

Embryology Course Materials

1951



EMBRYOLOGY 1951

MBL, WOODS HOLE

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7. Helen Byars
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## Induction of Exogastrulae in Arbacia and Echinarechinus

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

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

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

Treatment:- Obtain eggs and sperm of Arbacia or of Echinarechinus in the usual way. Inseminate a fairly large sample of the eggs in a finger bowl. At the time of first cleavage transfer a sample of concentrated eggs (with not more than 1 ml of sea water) to a finger bowl containing about 50 ml of the Li-sea-water and, at the same time, a similar sample to a control dish of sea water. After 2, 4, 8 and 12 hours transfer samples (ca. 0.5 ml) through three dishes of 10 ml of sea water and culture in half-filled, covered, stender dishes.

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

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

### References:-

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## Development of Isolated Blastomeres of Arbacia and Echinarachinus

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

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

Solutions:- 1 liter filtered sea-water  
100 ml hypertonic sea-water (30grams NaCl in 1 liter of sea water)  
100 ml of Ca-free sea-water (1000 ml M/2+22 ml M/2 KCl + 195 ml m/3 MgCl<sub>2</sub>.6H<sub>2</sub>O-103 ml M/3 Na<sub>2</sub>SO<sub>4</sub>+6 ml M/2 NaHCO<sub>3</sub>, adjusted to pH 7.9-8.3 (based on Lyman and Fleming 1940).  
50 ml of 5% formalin in sea-water

Removal of Fertilization Membrane. For the purpose of isolating blastomeres the fertilization membrane must first be removed. This is accomplished by shaking a suspension of freshly fertilized eggs. Obtain eggs and sperm of Arbacia or of Echinarachinus in the usual way. Inseminate (noting time) a sample of eggs, check for fertilizability and save to determine first cleavage time (necessary in part (a) below). Later (10 to 15 minutes) fill a test tube about four-fifths full with a freshly washed sample of eggs and inseminate, mixing by inverting the test tube once. About one-half minute later pour about  $\frac{1}{4}$  of the eggs from the test tube gently into a syracuse dish and examine for membrane elevation. When the membranes have separated from the surface of practically all of the eggs (about 1 to 2 minutes after insemination), shake the test tube ten times rapidly up and down using a full forearm swing and holding long axis of tube in direction of swing with thumb over open end. Pour about  $\frac{1}{4}$  of the eggs into a syracuse dish, immediately shake tube again ten times, remove another  $\frac{1}{4}$  of the eggs, repeat a third time and remove the remaining  $\frac{1}{4}$ . Examine the three dishes of shaken eggs and select the one containing the highest percentage of naked eggs. Wash twice with filtered sea-water.

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

Separation of Blastomeres. For this purpose the student may use either the Ca-free sea-water (Herbst, 1900) or the hypertonic sea-water (E. B. Harvey, 1940) method outlined below. Remove fertilization membranes from eggs and use the control sample of eggs fertilized 10 minutes before the experimental set to determine expected time of first cleavage of the membrane-less eggs.

A. Ca-free sea-water. At about five minutes before the time of first division, concentrate the membrane-less eggs in the center of the dish by gentle revolution of the dish (the center of the dish

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

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

Development. Observe and sketch the isolated blastomeres in their 4-, 8-, and 16-cell stages. How many micromeres are formed at what stage? Does the isolated blastomere cleave as though it were still part of a whole egg? At the beginning of gastrulation fix some "half"-embryos and whole embryos in 5% formalin and measure their respective diameters. What approximate ratios are obtained for their respective volumes, surface areas and wall-thicknesses? Examine and sketch the embryos in the completed gastrula, prism and pluteus stages. Do the "half"-embryos develop at the same rate as the controls? Are the "half"-plutei complete in regard to all structures seen in the whole plutei? Determine whether or not both members of the pairs of isolated blastomeres form normal plutei. (See Hörstadius 1936; Tyler, 1942, and experimental embryology texts for further analysis.)

References:-

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## FERTILIZATION

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

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

### ✓ NEREIS:

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

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

If possible, arrange to observe two or more eggs which are touching immediately after fertilization. 2 - 3 minutes after fertilization they will begin to be pushed apart by transparent jelly secreted by the eggs external to the vitelline membrane. By 20 minutes, the zone of jelly around each egg will be as wide as the egg diameter. The margin of the jelly can often be made out by observing supernumerary spermatozoa and other particles at the edge of the jelly. 5 or 6 minutes after fertilization the vitelline (fertilization) membrane will be noticeable due to the formation of the narrow perivitelline space upon jelly extrusion. At 7 or 8 minutes, the entrance cone begins visibly to form. Find an egg showing a profile view of the entrance cone and the sperm which is to enter. From 8 to 12 minutes or longer, the sperm is clearly visible outside the vitelline ~~changes, through~~ ~~circumference outside the vitelline~~ (fertilization) membrane at the tip of the

conspicuous entrance cone. In the course of the next 8 to 10 minutes, the vitelline membrane is indented slightly at its point of contact with the entrance cone. This tends to obscure the sperm from view to some extent, and at about 20 minutes after fertilization the egg wrinkles, becoming distorted and quite irregular in somewhat amoeboid fashion. The entrance cone has already flattened considerably, but is still present, and although the sperm is partially obscured from view, the entrance of its head into the egg is not completed until some time later (ref. #12, 15, 16). The final penetration of the sperm head through the membrane, leaving the middle piece and tail outside, may also be observed. At about 30 minutes, the egg rounds up again, but as the time approaches for 1st polar body formation, the egg elongates in a direction perpendicular to the polar axis. If no eggs lie so that the forming polar body is on the horizon, the dish should be shaken. The 1st polar body may form at about 36-40 minutes, and it lies in the space between the egg and the vitelline membrane. This space is wider in the region of the animal pole than elsewhere. The second polar body often forms at about 50 minutes and commonly does so immediately under the first polar body, which is thus lifted into perivitelline space. At perhaps 80 minutes the eggs will begin to divide into two unequal blastomeres. Observe 2nd and 3rd cleavages also, if time permits. The 3rd division, from 4 to 8 cells, produces 4 micromeres by spiral cleavage (ref. #24).

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

#### NEREIS: Exaggerated Entrance Cones:

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

#### Nereis: Centrifuged eggs:

If time permits, centrifuge some unfertilized Nereis eggs in the Emerson electric centrifuge (cover off) for 60 minutes. A layer of 0.95 molar sucrose (undistilled water) at the bottom of the centrifuge tubes prevents injury to the eggs. This is



somewhat hypertonic, but provides an adequate support for the Nereis eggs without injuring them in any way. This amount of centrifuging separates the various formed components of the egg into several strata (ref. #7). Inseminate the centrifuged eggs after washing off the sucrose with sea water, and observe asymmetrical jelly-extrusion. Is more jelly extruded at the centripetal or centrifugal pole?

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

#### Spawning habits of Nereis limbata

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

#### CHAETOPTERUS:

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

When the egg is taken from the female it contains a large immature nucleus (germinal vesicle), as does the Nereis egg, but unlike the egg of Nereis, it spontaneously undergoes partial maturation when placed in sea water, even if not fertilized. A number of species of eggs partly mature when they enter sea water and Pasteels (ref. #21) has shown that this is dependent upon the presence of Calcium in the sea water. Chaetopterus eggs develop quite rapidly. If eggs are fertilized just after the partial maturation in sea water has been completed, they develop as rapidly as eggs inseminated 12-15 minutes earlier when first placed in sea water (ref. #23). (time counted from fertilization): 1st polar body 14.5 minutes; 2nd polar body, 27 minutes; "pear" shaped stage, 46 minutes; polar lobe bulge, 52 minutes; cleavage with polar lobe attached, 58 minutes; completed cleavage with polar lobe resorbed into one blastomere, 62 minutes; 4 cell stage, 82 minutes. If the laboratory air temperature is about 24°C the development will be more rapid, and about at the rate indicated in the descriptive text below.

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

If eggs are now lightly inseminated and stirred, a few sperm may be seen adhering to the eggs almost immediately. Within 5 or 6 minutes, the vitelline membrane may be seen to separate from the egg surface, after which time it is called the fertilization membrane. It is not conspicuous and does not elevate much above the egg surface. By 10 or 12 minutes, the eggs, which had become almost spherical after fertilization, are seen to elongate in an axis perpendicular to the polar axis. This is preparatory to formation of the 1st polar body at about 12 minutes. In this division the egg thus assumes approximately the shape of a blastomere, although the polar body is a vestigial cell. After the first polar body forms, the egg

again rounds up (16-19 minutes), but it elongates again in the same manner to form the second polar body at perhaps 23 minutes. The second polar body often forms under the first, which is thus pushed away from the surface of the egg. The egg again rounds up (26-30 minutes); the egg pronucleus may now sometimes be seen migrating toward the center of the egg, and occasionally the sperm pronucleus may also be detected. By 35 minutes, the clear zone has extended from the polar region toward the equator of the egg, and at 37-40 minutes a typical "pear shaped" stage is reached. The polar bodies lie at a position corresponding to where the stem attaches to a pear, and the bulge which forms the polar lobe begins quite suddenly at the anti-polar end of the egg (40-43 minutes). When the polar lobe is fully developed, however, it corresponds to the stem end of the "pear", and the polar bodies are opposite.

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

#### CONCENTRATION AND ACTIVITY OF SPERMATOZOA:

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

#### CUMINGIA:

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

#### SPECIAL PROJECTS:

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

1. Fertilization of Platynereis. Collect Platynereis from the Caydetta Wharf floating stage at the right phase of the moon. Consult E. E. Just's papers on

breeding habits and fertilization. Compare your observations with those on *Nereis libata*.

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

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

## ECHINODERM DEVELOPMENT

### Arbacia punctulata

This species is usually ripe from mid-June to mid-September in the Woods Hole region. When stored in laboratory aquaria they may maintain their ripe condition even beyond the breeding season and supply apparently normal eggs and sperm. In general, however, it is safest to use animals within a few days after they are collected.

