. In Tubularia, for example, a planula-like embryo remains in the gonophore, where it develops lateral protuberances, the presumptive aboral tentacles. A gastral cavity then forms, oral tentacle buds appear, and a mouth breaks through. In this condition the larva becomes free, and is now called an Actinula. It creeps along the bottom for some time, oral end down. Then it turns over and becomes attached aborally.

The planula certainly, and perhaps also an Actinula-like organism, represents the ancestral coelenterate condition. It has given rise to the present taxonomic
diversity of coelenterates, and it probably also represents the starting point of flatworm evolution.

TURBELLARIAN LARVAE

MULLER'S LARVA

Only one order among free-living flatworms, the polyclads, are fully marine; and only the polyclads feature indirect, larval development. In all other orders development is direct. Moreover, the direct developers start from "ectolecithal" eggs, i.e., they do not possess yolk inside them but are surrounded by yolky nurse cells inside the egg shell. Thus the food supply is relatively large, and development can be direct. On the other hand, the polyclads have "endolecithal" eggs, where some yolk is within the egg cytoplasm. Here development includes a free-swimming larval phase, the so-called Muller's larva.

This larva could well have been derived from a planula. A third germ layer is represented by mesenchymatous cells between ectoderm and endoderm. As in coelenterates, a single gastric opening forms in Muller's larva, and this opening comes to be situated ventrally. It is surrounded by a fold of tissue, which will contribute to the formation of an eversible pharynx in the adult. The gastric opening leads into the gastric cavity, which in older larvae becomes branched increasingly, foreshadowing the adult condition. Externally, 2 to 12 eye spots (depending on the age of the larva) are situated anteriorly.

The most characteristic feature of Muller's larva is the presence of 3 preoral ectodermal folds or arms, their margins lined with a continuous ciliated band, (in the diagram, the 8th lobe is not shown -- it is situated dorsally) These folds serve a locomotor function.

Metamorphosis is gradual, involving a resorption of the ciliated folds and a lengthening and flattening of the larva.

In certain features, Muller's larva resembles both the Pilidiunm of the nemertines and the Trochophore of annelids. As an exercise in speculative biology try to estimate which of these resemblances might be (a) fortuitous, (b) indicative of parallel evolution, (c) indicative of close ancestral relationships.
In most proboscis worms development is direct. Two families of the Heteronemertini feature a larval phase, the Pilidium. This larva may either be free-swimming and planktonic (Cerebratulus), or modified for creeping, hence without locomotor appendages (Lineus; "Desor's larva").

The larva is helmet-shaped and the margin of the base is drawn out into two characteristic lappets. The entire margin of the base carries a ciliated band, the prototroph. A tuft of long cilia arises from an apical sensory plate. In early larvae, two ectodermal cells migrate into the blastocoele and give rise to loose mesenchyme. A wide mouth forms at the base of the larva, between the lappets. It leads into a blind-ended gastric cavity. (Note that the larva still features the 2-way alimentary system of coelenterates and flatworms, while the adult nemertean possesses a one-way system, with mouth and separate anus).

Metamorphosis is remarkable and complicated. Five ectodermal invaginations (their deep parts representing "imaginal discs") sink below the surface and eventually fuse one with the other, closing off a double-walled cavity deep within the larva. The outer wall is larval tissue, the so-called "amnion". The inner wall is formed from the tissue of the imaginal discs, and this is the ectoderm of the future adult. The adult mesoderm is represented by mesenchyme cells which have come to be included within the cavity. And the adult endoderm is the larval stomach, around which the amniotic chamber is formed. When the adult worm has matured it breaks out of the amnion, and the pilidium, having lost its stomach and unable to nourish itself, soon dies.

In its mesoderm and its alimentary system, the Pilidium resembles Muller's larva. The main evolutionary advance of the nemertines was apparently made in the adult, not in the larva, and the resemblance of the two larval types could indicate ancestral relationship. On the other hand, both larval types also resemble the Trophophore in ectodermal ciliation, and this could well be a matter of parallel evolution.
THE TROCHOPHORE

This most ubiquitous of invertebrate larvae is characteristic of Annelids, Molluscs, Ectopods, Entopods, and Brachiopods. It is possible that the trophophore was invented only once, but it is equally possible that it evolved several times independently. Given the requirement of ciliary locomotion in surface waters, of an alimentary system with mouth and anus, and of triploblastic origin, then a trophophore-like organism is the simplest answer and a virtual necessity, at least as a transient stage.