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

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

a) Cut around the peristome and remove the Aristotle's lantern. Pour out the body fluid and place the animal, aboral side down in a dish containing a small amount of sea water. The animals then frequently shed thru the gonopores. After 10 minutes remove any eggs that have been shed to a finger bowl (or other large flat dish) containing ca. 200 ml of sea water. Sperm should be kept in concentrated suspension or "dry" (ie. as it exudes from the testes). b) Cut around the testis about half way between the mouth and the equator and proceed as in a. Shedding is more frequently obtained by this method, but there is also more likelihood of cutting the gonads. c) Cut as in b, pour out body fluid and remove gonads (at gonoduct end) with blunt forceps, spatula or spoon. The ovaries should be placed in about 200 ml of sea water in a finger bowl and allowed to shed. If undisturbed the eggs are extruded in compact clumps or strings and may be readily removed to a fresh dish without ovarian tissue by means of a wide-mouth pipette. If large quantities of eggs are desired the ovaries should be allowed to shed for about  $\frac{1}{2}$  hour with occasional stirring, then poured gently thru washed (and sea water soaked) cheesecloth or being sick. d) Inject about 0.2 ml of 0.5 KCl into the peristomial cavity. Ripe animals will begin to shed within 2 minutes. The eggs can be collected by inverting the animal in a dish of sea water or by washing the eggs gently from the surface of the animal with a pipette. The sperm should be removed "dry" or in concentrated suspension.

Spermatozoa:- Upon dilution with sea water the sperm become temporarily intensely active. They lose their motility sooner in concentrated than in dilute suspension, due presumably to the more rapid accumulation of  $\text{CO}_2$ . On the other hand their ability to fertilize eggs is lost more rapidly in dilute than in concentrated suspension. (See F. R. Lillie, 1915; Cohn 1910; Hayashi 1945). When kept in the cold  $2^\circ\text{C}$ .) "dry" sperm may remain good for several days. At room temperature dilute sperm suspensions may lose their fertilizing power in an hour or less. It is advisable, then, to use freshly diluted sperm for fertilization.

The head of the sperm is comprised of acrosome, nucleus and mid-piece that are roughly 0.3 and 1 microns respectively in length and 0.3, 1.3 and 1.2 microns in greatest width. The tail is about 45 microns long and 0.1 micron in greatest width. Its axial filament protrudes a short distance beyond the end of the sheath. Examine under oil immersion and sketch a spermatozoon. Examine moderately

active spermatozoa under high-dry and describe their mode of swimming.

**Unfertilized Eggs:-** Arbacia eggs complete both meiotic divisions while still in the ovary and the polar bodies very seldom remain attached when the eggs are shed. Occasionally, especially from relatively unripe animals or after macerating ovaries, eggs may be found that are in the germinal vesicle (diakinesis of primary oöcyte) stage recognizable by the large clear nucleus (about one-half egg diameter) and nucleolus. Such eggs may exhibit some surface response to sperm but they do not develop upon insemination. The ripe egg (75 microns diameter) has a small clear nucleus. It contains uniformly dispersed pale yolk granules and slightly larger red granules containing a pigment called achinochrom which is a substituted naphthoquinone related to the K. vitamins. (Ball, 1936, Hartmann et al. 1939, Tyler 1939). Upon centrifugation mitochondria and oil sphules are also distinguishable. The nucleus is generally located excentrically. Since the polar bodies are not usually present the position of the nucleus with respect to the polar axis is not readily determined. Occasionally however, batches of eggs are obtained in which the polar bodies are attached. In these observations (Hoadley, 1934) have shown that the nucleus may lie in any part of the cytoplasm between the cortex and the center. In the transparent gelatinous coat (about 30 microns wide) of the egg there is a funnel-shaped space which generally lies in the polar axis. The funnel is rendered visible by staining the jelly with Janus green or by placing the eggs in a suspension of Chinese ink. For this purpose the eggs should be taken immediately after shedding since the micropyle (funnel) may disappear as the jelly swells. Examine and sketch some unfertilized eggs under high power noting features described above.

Centrifuge a sample of unfertilized eggs at about 10,000 g for  $\frac{1}{2}$  hour and sketch one in "side view" noting the following five layers of stratified material:- oil cap (centripetal end), hyaline zone, mitochondria, yolk zone and pigment layer (centrifugal end). Where is the nucleus located? Have the granules in the cortex of the egg (see second paragraph below) been displaced?

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

The spermatozoon enters the egg within a few seconds after attachment. To observe the process place a drop of eggs in the center of a vaseline-ringed slide and add a drop of sperm of just sufficient concentration to fertilize all of the eggs. Add a coverslip and locate as quickly as possible an egg that shows only one spermatozoon on its surface. Note the changes that occur upon penetration of the sperm. Where does membrane elevation first begin? In the cortex of the unfertilized egg there is a single layer of granules which disappear (Moser, 1939) upon fertilization and contribute (according to Runnström, 1944) to the formation of the fertilization membrane. These are best

seen in the hyaline zone of the centrifuged eggs. Inseminate a sample of centrifuged eggs on a slide, as described above and observe the behaviour of the cortical granules. Dark-field illumination shows a bright reddish "luminous" layer on the surface of the unfertilized egg. The luminosity diminishes and becomes paler upon fertilization (Runnström, 1928; Ohman, 1945) Using the dark-field stop disc for the condenser of your microscope examine a sample of unfertilized and fertilized eggs.

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

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

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

Cleavage:-	First	Second	third
Minutes after fertilization:-	42 (25°)	113 (15°)	107 (20°) 145 (20°)

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

$$\log (\text{time at temp. } t) = \log (\text{time at } 20^\circ) - (t-20) 10. \log 2.6$$

Temperatures above 30-32° are lethal for Arbacia eggs. The first three cleavages divide the egg into eight equal sized blastomeres. The planes of the first two cleavages are meridional (in the polar axis), that of the third is equatorial or horizontal (at right angles to the polar axis). Follow the progress of the cleavage furrow in dividing eggs. Note that the hyaline layer forms the surface of the furrow, and later, when the cells flatten against one another, that it forms the boundary between them.

At the fourth cleavage the upper four cells divide meridionally forming eight equal cells called mesomeres, while the lower four cells divide unequally and horizontally forming four large cells called macromeres and below them, at the vegetal pole, four small clear cells called micromeres. At the fifth division the eight mesomeres divide equally and horizontally forming two tiers of cells termed  $a_1$  and  $a_2$  cells divide in more or less radial direction while the macromeres divide horizontally forming two tiers termed  $veg_1$  and  $veg_2$ .  $veg_2$  is next to micromeres which have also divided at this time but which do not form distinct layers. Layers of cells are not readily distinguished in later cleavage stages and no special designation is applied to the cells after the 64-cell stage. It has been shown (see Horstadius, 1939)

that the  $an_1$ ,  $an_2$ , and  $veg_1$  cells form the larval ectoderm; the  $veg_2$  cells form endoderm and part of the mesoderm (coelom and 2nd mesenchyme); the micromeres form the mesoderm (primary mesenchyme) which produces the skeleton. Sketch the various stages up to 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. At about 6 hours after fertilization a smooth-surfaced spherical young blastula is formed, the wall of which is one-cell thick. Cilia soon develop on the surface and the blastula is rotated by their action within the fertilization membrane. At about 10 hours the blastula hatches out of the fertilization membrane. It has been shown (Kopac, 1911) that the blastula releases a "hatching enzyme" at this time that weakens and dissolves the membrane sufficiently for the blastula to break thru. A small tuft of long cilia develops at the animal pole of the blastula which is the forward end when it is swimming. At the base of this apical tuft the blastula wall is thickened, forming the apical plate. At the vegetal pole the blastula wall becomes flattened and the micromeres migrate into the blastocoel, forming the primary mesenchyme which gives rise to the skeleton. Sketch early and late blastulae.

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

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

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 specules 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 con-

tinues to elongate in the dorso-ventral direction and becomes pointed at the postero-dorsal end where the body rods meet. The axis running thru oesophagus, stomach and intestines becomes J-shaped. The stomach expands to form a spherical structure that fills a large part of the body of the pluteus and sphincter muscles connect it with oesophagus and intestine. The two coelomic sacs extend postero-laterally 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 Echinopodium 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.

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Asterias forbosii (or A. vulgaris)

A) Obtaining gametes, maturation and fertilization:

The sexes are separate in Asterias, but it is not possible to distinguish them on the basis of external characteristics. Only animals with soft, bulging arms are fully ripe, and it is a waste of material to open small, hard-skinned starfish in an attempt to obtain gametes. Fill two 1750 cc. finger bowls with clean sea water from a 2-liter flask in which the sediment has been permitted to settle by about fifteen minutes of standing after withdrawal of the sea water from the tap. With large scissors, make a small puncture in one arm close to the disc, and pipette a few drops of cells from the gonad to ascertain the sex of the animal. If the animal is a female, remove this arm completely, and slit it along the mid-dorsal line to expose the bulging pair of ovaries, of a typical pale salmon color. Then with a pair of forceps carefully detach each plume-like ovary by grasping it near its point of attachment at the disc end, closing the gonaduct, and rinse it with as little injury as possible in the first bowl of sea-water, then transfer it to the second bowl. The animal from which the arm has been separated may be returned to a separate aquarium of running sea water, and other arms may be used for gametes later in the day. Such an injured female will not keep indefinitely, however, and gametes are rarely usable at the time the animal begins to show autotomy. Do not cut up to ovaries in the bowl of sea water, merely allow the eggs to exude from the blunt end of the ovaries for a period of five minutes. At the end of this time, remove

the ovaries to another container, or discard them. The best eggs are those first shed. Gently stir the water in the large finger bowl and allow the eggs to settle. Settling occurs very slowly. Then pour off the supernatant sea water and carefully replace with an equal volume from a 2-liter flask. Then leave the eggs undisturbed, without shaking or stirring, for about 20 to 30 minutes. During this time small samples may be removed with a pipette for examination under the microscope, and the steps in germinal vesicle breakdown observed. Note the jelly-hull about the eggs. This may be demonstrated more readily in dim illumination or by adding a trace of Janus Green to one slide preparation. Eggs from a ripe female which was kept under proper conditions of coolness and adequate oxygen supply from the moment of collection, and properly manipulated in obtaining gametes, should show 85 to 94% germinal vesicle breakdown at approximately the same time. Retain a good sample of eggs in a small finger bowl to follow the maturation stages through the second polar division in the uniseminated eggs.

If the animal opened is a male, the testes will be white or ivory. Since it is important to use a fresh sperm suspension, this animal may be placed in a dry fingerbowl until the eggs are ready for fertilization. Then a single testis is removed, rinsed in clean sea water, and a small piece from the blunt end cut off and placed in 200 cc sea water. Two or three pipettes of this suspension should be added to a 1750 cc. finger bowl of eggs, with an immediate but not violent rotational movement to ensure complete mixing. The optimum period for fertilization is after the breakdown of the germinal vesicle and before the 1st polar body has been extruded. It is, therefore, convenient to inseminate when the distal end of the first maturation spindle begins to protrude above the previously smooth surface of the oöcyte, in a fair percentage of the eggs showing germinal vesicle breakdown. Eggs inseminated in the stage of the intact germinal vesicle are non-fertilizable. Even though they may elevate a fertilization membrane they do not develop further. The details of sperm penetration may be readily studied, if the observer examines the eggs without delay. It was in the egg of the starfish that Fol (1876) first observed the actual penetration of an egg by a sperm. Chambers (1930) has confirmed these early observations. A microscope with clear objectives, clean slides and covers, and good illumination are prerequisites for observing the finer details of this process in the laboratory.

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

#### Cleavage:

The blastomeres of *Asterias* are rather loosely connected, because the perivitelline space is wider and the hyaline plasma membrane thinner and weaker than in the *Arbacia* egg. Note the relation between the first and second polar bodies and the fertilization membrane. Chambers has pointed out that in the absence of fertilization membrane, the blastomeres tend to separate completely. Because of the relative transparency of the yolk of this egg, details of living asters may be seen.

A detailed study of the cleavage of this form is usually not undertaken by students of the course unless they have a special interest in this material, but the later stages are of considerable significance.

### Later Stages:

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

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### *Asterias Forbesii*

#### B. Development

Prepare your own cultures for early stages and use those prepared by the assistant for stages from blastula on. Development up to the early Bipinnaria (Dipleurula) can be followed on living material. Older Bipinnaria, Brachiolaria larvae and metamorphosis stages will be studied on stained whole mounts. Whole mounts of early Bipinnaria are also available for comparison. It is advisable to begin with early gastrula stages and follow the development through to the Dipleurula.

Study fertilization, cleavage and blastula later on, using your own cultures. Consult the illustrations in MacBride, Agassiz (1877) and Gemmill (1914) See time table in appendix.

1. Fertilization (see lab directions for "Fertilization")
2. Cleavage. The first two cleavages are meridional, that is, they go through animal and vegetal poles and are perpendicular to each other. The third cleavage is horizontal, the eight cells of this stage are approximately equal in size. In the 16-cell stage, no definite arrangement of cells in rows takes place, and cleavage from now on is irregular. Throughout these early cleavage stages the blastomeres have a tendency to assume spherical shape, resulting in a rather loose arrangement of cells.
3. Blastula. Eventually the cells arrange themselves in an epithelial wall enclosing the blastocoel. The surface cells acquire cilia, and the blastula rotates within the vitelline membrane. The two polar bodies are still visible, either attached to the animal pole, or detached from the embryo. The embryo hatches in the late blastula stage.
4. Early gastrula. The vegetal pole area thickens and flattens and invagination begins. The blastopore is destined to become the anus. The larva elongates along the animal-vegetal axis.
5. Middle and late gastrula. The gastrula becomes pear-shaped. The blind inner end of the archenteron becomes thin-walled and expands. From this end mesenchyme cells wander out into the blastocoel. In a slightly later stage, two outpocketings of the distal end become distinct, the primordia of the coelomic sacs. At the same time, the archenteron bends towards one side which is the future ventral side. This is the first sign of the change of radial into bilateral symmetry. Note the ciliation in the archenteron.
6. Transition to Dipleurula-larva. The blind end of the archenteron bends sharply towards the ventral body wall, makes contact with an ectodermal depression, the stomodaeum, 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 pericanal.
7. Fully formed Dipleurula (early Bipinnaria) larva. This larva represents an early larval type common to Asterozoa, Echinozoa, Ophiurozoa and Holothurozoa (see Korschelt, vol. 1 p. 409). Study carefully a ventral, dorsal and lateral (preferably left) view

Observe the following:

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

## Locomotion

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

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

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

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

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

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

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

## Intestinal Tract

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

9. Brachiolaria and metamorphosis stages. 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 (brachia) are short. They contain diverticula of the coelom. They are not ciliated but their end discs differentiate small papillae and can adhere to the substrate.

A sucker, the gland cells of which secrete a sticky substance, is formed between the brachia. Brachia and sucker serve for attachment of the larva to the substrate in later stages of metamorphosis. (Gerrill, p. 250).

### Intestine

Coelom, in different stages of subdivision

Disk, or developing starfish, on left side.

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

### Echinarachnius parma

#### Obtaining gametes

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

#### Fertilization

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

penetration occurs from fourteen to forty-five seconds after insemination, both processes (i.e., sperm penetration and membrane elevation) may be completed within about 40 seconds after insemination (Just, 1919).

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

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1. Normal development and metamorphosis

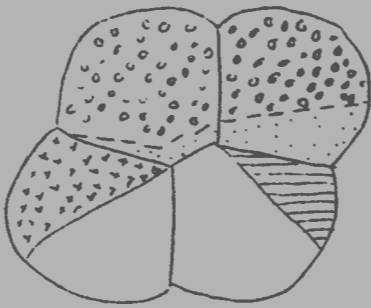
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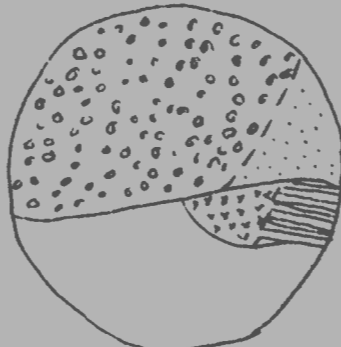
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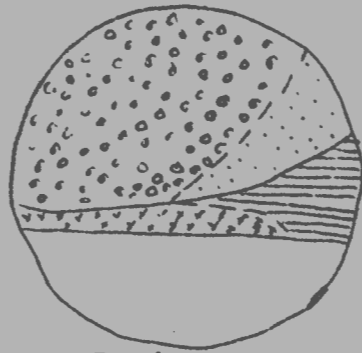
DIAGRAMS OF PRESUMPTIVE TERRITORIES BEFORE GASTRULATION  
 IN CHORDATES (Modified from Pasteels, 1937 and Vogt, 1929)



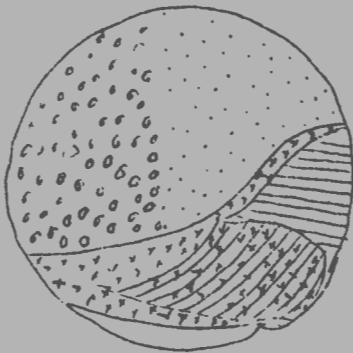
1. Ascidella



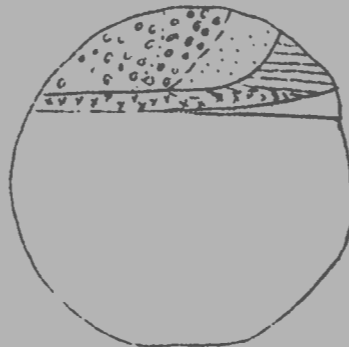
2. Petromyzon



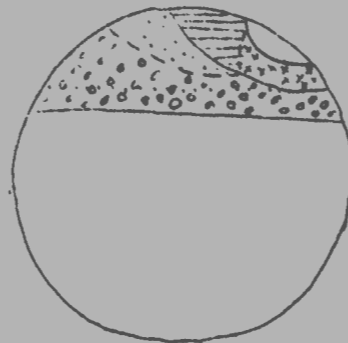
3. Anuran



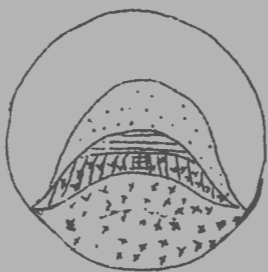
4. Urodele



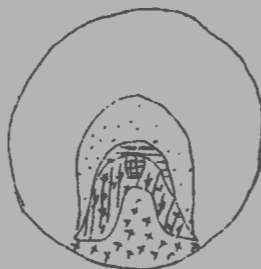
5. Teleost



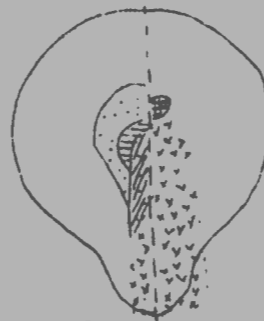
6. Reptile



7.

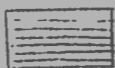


8.

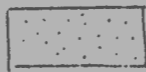


9.

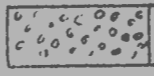
7,8,9.-Chick



Notochord



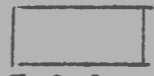
Nervous Syst.