The larva carries an apical tuft of long cilia on an anterior sensory plate, a preoral ciliated bend, the prototroch, and in many trophophores a postoral metatroch is also present, together with a telotroch, a posterior ciliary tuft. Internally, in addition to a complete alimentary canal, there is an archinephridium, running diagonally from the mouth region to the anus region.

The trophophore arises from the gastrula by increased relative proliferation of dorsal posterior cells (descendants of 3d), which shifts the blastophore from the vegetative pole ventrally, to the mouth position. In some cases the blastophore becomes oval, constricts in the middle, and so gives rise to both mouth and anus. Otherwise a proctodaeum breaks through independently. Alongside the intestine are the mesoblast cells, descendants of 4d, the larval mesoderm.

In Annelids, the trophophore gradually metamorphoses by posterior longitudinal proliferation, and subsequent segmentation, of the posterior ectoderm, the mesoblasts, and the intestine, and by transformation of the remainder of the larva into the adult head.

In Molluscs, the trophophore transforms into another larva, the VELIGER, which metamorphoses into the adult later. The Veliger is characterized principally by three structures: the velum, a ciliated flap developed from the prototroch and serving as locomotor organ; the shell gland, differentiated from the dorsal postprototrochal ectoderm, which forms the mantle and shell of the adult; and the anlage of the adult foot, originally an ectodermal thickening just behind the mouth of the trophophore, later backed increasingly by proliferating mesenchyme. In the metamorphosing Veliger, the velum is sloughed off and anterior larval tissues disintegrate, so that the mouth and the emergent foot are drawn relatively forward. The shell by this time is typical of a given molluscan class.
CRUSTACEAN LARVAE

The development of certain decapod crustaceans includes the following series of sequential larval stages:

NAUPLIUS → METANAUPLIUS → PROTOZOA → ZOA → MYSIS → (ADULT)

This sequence may be regarded as the basic larval series for Crustacea as a whole, but only in relatively few species is the entire series explicit:

1. Hatching may occur at any of the above stages, in different species. In one extreme the egg is shed, and such forms are truly oviparous. In the other extreme, small adults hatch viviparously.

2. Abridged development may occur either before or after hatching. If hatching occurs at the Protozoa stage, for example, some or all of the preceding stages may be indistinct, may be abbreviated in various ways, and some may be skipped altogether. Most crustaceans however pass through a Nauplius-phase, either in ovo or pelagically, and the Nauplius may be regarded as the basic larval form of the group. Post-hatching larval phases may also be skipped or abbreviated in various ways. In primitive crustaceans (Branchiopods), hatching tends to occur in the Nauplius or Metanauplius stage, with later gradual transformation into the adult and without other distinct larval stages. In advanced types (Decapods), some or all of the later larval stages do tend to become distinct, regardless of whether hatching occurs early or late.

3. Precocious or retarded development of some structures may obscure the typical larval series further: thus Ostracod Nauplii already possess an adult bivalved carapace. In decapod Zoaeas, thoracic development lags behind abdominal development. Moreover, many larval types differ in minor ways from "typical" larval stages, and different unique designations have been applied here (e.g. the zoea-like "cyclops" larva of copepods, or the zoea-like "Cypris" larva of cirripeds).

Each stage of the basic larval series is defined by features not yet present in the preceding stage, as follows:

NAUPLIUS: 3 pairs of appendages present (1st & 2nd antennae, and mandibles); non-segmented; median occlud. Example: newly hatched Artemia (1st molt produces Metanauplius)

MATANAUPLIUS: 1st & 2nd maxillae present; segmentation starting. Example: newly hatched Branchiopus

PROTOZOA: 1st and 2nd maxillipeds present; compound eyes begin to differentiate. Example: newly hatched Squilla

ZOA: 3rd maxillipeds and variable number of thoracic appendages present; compound eyes fully developed. Example: newly hatched crab