Ectoderm



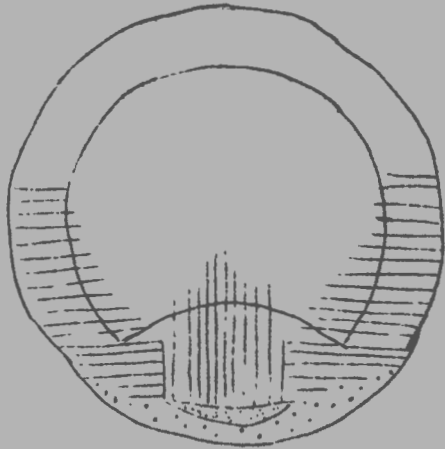
Lat. mesoderm  
& somites



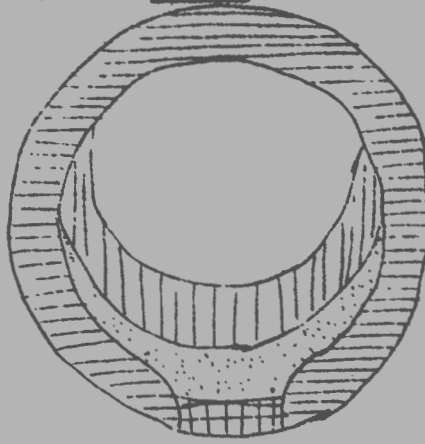
Endoderm  
& yolk

PROSPECTIVE FATE MAPS AND GASTRULATIVE MOVEMENTS IN TELEOSTS  
 (After Oppenheimer, QRB, 1947; and Pasteels, 1936)

1. Fundulus

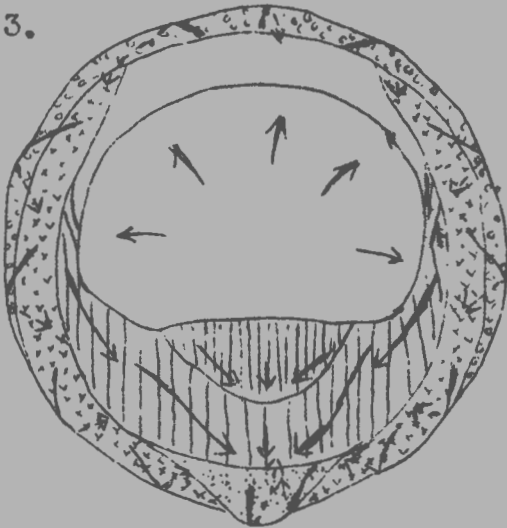


2. Salmo

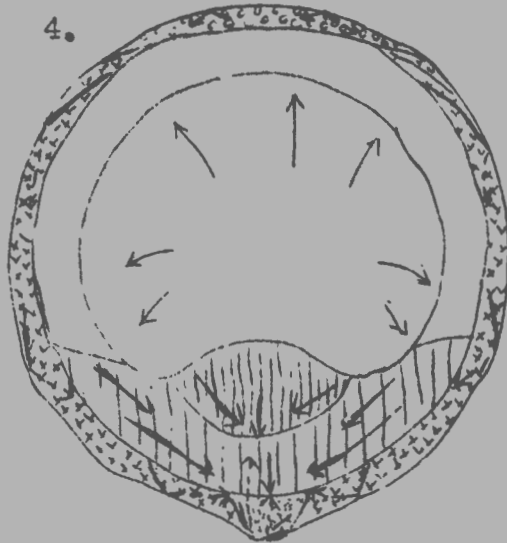


mesoderm    nervous system    endoderm    notochord    prechordal plate

3.



4.

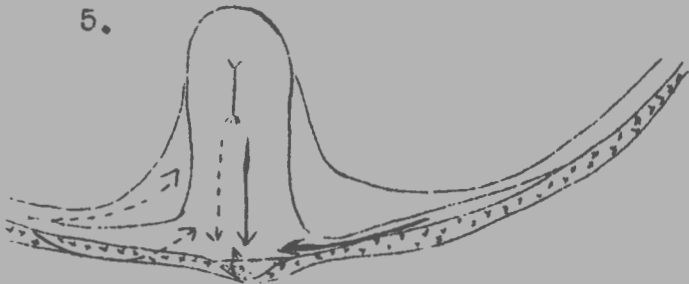


1. CONVERGENCE  
 2. EPIBOLY  
 3. EXPANSION  
 4. DIVERGENCE

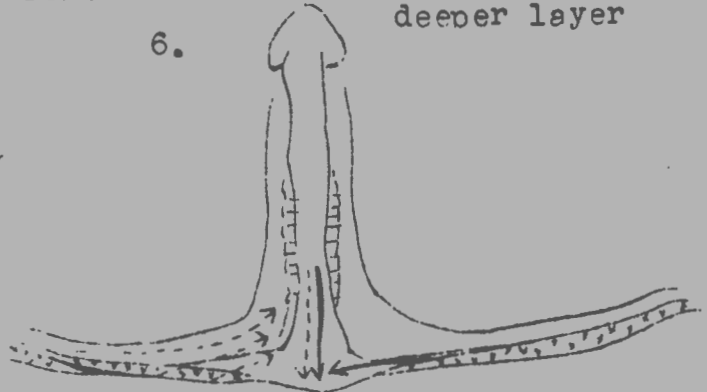
somites    lateral & brain ventral mesoderm    brain

Unbroken arrows - movements on the surface layer  
 Broken arrows - movements in deeper layer

5.

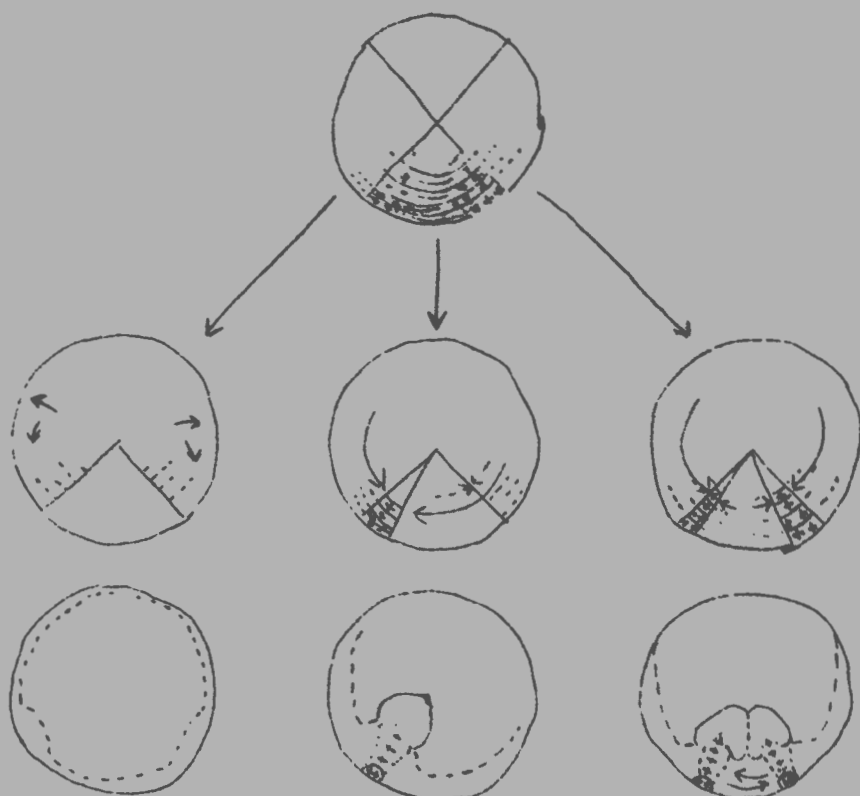


6.

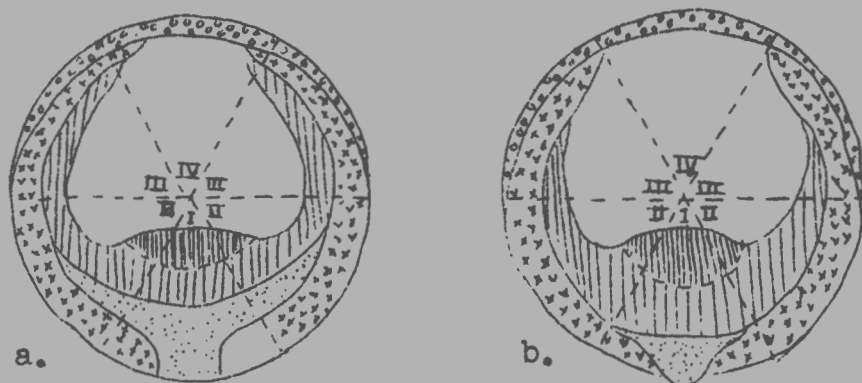


Arrows on left (broken) indicate movements of mesodermal elements; those on right (unbroken) indicate movements in the morphogenesis of the nervous system. Figs. 3, 4, 5, 6, Salmo (Pasteels, 1936)

(not complete agreement on movements -



Luther, fig.9, 1937a; Schematic representation of three theoretically possible types of embryogenesis after substitution of embryonic sectors with extraembryonic material.



Luther, fig 2 (part), 1936a; Sectors of the blastoderm of the earlier (a) and later (b) gastrulae of *Salmo* isolated on the yolk-sac epithelium. Symbols as before (cf. Pasteels, 1936)

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tion of cells

the type of...

Microscopic Examination

The leucocyte count is made by...  
place in a horizontal...  
slide & certain...  
pipette, or better...  
the side of the...  
to determine the...  
the count from the...

count

## Qualities of the material

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

## b) Types of Life History Illustrated

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

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

## c) Order of Study

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

## Laboratory Procedure

### 1. Study of Forms with Perfect Medusa

Examples: Bougainvillia, Obelia, Podocoryne.

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

### 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 thruout the colony, there being no orderly arrangement according to age. Select buds that show various stages of medusa development and mount them under cover slips, and study their unfolding structure. Draw off water from under the coverslip with absorbent paper to produce a slight pressure on the buds. Sketch three stages in medusa development.

When all of its parts except the gonads are fully formed, the medusa breaks loose and swims away. It lives independently one or two months, the gonads gradually maturing. Find a well-developed specimen that is swimming actively and sketch its diagrammatic



structure. Identify manubrium, radial and circular canals, velum, oral tentacles, & groups of marginal tentacles.

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

OBELIA (June, July, August)

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

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

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

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

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

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

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

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

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

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

The highly specialized colony grows in an encrusting mat on snail shells, etc., and is almost exactly like hydractinia. Both have three types of individuals: feeders, gonosomes, stingers (Cf. descriptions of Hydractinia below, p. 6 ) Podocoryne is included here because of its startling metagenetic contrast to Hydractinia.

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

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

### B. Study of Forms with Imperfect Medusae

Examples: Pennaria, Tubularia.

Life Histories: Zygote shed from short-lived imperfect medusa (Pennaria) or retained in reduced sessile medusa form.

(Tubularia): Development to planula larva and metamorphosis to polyp (Pennaria) or development to Actinula larva and growth to polyp (Tubularia): Asexual multiplication of polyps by budding to produce colony; Gonophores formed by special buds on hydranths; Maturation of gonophores (imperfect medusae) and fertilization either in situ (Tubularia) or within the limits of the colony during their detachment (Pennaria)

PENNARIA (July, August, September: begins to ripen middle of July)  
Gonophores bud off singly around the lower portion of the hydranth. They form slightly reduced medusae with rudimentary tuftlike tentacles. Before opening out as transparent bell-shaped forms they suggest coconuts. A single colony bears gonophores of one sex only, but in the living individuals, sex cannot be diagnosed only with difficulty until they mature, when the pinkness of eggs and the whiteness of sperm appear. (Smallwood '89) "Male" and "female" colonies are actually asexual, bearing male and female gonophores respectively.

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

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

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

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

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

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

TUBULARIA (June, July)

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

Examine a ripe male gonophore, considering it as a very degenerate medusa. Notice and sketch its mode of attachment, its shape, the structure of its free end and the position of the sperm surrounding the dark red manubrium. Crush it on a slide and inspect the motile sperm under high power.

Examine and sketch a ripe female gonophore. Usually the tentacles at its distal end appear only as four short blunt knobs, but one or more of them are sometimes slightly elongated. The eggs come from favored oocytes that progressively swallow up their neighbors, lying in the space around the spadix (manubrium).

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

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

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

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

C. Study of Forms with Degenerate Medusae.

Examples with blastostyle inside gonotheca: Campanularia, Gonothyrea.

Life Histories:

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

b) Hydractinia, Eudendrium: Zygote develops into planula larva either inside gonosome (Eudendrium) or after being shed from gonosome (Hydractinia); Planula metamorphoses into polyp; Asexual multiplication by buds; Colony

formation; Gonosomes formed from *Hydrorhiza* (*Hydractinia*) or by transformation of hydranths (*Eudendrium*); Gonophores (highly reduced medusae or sporosacs) borne on gonosomes; Eggs and sperm formed in the sporosacs; Eggs fertilized in situ (*Eudendrium*) or during shedding (*Hydractinia*).

CAMPANULARIA (June, July)

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

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

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

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

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

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

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

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

GONOTHYREA (July, August)

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

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

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

### HYDRACTINIA (June, July, August)

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

#### a) Gonosomes and Gonophores:

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

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

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

#### b) Cleavage and Development

If a number of "male" and "female" colonies of Hydractinia are put together in a large dish of sea water (or a pair of prime colonies in a fingerbowl) and left overnight, eggs should be shed and fertilized between 7 and 9 a. m. WST. The shedding can be controlled by light. If fertilization and cleavage stages are needed later in the day or in the evening, the colonies may be kept illuminated during the preceding night, put in the dark for a couple of hours and reilluminated one hour before the time when shedding is desired. Eggs are shed in 55 minutes, sperm in 50 minutes. Cf. Ballard '42.

Materials for the study of the entire development of Hydractinia from egg to polyp will be made available. If possible, observe the shedding of eggs and sperm. Sketch eggs undergoing first three cleavages, elongated gastrula, swimming and attached planulae, metamorphosing form and young polyps.

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

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

of drying up and concentration of sea water, or other unfavorable circumstances.

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

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

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

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

#### EUDENDRIUM (July, August)

The gonosomes of this genus are degenerate sessile medusa-forms or gonophores, strikingly different in the two sexes, borne at the bases of special hydranths which lose their tentacles and degenerate while the gonophores are ripening.

"Female" colonies bear loose irregular tufts of sporosacs attached to the stems, each fine sporosac being bright orange in color. "Male" colonies bear light pink sporosacs arranged in groups of two to four or more in a line, the lines radiating from a common point on the base of the degenerated hydranth. The ripest male sporosacs occur at the periphery of the cluster and are white with sperm. Sketch both male and female sporosacs.

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

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

#### Development of Scyphozoa

#### SHRELIA OR CYANEA (April, June)

Both these jelly fishes have oral lobes extending downward. In mature specimens granular material will be found entangled on the lobes or contained in small brood sacs in the lobes. Tease off some of this material into a drop of sea water on a slide, and examine under the microscope. Embryos of different stages can be found, from spherical cleaving eggs to oval gastrulating forms and fully formed stocky, active planulae. (Hargitt, G. T. '11) Hein '00/ Is cleavage regular? Sketch the embryonic stages that are available.

...in the ...  
...of many months.

...life history illustrated in the texts of ...  
... 1911.

## Reconstitution in Animals

The processes by which organisms replace lost parts have many points of similarity with those that take place in embryonic development. Thus, in the ontogeny of most species there is a period of cell multiplication with little or no cellular differentiation (cleavage stages) followed by the period in which cellular differentiation occurs. In regeneration the first of these two periods is represented by the formation of the blastema, and the second, by its differentiation.

Coelenterates replace lost parts with great facility but the process differs from true regeneration because no increase in cell division has been demonstrated and there is no blastema from which the new hydranth differentiates. Likewise, there are no special reserve cells to become activated by the injury and give rise to the new structures. Instead, the cut surface is healed by the expansion and migration of adjacent cells, and, without any new growth, transformation of cells of the stem into parts of the hydranth takes place. This type of replacement of lost parts is known as reconstitution instead of regeneration.

The study of reconstitutive development has some inherent advantages over embryonic development, chief of which is that in reconstitution one can study cell differentiation without having cell division as a serious complicating process. Another advantage is that of size. Reconstituting organs are generally considerably larger, and consequently easier to observe and manipulate, than are those in the embryo. Since the analysis of this process has been carried farther in Tubularia than in other forms, we shall study reconstitution in this genus in the laboratory exercises. It must be born in mind that we are studying the transformation of "adult" cells; from one type of organization and differentiation (i.e. oenosare) to another (i.e. hydranth).

Most studies of reconstitution of a hydroid stem may be grouped roughly into two categories: one which deals with the physiological properties imposed upon the stem by the original organization of the intact stem (gradients, dominance, polarity). The other involves a study of the manner in which changes in the environment may alter these properties, i. e. enhance or inhibit them.

The intact hydroid consists of a hydranth, stem and base. The hydranth is the most active region of the organism. When it is removed it may be seen that a gradient of activity exists along the stem with the high point at the distal end. Such a gradient may be revealed by differential dye reduction (Child, '41) and by the behavior of the stem during reconstitution. In the latter, two of the chief expressions of the gradient are in the polarity relations of the reconstituted stems (the hydranths tend to form at the distal ends of the stems, especially in small pieces) and in the phenomenon of dominance (the ability of a distal developing hydranth to inhibit hydranth formation in more proximal regions of the stem). Similar dominance relationships are encountered in embryos. While the events resulting in polarity in embryonic fields are much more complex than in hydroid stems once the polarity is established the phenomenon of dominance is also revealed, e. g. the ventral differentiating region of a limb-bud field inhibits adjacent tissue, potentially capable of forming limb structures, from becoming a part of the limb.

One advantage of the hydroid material is the ease with which the processes of reconstitution may be altered. They may be completely inhibited (by covering the reconstituting region with sand (Morgan, '03), with a glass tube, Barth, '36), by a ligature (Morgan, '02, Peebles, '31) (see Barth, '36), by oxygenated sea water (Miller, '37), by lowered pH (Goldin, '42 a & b) and by an inhibitor released by the hydroids themselves (Rose & Rose, '41, Miller, '42). Polarity and dominance relations may be changed by altering the supply of oxygen



or removing barriers to free diffusion (Miller, '37, '39, Zwilling, '39, Nakamura, '39).

After a hydranth is removed and the stem of a Tubularian is isolated the first visible indication of reconstitution is the appearance of a pink pigment. As the process progresses longitudinal striations, which represent future tentacles, become evident in the pink region. The next marked change is the appearance of a constriction which separates the newly forming hydranth from the coenosarc. This occurs in 20-30 hours from the initiation of reconstitution. Within the next 10-30 hours the hydranth emerges. The time of appearance of the constriction and the length of the stem from the free end to the constriction have been used as indices of rate of reconstitution. See Barth, '38b, Miller, '42, Child, '41, and Spiegelman and Moog, '44 for a discussion of measurements of rates.

### Collection and Care of Tubularia

Theoretically Tubularia is a solitary form but actually so many individuals grow together that a dense tangled mass usually results in the older forms. Young short stems are the best for experimental work and can be obtained from floats and rocks where the current is swift. In general it is best to collect your own stems. Since the stems need running water and a low temperature they do not keep well in the laboratory. In nature the hydranths drop off about the end of July and the stems remain dormant until the water cools down in the Fall. At Woods Hole the stems appear in mid June and can be used until August. However, since the waters of Cape Cod Bay on the north shore are much colder, Tubularia may be obtained from the north end of the canal throughout August. The best method of keeping them in the laboratory is to place each bunch in a 300 ml beaker on steps which allow the water to cascade from one beaker to the next below to insure vigorous circulation.

The stems as collected vary in length, thickness and in general physiological condition (some are crushed, some starved, others old with large gonophores). Therefore, we cut the stems off and sort them out in a large finger bowl being careful not to crush them. For most work stems about 10mm in length are suitable. These are selected for uniform diameter and appearance. The hydranth is cut off a few mm from its base. It is necessary to cut off 3-5 mm of the stem with the hydranth as this part of the stem does not regenerate consistently, especially in older stems.

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

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

The instruments used for cutting and handling are a sharp scalpel, a pair of sharp scissors, a pair of forceps and a medicine dropper. In using the forceps care must be taken that only the parts which are finally cut off and discarded are handled. Stems are most easily trimmed to size by cutting them on a glass plate

over a black background with a ruler along which the proper length may be measured. After the stems have been cut to size they are transferred with a pipette and must not be handled with forceps.

When the temperature of running sea water gets above 25° C it is necessary to keep the stems in a refrigerated bath or in stoppered flasks with an atmosphere of oxygen.

Experiments

✓1. Gradients in Reconstitution

There are quantitative differences along the stem which can be demonstrated in a variety of ways, but perhaps the most significant is the difference in rate and size of the products of reconstitution at different levels of the stem. You may demonstrate this gradient by cutting long stems into thirds and recording the time of appearance of the constriction separating the future hydranth from the neck region (Barth '38). Since this constriction appears from 24 hours after cutting and the still unconstricted stems should be examined every two hours until it appears, it is well to begin this experiment as early in the day as practical.