MYSIS: all thoracic appendages present, plus variable number of abdominal appendages; segmentation complete. Example: newly hatched lobster
ADULT: abdominal appendages mature.
Example: newly hatched fresh-water shrimp

The following summarizes the typical larval history of the different crustacean classes:

Branchipoda:
Nauplius $\rightarrow$ Metanauplii $\rightarrow$ Adult (Artemia); or Viviparous adults (Daphnia)

Ostracoda:
Nauplius $\rightarrow$ Adult; or Viviparous adults

Copepoda:
Nauplius $\rightarrow$ Cyclops (Zoea-oid) $\rightarrow$ Adult

Cirripedia:
Nauplius $\rightarrow$ Cypris (Zoea-oid) $\rightarrow$ Adult

Malacostraca: hatching at all larval stages, and complete larval series in some, abbreviated series in others.
ECHINODERMAE LARVAE

Large, heavily yolked echinoderm eggs typically gastrulate by epiboly, form a schizocoelous body cavity, develop directly or with more or less abridged larval phase, and tend to be viviparous. By contrast, small yolkless eggs typically gastrulate by invagination, form an enterocoelous body cavity, develop indirectly via more or less protracted larval phases, and tend to be oviparous. Only Echinoids are predominantly indirect developers. In all other groups, one developmental type is as "typical" as the other. Moreover, beautifully graded series of intermediate types can be distinguished, particularly among Ophiuroids. Indirect, larval development is almost certainly primitive.

The first three types of larvae described below develop from the gastrula in roughly the same manner. The blastopore becomes the anus, a mouth breaks through separately, and the ciliation of the blastula disappears, except for a continuous rectangular band on the presumptive ventral side, where the cilia become longer and stronger. This band, growing more rapidly than the rest of the ectoderm, becomes thrown into folds, so producing larval arms. The manner of growth, relative size, and the topographical arrangement of the arms is characteristic for each echinoderm class, so that several larval types can be distinguished.

"early larva"

pluteus

auriculata

bipinnaria

PLUTEUS

Characteristic of some of the Ophiuroids and all known Echinoids. The post- anal portion of the "early larva" (described above) becomes elongated and conical, and the ciliated band on the ventral side comes to border 2 to 8 larval arms, the number depending on the age of the larva. The arms are supported by internal calcareous rods. In Ophiuroids, metamorphosis in different species has been shown to occur at different larval stages. Small-egged forms metamorphose late, 8-armed larvae being common. Large-egged forms metamorphose early, and 2-armed or even no-armed larvae are common. In the extreme, there is no larval period at all, and metamorphosis occurs right after gastrulation. These extremely large-egged forms are viviparous.
BIPINNARIA

Characteristic of Asterias. In the early larva a pair of outgrowths develops posteriorly, giving rise to the "bipinnate" condition. These "arms" are bordered by the ciliated band. Anteriorly, a portion of the band nips off and comes to border a separate preoral lobe.

AURICULARIA

Characteristic of some of the Holothuridae. This larva resembles the Bipinnaria in all essential respects. In the Auricularia, however, the ciliated border of the preoral lobe remains continuous with the rest of the ciliated band.

VITELLARIA

Characteristic of all known Crinoids, some Ophiuroidea, and some Holothuridae. Whereas all other echinoderm larvae are derivable from a common "early larval form," the Vitellaria on the contrary derives independently from the gastrula. As the name suggests, this larva occurs in species with (moderately) yolky eggs. The larva is barrel-shaped, and it possesses separate ciliated bands. These bands develop in situ from the general ciliation of the blastula. The larval alimentary system is incompletely formed or it may develop very late, as might be expected in a yolky organism.
TORNARIA

In many of the Hemichorda, eggs are large and yolky, and development is direct. In other forms, development passes through a pelagic larval phase, the Tornaria. This larva resembles both the Trochophore and the Bipinnaria. The apical sensory plate with its ciliary tuft, the telotroch, and the alimentary system are reminiscent of the trophophore; general shape, coelum formation, and the anterior ciliated band are reminiscent of the Bipinnaria. The chordate character of the Tornaria is revealed early, principally by the appearance of four, and later more, pairs of esophageal evaginations, the early gill pouches. These acquire openings through the larval skin comparatively late. A portion of the dorsal coelum (which originally opens to the exterior through the dorsal ectoderm) becomes the pericardium. In the Bipinnaria, the comparable part of the coelum becomes the madreporic vesicle, a portion of the water-vascular system.