Select 25mm stems for homogeneity with regard to diameter and appearance and after removal of the hydranth cut each into three pieces of equal length (6mm) and discard what is left. Place them in separate finger bowls marked Distal, Middle and Proximal and keep on a water table until they have been recorded. (If you have an ocular micrometer the length of the hydranth can be accurately measured also). After they all have reconstituted average times (and lengths) should be calculated. What factors can you suggest which might play a role in the differential which your experiments reveal?

✓2. Dominance 1/2

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- Four lots of 10 stems each will be used.
- Lot 1 long stems (12 mm long after cutting)
- Lot 2 short stems (6 mm long after cutting)
- Lot 3 short stems (6mm) ligatured in the middle
- Lot 4 very short stems (2 to 3 mm long after cutting)

In preparing these lots be very careful to remove 3mm or more of the stem with the hydranth. Cut the stems in such a way that the two ends of the stem can be distinguished. This may be accomplished very simply by making the distal cut at an oblique angle and the proximal cut at a right angle to the stem.

With regard to the proximal hydranth this is an all or none type of experiment. If taken from healthy colonies the developing distal hydranth either will completely prevent hydranth reconstitution at the proximal end of the piece or will have little effect upon it.

Place the stems in Syracuse dishes in a large finger bowl through which a current of sea water is flowing gently, or in finger bowls kept on the sea water table. At 48 hours the reconstituted hydranths should have emerged from the perisarc. Record the number of distal and proximal hydranths in each lot.

### 3. Effects of Oxygen upon Reconstitution

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Cut 20 or more 6 or 8 mm pieces. Place half of them in a 200cc Erlenmeyer flask filled to the top with oxygenated sea water and stopper tightly so that no air is trapped in the flask. Place the other half of the stems in a similar flask filled with sea water through which nitrogen has been bubbled. After 36 to 48 hours count the number of hydranths reconstituted in each flask. If you wish to continue the experiment, the stems from the nitrogenated flask may now be transferred to the oxygenated flask and their ability to reconstitute can still be elicited (Cf. Barth '38).

### 4. Effects of Oxygen Upon Scale of Organization and Upon Bipolarity (Cf. Miller '49)

Cut 60 or more very short pieces (1-1.5mm long), select 50 which are most nearly the same size and divide into two lots of 25 each.

Lot 1. Place the pieces of this lot in the flask of oxygenated sea water you prepared for experiment 3. (Since they are very small there is no danger of confusing these pieces with the 6 or 8 mm pieces you already placed in it).

Lot 2. Place these pieces in a covered fingerbowl or Erlenmeyer flask on the water table.

Count the number of complete hydranths reconstituted and also the numbers of various partial hydranths beginning about 48 hours after the experiment was started. Since the partial hydranths are unable to emerge from the perisarc, it will be necessary in most cases to squirt them out by drawing them up into an eye dropper and squeezing the bulb rapidly.

Note that Oxygen increases:

- (1) the percentage of pieces that reconstitute.
- (2) the number of partial hydranths (a result of its effect upon the size of the primordia when there is not enough tissue in the piece to form a larger hydranth)
- (3) the percentage of bipolar types. Can you suggest an explanation for the increased frequency of bipolar types in short pieces as a result of oxygenation, when it decreases bipolarity in longer stems?

### 5. Effects of Reducing Metabolic Exchange Through One End of the Stem.

Cut 20 10mm long stems in such a way that the two ends of the stems can be distinguished.

Insert the distal ends of 10 of these into some washed sand in a finger bowl filled with sea water and insert the proximal ends of the other 10 into the sand. After 36 to 48 hours remove and count the hydranths which have developed at distal and proximal ends (Cf. Morgan '03).

### 6. Effect of Acidity Upon Reconstitution

Prepare acidified sea water by first adjusting about 100 cc to pH 3-4 with concentrated HCl. Bubble air through this water vigorously for 3-6 hours. Adjust again to pH 5.9 with McIlvaine's buffer (citric acid, 1M- secondary sodium phosphate .2M). Place 10 stems (10mm) into a flask or finger bowl in the adjusted sea water. Place a similar group of stems in untreated water. Keep these on the

sea water table. When all of the control stems have become constricted note the number which have reached the same stage in the acidified sea water. Check to pH of the water in each vessel at the end of the experiment (Goldin, '42 a&b).

7. Inhibitors Released By The Stems. *Moff.*

Take a small colony of Tubularia (200-250 long stems) and cut it up into small pieces (8-10mm). Allow these to heal for 1-2 hours, then wash them carefully and place them in a flask with 250 cc of filtered sea water. Let them stand for 12 hours with air bubbling through the water. After this interval place two groups of 10 freshly prepared 10mm. stems in some of this water in separate containers. Place 10 similar stems in an equal quantity of untreated sea water. Bubble air through one of the two vessels which have "colony" water for the duration of the experiment.. Keep all vessels on the sea water table until most of the control stems have reached the constriction stage. Note the number constricted in each. Check the pH of the water in each vessel at the termination of the vessel. Inhibited stems may be further tested by placing them in fresh sea water at the end of the experiment. If they reconstitute they have been inhibited and not killed. (See Rose and Rose, '41).

8. Independence of time for reconstitution and length of hydranth.

Prepare 2 groups of 10 stems each (10mm). Place them in separate vessels. Keep one of the vessels on the sea water (note temperature) table (one of the control groups in exp. 6 or 7 may be used for this experiment). Place the other in a refrigerator at approximately 7°C. Note the time for appearance of the constriction and the length of the hydranth primordium in each case. Plot the results by converting 1/t (rate) and length of the refrigerated stems to percent of the controls. Temperature is the abscissa. (Spiegelman and Mugg, '44).

If time permits perform the following experiments in addition to the above.

9. Demonstration of the liberation of inhibitors of reconstitution by the cut ends of stems.

Fill 10 pieces of 1mm glass tubing 15mm long with oxygen. (This can be done very readily under water). Affix one end of each to a small amount of neutral plasticine attached to the bottom of the finger bowl and insert into 5 the distal end (cut obliquely) of a 10mm piece of stem. Into the other five insert the proximal ends (cut transversely) of similar pieces. After 36 hours record the hydranths reconstituted at the exposed ends and those at the ends inserted in the O<sub>2</sub> filled tubes (Cf. Rose and Rose '41, Miller '42).

10. To Demonstrate Effects of Acidity upon Reconstitution

Tubularia has been found to be very sensitive to acidity. (Goldin'42a, '42b). An external pH of 6 will completely prevent reconstitution. Observe stems which have been injected with phenol red 30 hours earlier and placed in glass tubes which interfere with the release of those pH lowering substances. Compare these with stems which have been injected but have not been placed in long tubes. Draw and color one stem from each lot. After the hydranths have emerged they may be drawn and colored again (Cf. Miller '49).



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*Springer*

Development of  
Non-Pelagic (Demersal) Eggs  
Type - Fundulus sp.

Breeding Season: Material is best and most abundant during the first three weeks of June but small numbers of fertilizable eggs can be procured through July 15.

Equipment:

Living Material - Fundulus heteroclitus and/or Fundulus majalis

Classware, etc. -

General Class Equipment - 3 large aquaria  
Individual Equipment - 3 clean finger-bowls (4 by 2")  
3 glass plates to cover finger-bowls  
2 syracuse dishes  
3 ordinary pipettes and bulbs  
1 fine-tipped pipette and bulb  
paper toweling or filter paper  
lens paper  
2 syracuse dishes  
hair loop  
culture slide - 1.7-1.8 mm. depression  
plain glass slides  
thin sheet of mica  
cover slips

Solutions - stock 0.5% Neutral Red solution  
Dilute sea H<sub>2</sub>O (70% sea H<sub>2</sub>O, 30% fresh water)

Additional Reagents needed if eggs are to be fixed for sectioning or total preparations:

1- Total preparations:

Stockard's solution: formalin - 5 parts  
glacial acetic - 4 parts  
glycerine - 6 parts  
distilled water - 85 parts

2- For Sectioning:

Bouin's or Zenker's Fluids  
Graded series of Alcohols  
amyl- acetate  
56-58 degree paraffin

Technique of Preparing and Handling Material:

A. Care of Adults

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 dozen broad, dark, transverse bars. The black patch on the dorsal fin is striking.

B. Procuring 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 drawn 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.

C. Preparation of Cultures

Strip eggs into a clean finger bowl which has been moistened with sea water. Strip milt into a small amount of sea water, mix with eggs, and allow to stand in  $\frac{1}{4}$ " of salt water. Neither eggs nor sperm should be allowed to stand before fertilizing. After 30-45 minutes change the sea water and leave the eggs in  $\frac{1}{2}$ - $\frac{1}{2}$ ". Keep bowl covered with a 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 at least twice a day.

D. 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 outer membrane 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 where absolutely normal development is essential, eggs are usually examined uncovered in shallow depression slides 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 1.7-1.8 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 mica. 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.

E. 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.

F. Preparation of Eggs for Sectioning

Eggs must be dechorionated before fixation to allow fluids to penetrate the interior. (For details of this process see Nicholas, '27). The following method of embedding is that of

Barron '34, with some modifications of timing suggested by J. Oppenheimer.

- 1- Fix in Bouin's or Zenker's solution 12-24 hours.
- 2- Run up in ordinary manner through the alcohols leaving eggs in each through 95% for one hour.
- 3- Place in absolute for 2 hours-running through several changes.
- 4- Place in equal parts absolute and amyl-acetate for 2 hours.
- 5- Place in amyl-acetate for 24-48 hours.
- 6- Place in equal parts of amyl-acetate and paraffin and incubate at about 30 degrees for about 12 hours.
- 7- Transfer through three changes of paraffin (15 minutes in each) and embed in 56-58 degree paraffin.

#### Observations of Normal Development

- 1- The Unfertilized Egg: Strip the eggs from a female into diluted sea water (70% fresh water, 30% sea water). Keep them in this solution to retain the morphological characteristics observable at time of extrusion. Note the details of structures of the unfertilized ripe ovum. These include platelets, oil drops, protoplasm, membranes, micropyle, etc. (The micropyle must be observed before removal of chorionic jelly). If young ova are present compare with ripe ova.
- 2- The Sperm: Sperm may be stripped into sea water and a drop of the suspension examined under a cover slip under high power. Note the general structure and the enormous size difference between eggs and sperm.
- 3- Fertilization: Prepare a culture of fertilized eggs according to the method outlined in part C of the section on technique. Be sure to record exact time of insemination. Be prepared to transfer eggs immediately to a slide for observation. Record time of fading of platelets, of the formation of the perivitelline space. What are your conclusions in regard to the rapidity of activation of the egg? If practicable, place a number of newly-extruded unfertilized eggs in a depression slide and partially cover the depression with a cover slip leaving uncovered a space large enough to permit the introduction of a fine pipette. Rotate eggs until the micropyle of one comes into view. Introduce a drop of sperm suspension into the depression without disturbing the cover slip and watch the entrance of the sperm and the spread of the fertilization reaction from the locus of the micropyle. Since polar bodies have not been seen in Fundulus it has not been determined at what stage of maturation the sperm enters the egg.
- 4- Formation of the Blastodisc: Note the gradual accumulation of the protoplasmic cap. This is the blastodisc or germ disc. Compare polar and lateral views. What is the relation of the pole of the egg to gravity? How does this compare with the condition in the frog egg; with the chick egg? Do any processes take place in the unfertilized egg similar to those in the fertilized egg?
- 5- Cleavage: Watch for the appearance of a groove on the surface of the blastodisc- the indication of the first cleavage

plane. This usually occurs within 2-3 hours after fertilization. Note the geometric relation, and time sequence of the subsequent cleavages. Do the cleavage planes divide the entire ovum? The entire blastodisc? During interkinesis the nuclei are sometimes visible. Distinguish between central and marginal cells. Follow cleavage carefully to the 32 cell stage. Note irregularities. When do horizontal cleavage planes first occur? Does the blastodisc increase in size or alter in form? Note that cleavages continue for a considerable period without much change in form from that of the original blastodisc. This is called the period of the high blastula. When does the change of form to the flat blastula occur? (See Oppenheimer '37 for chronological terms).

- 6.- Time Table of Development: The rate of development varies with temperature and other external environmental conditions. The approximate developmental stages which may be expected under various conditions of temperature and salinity may be seen in chart 1. The stages are numbered according to the chronology established by Oppenheimer '37.
- 7.- The Periblast: (Appears 16-24 hours after fertilization) The uncleaved protoplasm around the margin of the group of blastomeres is called the marginal periblast; that beneath the blastodisc (only visible in sections) the central periblast. In the late blastodisc (18-20 hours) observe particularly the behavior of the marginal cells and distinguish between circular and radial cleavages. The large pinkish nuclei of the periblast are easily visible. Note how the nuclei of the marginal row of cells become free of cell outlines, continue their divisions and migrate into the marginal periblast, converting it into a nucleated but non-cellular structure. Follow the periblast structure in later stages.
- 8.- The Germ Ring and the Extension of the Blastodisc (18-48 hours) Subsequent to the nucleation of the periblast note the change in form and size of the blastoderm. The embryo is now referred to as a blastula. Soon the margin of the disc appears relatively thicker. This thickening is termed the germ-ring and is due both to an actual peripheral increase in cells and to a thinning of the central part of the disc. This germ ring can best be observed in F. majalis. During the next few hours the germ ring grows completely over the surface of the yolk mass. The uncovered portion of the yolk is the blastopore. The final covering of the yolk or the closing of the blastopore occurs after the first stages of the formation of the embryonic axis. Under favorable conditions the beginning of gastrulation may be observed in the appearance of a slight indentation at the edge of the germ ring at the time when the yolk is about  $\frac{1}{4}$  covered. Staining with neutral red will aid in identification of the germ ring and periblast. (Add 1 or 2 drops of stock solution to a syracuse dish of sea water).
- 9.- The Formation of the Embryo (Begins in 24-36 hours) While the germ ring is extending around the yolk the embryonic axis is being established. Its first indication is a cellular

thickening known as the embryonic shield caused by a more active movement of cells in one part of the germ ring. This formation is initiated when the blastoderm has covered from one-quarter to one-third of the surface of the yolk. By the time that the blastoderm has covered about one-half of the yolk the embryonic shield has become a bluntly triangular area extending from the margin of one portion of the blastoderm to near the center of the blastoderm. The shield can best be identified in profile view. As the blastoderm spreads over the surface of the yolk the embryo grows rapidly in length.

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

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

During this period the embryo becomes segmented. This segmentation is confined to the mesoderm which lies on each side of the axis of the embryo forming mesoblastic somites. How many somites are present at the time of the closure of the blastopore?

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

10. Later Development Obtain series of embryos of 2,3,4,5,6 days and make a detailed comparative study. It is suggested that drawings be made at 24 hr. intervals and that a chart be made showing the first appearance and later development of the organ systems. The following method of chart construction may be used:

	Days after fertilization				
	2	3	4	5	6
Somite Number					
<u>Nervous System</u>					
Brain Divisions					
Eye					
Otocyst					
Olfactory Pit					
<u>Circulatory System</u>					
:					
:					
:					
etc					

If you are unfamiliar with the form of chronological charts good examples may be found in the following texts:

Lillie, F. R. 1940 The development of the chick. New York: Holt. (opposite page 68)  
 Arey, L. B. 1940. Developmental anatomy, Philadelphia and London: Saunders. (opposite page 148)

It is suggested that embryos be removed from their chorions in the later stages, for better observation of structural details. Although this is difficult to perform in early stages and requires special instruments (See experimental section, page...) in later stages the loose chorion may be torn off with sharpened forceps, or with the beading needles. Be careful to avoid injury to the yolk sac.

The following developmental features should be observed and incorporated into drawings and chart;

- a. Sonites. Note first appearance and determine numbers formed on successive days.
  - b. Nervous system Find in early stages the optic vesicles, lens neuromeres, mid-, fore-, and hind-train regions and trace the development into cerebrum, optic lobes, cerebellum, medulla, etc. Study the development of the eye, olfactory pit and otocyst. How is the lens of the eye formed?
  - c. Circulatory system. Note the extra-embryonic body cavity; formation of the pericardium; first blood vessels (and especially their mode of formation from wandering mesenchyme cells); the first action of the heart; form and position of the heart. Compare the course of circulation on the 4th and 6th days. Consult chart No. 2 for an outline of the development of the circulatory system.
  - d. Mesenchymal cells. Note wandering mesenchymal cells, especially abundant beneath the posterior part of the embryo on the 2nd, and 3rd days. Can you distinguish various types? By successive observations at brief intervals, the change in form, migration, and division of these cells may be noted.
  - e. Note first appearance of fins; of the urinary vesicle or bladder (a bi-lobed outgrowth of the hind-gut); the liver posterior to the left fin bud.
11. After Hatching. The young fish may be studied just after hatching by anaesthetizing with chloroform. Consult paper by Oppenheim '37 for further details of developmental stages.

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DEVELOPMENT OF THE TELEOST

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IX Technique

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Technique of Handling Pelagic Eggs

Type - Tautogolabrus adapersus, the  
American cunner or Chogset

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.

Pelagic eggs may be obtained from the scup (*Stenotomus chrysops*, Linn.) and the mackerel (*Scomber scombrus*, Linn.), but must be stripped and fertilized as the fish are taken fresh from the live car. The cunner will prove far more useful, particularly for the study of early stages, for it may be brought to the Laboratory aquaria and stripped as needed.

Cunners should be caught the same day as needed; females are ordinarily obtained only after 12 M. 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 for holding the fish while they are being stripped, because they are extremely slimy and have sharp spines in the dorsal fin.

As soon as possible after stripping, the sperm-suspension should be poured into the egg dish and the time recorded. Let the mixture stand for half a minute; 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 to the top and collect principally at the edge of the meniscus. They should be pipetted off and placed in covered finger-bowls containing  $\frac{1}{4}$ " 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 16-18°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.

### Stage of Development

	11	12	13	14	15	16	17	18	19	20	21	22	23
<b>15°C</b>													
Sea water		1		2			3			4	5	6	
1/2 sea													
1/2 distil.		1		2		3		4	5	6	7	8	
Tap water			1			2		3			4		
Dist. water		1		2		3	4	5	6		7	8	9
<b>20°C</b>													
Sea water			1						2			3	
1/2 sea													
1/2 distil.			1				2		3			4	
Tap Water				1				2			3		
Dist. water			1				2		3			4	
<b>25°C</b>													
Sea water					1						2		
1/2 sea													
1/2 distil.				1				2				3	
Tap Water						1						2	
Dist. water						1					2		
<b>30°C</b>													
Sea water								1					2
1/2 sea													
1/2 distil.								1					2
Tap water									1				2
Dist. water									1				2

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

Daniel Herriman, Osborn Zoological Laboratory and Wingham Oceanographic Laboratory, Yale University.

24 25 26 27 28 29 30 31

7 8 9 10 11 12 13 16

9 10 11 12 13 17 20 23

5 6 7 9 10 11 13

10 11 12 14 16 19 23

4 5 6 7 8 10 12

5 6 7 9 12 14

4 5 7 8 10 11

5 6 7 9 11 14

3 4 5 6 7 8

4 5 6 7 8.5

3 4 5 6 7 8

3 4 5 6 7 8 9.5

3 4 5 6

3 4 5 6.5

3 4 5 6

3 4 5 6 7







# EMBRYOLOGY OF MOLLUSCA

## Gastropoda

### 1. The Veliger Larva

Study the typical Veliger larva of Crepidula fornicata. Obtain material by breaking the animal from the substrate to which it is attached. You will find the yellowish eggs (enclosed in transparent capsules) attached to the substrate or in the shell of the mother. Tease the embryos out of the capsules. Obtain swimming larvae in different stages, particularly old ones with the yolk resorbed. They are transparent and show the inner organs. Consult Conklin (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 Korschelt 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.

### 2. EARLY STAGES OF THE VELIGER

There is no typical trochophore stage in Crepidula. 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.