Beyond the strong probability that the Hemichorda belong to the Echinoderm-Chordate line of evolution, their ancestral relationships are obscure. Berrill suggests that the group may be extremely ancient, having evolved independently and separately from the main echinoderm and chordate stocks. In that case larval resemblances would be largely fortuitous, or at most indicative of parallel evolution.

TUNICATE LARVAE

THE ASCIDIAN TADPOLE

This larva probably gave rise, through neotenic macroevolution, to (a) the pelagic tunicates, the Thaliacea and the Larvacea, (b) the Cephalochorda (Amphioxus), and (c) the vertebrates. The ascidian tadpole thus is important not only for its own sake but also as the clearest and the original representative of the chordate prototype. Berrill regards this larva as an independent, original invention of sessile ascidians.

In the Ascidiae as in other groups, large eggs, abridged development and/or absence of larval phases, and viviparity go together. In more primitive small-legged forms, pelagic non-feeding larvae may be formed within 24 hours after fertilization. The free-swimming period may be as long as a few days or in
large-agged forms as short as a few minutes.

The structure of the primitive tadpole varies relatively little. The tail, about 4 times as long as the trunk, contains: a notochord; a hollow dorsal nerve cord; lateral muscle bands; mesenchyme cells; and an enveloping test, median flaps of which serve as gills.

The trunk consists of: brain (expanded anterior portion of nerve cord), with ocellus and otolith; dorsal mouth, covered by test, hence non-functional; pharynx, with endostyle, and originally a single pair of gill slits leading to ectodermal pouches (peribranchial siphons) opening to outside (later the pouches fuse dorsal to pharynx, forming the atrium and the atrial siphon); U-shaped digestive tract, opening into atrium; pericardium, a ventral pharyngeal outgrowth which forms the heart late in larval period; holdfast papillae anteriorly; and an enveloping test.

Such variations of tadpole structure as occur are generally associated with abridged larval development (e.g., absence of ocellus; modification, reduction, or absence of tail). Moreover during later larval stages emergent adult structures develop alongside the larval structures, and different relative rates of growth in different species may produce further variations of the basic tadpole organization.
REFERENCES

The following books contain textbook accounts on invertebrate larvae. Most of these books also cite references to the original descriptive papers (the majority of which is written in Mittelhochdeutsch). The accounts in the older books are generally more reliable and more accurately illustrated than those in the later ones.

Following the list of books is a selected group of key references on particular phases of invertebrate larval biology.

General Books


(good on bibliography but not much else)


HYMAN, LIBBIE The series of volumes on invertebrates.

Special references


COE, W. R. 1899 On the development of the pilidium of certain nemerteans. Transact. Connecticut Acad. 10


Embryology Course

Marine Biological Laboratory

June 16, 17, 1959

Teleost Development

I. Introduction

II. An outline of teleost development
   A. Fertilization
      1. Entrance of sperm
      2. Barriers to fertilization
      3. Role of calcium in fertilization
   B. The sequence of developmental changes
      1. Cleavage
      2. Blastulation and periblast formation
      3. Formation of germ ring and embryonic shield
      4. Epiboly
      5. Origin of the embryonic axis

III. The Experimental analysis of development
   A. What is the relationship of the first cleavage plane to the embryonic axis?
   B. Does cleavage segregate cells of different developmental potencies?
   C. What are the normal fates of the parts of the blastula?
   D. An analysis of the mechanics of gastrulation.
      1. Superficial changes
      2. Endoderm and mesoderm formation
      3. Direction of cellular movements during gastrulation
   E. An analysis of the origin of the embryonic axis.
      1. Axial elongation
      2. The source of cells for axial elongation
         a. Mitotic index
         b. Does the embryonic axis receive contributions from the germ ring?
   F. Correlative analysis of morphogenesis.
      1. Is the volk necessary for development?
         a. Is there an "organizing" factor in the volk?
      2. The "dorsal lip" and "archenteron roof"
         a. Inductive and field properties
            i. Grafts of the "dorsal lip"
            ii. What determines the position of the "dorsal lip"?
            iii. Does the "organizer" show field properties?
      3. The analysis of epiboly
         a. The surface gel layer or "surface coat"
         b. Does contraction of the gel layer contribute to epiboly?
EMBRYOLOGY OF TELEOSTS

References


Rother, K., 1935. Entwicklungsphysiologische Untersuchungen an Fossillenkaimi.


Biol. 22: 105-118.


J. W. Saunders, Jr.
June 1958
REGENERATION IN HYDROIDS

Selected References


Trembley, A., 1744. Mémoires pour servir à l'histoire d'un genre de polypes d'eau douce, à bras en forme de cornes. Leide: Verbeek.


CMF

1959
progressive transformation: multiplication and diversification at all levels of organization.

Proposition:
- each level develops and behaves in the context of the levels above it; with limitations imposed by its own level and levels below it.
- Organ system
  - Organ
  - Tissue
  - Cell
  - Cytoplasm
  - Gene

organism

Problems:
1. How do the diverse cell types arise?
2. How stable are the initial, intermediate and final cell types?
   To what extent does stability of cell type depend upon system-independent (intra-cellular) and system-dependent (extra-cellular) mechanisms?

I. MECHANISMS OF DIVERSIFICATION

A. Inherited Properties (genetic and paragenetic)
   Cytoplasmic segregation in mosaic eggs
   System-independent cytodifferentiation

B. Acquired Properties (general and microenvironment)
   Diverse properties arise in response to diverse environments.
   System-dependent cytodifferentiation
   1. Position (originally homogeneous aggregate)
   2. Cellular interactions (heterogeneous aggregate)
   3. Cell population size and density (critical aggregate size)
   4. General systemic factors
      a. Specific chemical agents (vitamin A, hormones)
      b. Nervous system
      c. In vivo vs. in vitro nutritional factors
      d. Biophysical factors (pressure, tension, etc.)
II. MAINTENANCE OF CELL STABILITY

The successful organism has imposed upon it the simultaneous requirement of stability (genetic continuity) and lability (adaptability). It achieves this by building up (during development) a population of cell's heterogeneous in respect to relative stability, together with the construction of a heterogeneous and remarkably stable pattern of internal microenvironments.

A. System-independent stability
   1. Terminal differentiations
   2. Genetic properties
   3. Mosaic eggs
   4. Evidence from tissue culture
   5. Behavior of dispersed and reaggregated cells
   6. Clone cultures

B. System-dependent stability
   1. Overt dedifferentiation in tissue culture
   2. Cell culture
   3. Role of serum proteins and hormones
   4. Metaplasia

III. PROPAGABILITY OF CELL TYPE

Does a dividing cell transmit its differentiated properties to its offspring or are the offspring like the parent because they occupy the same environment?

Never certain techniques of cell culture, cloning or other assurance of absence of population effects will be necessary before the question can be answered.

Conclusion:

In the initiation and maintenance of cytodifferentiation environment may prove to be more important than heredity.
Selected References


Added:


Tissue Mass and Density

Selected References


Thompson, D'Arcy W. On Growth and Form. Cambridge (latest edition)


NTS '59
Bibliography


Growth of Single Cells


Synchronous Cell Division

Campbell, A. 1957 Synchronization of cell division. Bact. Rev. 21, 263.


Zeuthen, E. 1953 Growth as related to the cell cycle in single-cell cultures of Tetrahymena pyriformis. J. Emb. and Exp. Morph. 1, 239-249.


Synchronously Developing Eggs


Unbalanced Growth


SYNTHESIS OF DNA


DNA AND DNA PRECURSORS IN EGGS AND EMBRYOS.


Bibliography -- Addendum

Growth, Cell Division and DNA Synthesis


Enzyme Regulation

Selected References


Pollack, M.R. 1953 Stages in enzyme adaptation. in Adaptation in Microorganisms.


Ephrussi, B. 1956 Enzymes in cellular differentiation. in Enzymes, Ude of Structure and Function.


P. Grant
1959
EMBRYOLOGY CLASS 1959

9. Gerald Merson 18. Ted Grant

Not in picture: Fred Bergman, Ned Holt, Barry Pierce, Irwin Rose, Lionel Rebhun