CREPIDULA -- DERIVATIVES

1st quartette of micromeres:

- a. all ectoderm cells of head vesicle
- b. apical plate of ciliated cells
- c. posterior cell plate
- d. dorsal portion of functional velum and portion of first velar row on ventral side
- e. supraesophageal 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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## Lamellibranch (Pelecypod) Development

Type: Lactra solidissima

### 1. Normal Development:

- A. The Unfertilized Egg: Obtain a sample of unfertilized eggs in a stendorf dish and transfer a few to a depression slide for study. When shed, the eggs are irregular in shape due to the pressure within the ovary, but they become spherical on standing. They are small (53 micra in diameter) 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 the eggs are inseminated, they will retain 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.
- B. Fertilization and Maturation: 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 by 30-35 minutes after insemination. Note the position of the polar bodies, for they mark the plane of the coming cleavage.
- C. 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 large cell and three smaller cells. Compare the four cell stage of *Crepidula* and *Nereis*. The following cleavages are rapid, perhaps only ten minutes intervening 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.
- D. Time Table of Development: There is much variation in the developmental rate depending on temperature and other environmental conditions, but the following table will give some idea of the chronology at 25°C. (Schechter, 1941).

Times are recorded from insemination:

10 minutes	Germinal vesicle reaction
30 "	Polar bodies formed
50 "	Pronuclei visible
1 Hr 5 min.	First cleavage
1 Hr 35 min.	Second cleavage
5 Hrs.	Swimming forms

- E. Later Development: The figures of the development of *Dreissensia* (Leisenheimer, 1900) will prove very helpful, for the embryology of the two forms is very similar.
1. Gastrulation and early Trochophore stages: Remove samples 4-9 hours after insemination. If forms are moving too rapidly, add a drop of Janus green to mounts. 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 epiboly. 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 appearance 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 blastopore is visible on the ventral side as a conspicuous concavity on the dorsal surface. By 14 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.
  2. 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 apical flagellum, telotroch, and the long cilia of the developing velum.
    - d. The stomodeal invagination on the ventral side, just below the velum. The proctodeal invagination appears later (about 23 hours).
    - e. The internal structures are difficult to recognize at this time, for a large, dark mass of undifferentiated endodermal and mesodermal cells fills most of the post-velar area.

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ALPHINEURA

Chaetoploura apiculata (the Chiton)

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. Living larvae can be mounted in a dilute solution of Janus Green for observation.

Study of Normal Development:

1. 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.
2. 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 dexiotropic 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 stomadeum, 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.
3. 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 seemed to occur early in this batch, the usual time being from 7-12 days. Time is recorded from the time of insemination:

1st polar body	30 min
2nd " "	55 "
1st cleavage	1½ hours
2nd " "	2 "
3rd " "	2 hrs. 40 min
gastrulation	ca. 13 hrs.
beating cilia	14 hrs.

rotation in capsule	20 hrs.
hatching	36 "
free swimming trochophores	2½-3 days
metamorphosis	4 days

4. Later Stages of Development and Metamorphosis:

- A. Young Trochophores 40-60 hours 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. Crowning the pretrochal 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 protrochal 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.

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which have been observed. Eggs are visible at the (top of foot). After the egg is removed gently, and the capsule transferred to filtered sea water with a pair of fine forceps, it is seen to prefer to deposit eggs on the wooden sides of the dish in preference to the glass. Sometimes suitable 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 egg to be determined before removal from the capsule with the aid of a binocular microscope). When the proper stage is found, pierce the side of the capsule furthest from the eggs with a clean needle. With another needle, tear off the section that is attached to the dish, making sure that a very large hole is made. While pressure is not applied to the capsule, the eggs will come out in a mass of jelly. Make sure that all of the eggs are out of the capsule and that the capsule is rather well torn before releasing the pressure, or else the thick jelly will run back into the capsule, carrying the eggs with it. All of these operations must be carried out under water, for the eggs will die 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 "	(1st polar body off)
2nd lobe appears	1 hr 12 min.	
2nd lobe gone	2 hrs 12 "	
3rd lobe	2 " 42 "	
1st cleavage	3 " 42 "	
4 cells	4 " 52 "	

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

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## CELL LINEAGE

### Maturation, Fertilization, and Cleavage through the 25-cell Stage in Crepidula.

Due to the opacity of the living eggs, the details of maturation, association of the sperm 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 spermatozoa 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 dextrotropic and which are leiotropic. 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 slides 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 Syracuse 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 micro-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-alun, 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.

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## Intercourse of animals

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

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

Cultures of advanced stages will be prepared. If you wish to prepare your own cultures proceed as follows: Hydroides both male and females will spawn immediately after being removed from their calcareous tubes. Remove several and place them in finger bowls (one worm per dish to keep the sexes separate). Remove the animals after they have spawned. Let sperm stand for about  $\frac{1}{2}$  hour. Add a few drops of sperm suspension to a dish of eggs. (Extrusion of polar bodies and cleavage may be easily studied). The blastula stage is reached after 5-6 hours, gastrulation after 8-12 hours; the trochophore stage lasts from 20 hours to two weeks. The trochophores are best for study when 2-5 days old. Sabellaria may be treated in the same way. Fertilization of Nereis has been studied in a previous lab period.

### 1.

#### The Trochophore of Hydroides.

The Trochophore is a typical Annelid trochophore. Consult the excellent figs. in Hetschek ('86) and Shearer ('11). The larvae show positive phototaxis and gather at the window side of the dish. Mount trochophores, 3-5 days old, on a slide on which a few shreds of lens paper have been placed to entangle them and hold them quiet. Narcotics, e. g., a few drops of chloroform or of  $MgSO_4$ , are not very effective but may be tried. Vital staining obscures rather than clarifies the structures. The larvae are transparent, and proper adjustment of the illumination by moving the mirror and the Abbe condenser will bring out all structures. Study animals in lateral and in polar views (both from animal and from vegetal pole). The apical tuft and the anal vesicle are landmarks for the poles, the mouth is on the ventral side; the eye is on the right side. Observe the locomotion first.

#### Observe:

1. Shape of the trochophore
2. Apical tuft (several long cilia, probably functioning as a sense organ).
3. Apical organ, a thickening of ectoderm at the animal pole; a nerve center and probably the primordium of the cerebral ganglia.
4. The Prototroch, an equatorial band of large cilia. In older 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 (proctodaeum). 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 posttrochal hemisphere.
6. A ciliated groove on the midventral line connecting the mouth and anus. This groove is interesting in that it marks the line of closure of the blastopore. The mouth is the remnant of the blastopore; the anus is a secondary opening at the lower end of the original blastopore slit.
7. One eye on the right side of the pretracheal hemisphere. Note the red eye pigment.
8. Two statocysts on the ventral side.
9. The digestive tract, consisting of: mouth opening, stomodaeum (= oesophagus; ectodermal), enlarged stomach (entodermal), narrow intestine (entodermal except for the end portion which is invaginated ectoderm = proctodaeum), and the anus, an opening behind the vegetal pole. All parts are beset with cilia. Feed India ink and study the mechanism of food intake.

10. The anal vesicle, a large vacuolated cell at the posterior end, not found in other trochophores.
11. The cavity between intestine and outer body wall is not a true coelom but a primary body cavity, the persisting blastocoel.
12. The larval kidney (paired) are typical protonephridia with flame cells; they open near the anus. They appear as slender cords near the statocysts, extending between oesophagus and anus. They are best identified in animals with vegetal pole up (consult figs. in Batschek and Scheerer)
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 ectomesoderm cells, single or in small groups, will be seen attached to the stomach, to the inner body wall, near the apical organ, etc.
15. The important entomesodermal cells (derivatives of 4d=Teloblasts) which will give rise to the mesodermal structures of the worm body are difficult to distinguish. They are small groups of cells near the lower end of the head kidney.

Draw lateral and polar views.

11.

### Metamorphosis of the Nereis Larva

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

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

Observe:

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

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

Identify all structures found in A. In addition observe:

1. Eye spots
2. A third pair of seta sacs has appeared behind the first two. All three are lined up in a row indicating the first 3 segments. Setae can be seen in

the process of formation, inside of these sacs; in slightly older stages, they will be seen projecting from the sacs.

3. Pigment appears in the prototroch cell.

C. Metamorphosis, 2 $\frac{1}{2}$ -3 days (Wilson, figs. 90-91)

Notice the change of shape and the gradual demarcation of the first three segments.

5. The head, a fold overhanging the mouth
6. Very long bristles develop in seta sacs. Study their fine structure. They appear one pair after another, eventually 10 pairs. They will be replaced at metamorphosis by ordinary setae.

IV. Gastrulation and Formation of the Trochophore in Hydroids

Prepare your own cultures (see p. 1). Gastrulation by invagination occurs approximately 7-10 hours after fertilization. Consult the figs. in Schearer ('11) and Hatschek ('86).

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## ARTICLE 1. GENERAL PRINCIPLES

### Section 1. Purpose

The purpose of this Act is to provide for the development of a national system of standards for the production and distribution of goods and services, and to ensure that such standards are maintained and improved.

### Section 2. Definitions

In this Act, the following definitions shall apply: (a) "Goods" means any tangible, movable property, whether or not it is attached to realty, and whether or not it is a chattel; (b) "Services" means any intangible benefit or advantage, whether or not it is a chattel; (c) "Standard" means a written specification or requirement, whether or not it is a law, which is intended to regulate the production or distribution of goods or services; (d) "Producer" means any person, firm, or corporation who produces goods or services; (e) "Distributor" means any person, firm, or corporation who distributes goods or services.

The method of obtaining eggs and sperm from a number of large individuals in a population is all stages in the natural history of the individual, whether the egg is fertilized or not.

### Section 3. Development of Standards

The Commission shall develop a national system of standards for the production and distribution of goods and services, and shall publish such standards. The Commission shall also develop a national system of standards for the production and distribution of goods and services, and shall publish such standards.

### Section 4. Unfertilized Egg

The unfertilized egg is a cell which is a part of the body of the mother.

(1) Chorion, a tough membrane which is attached to the outer surface.

(2) Shell, a tough membrane which is attached to the inner surface.

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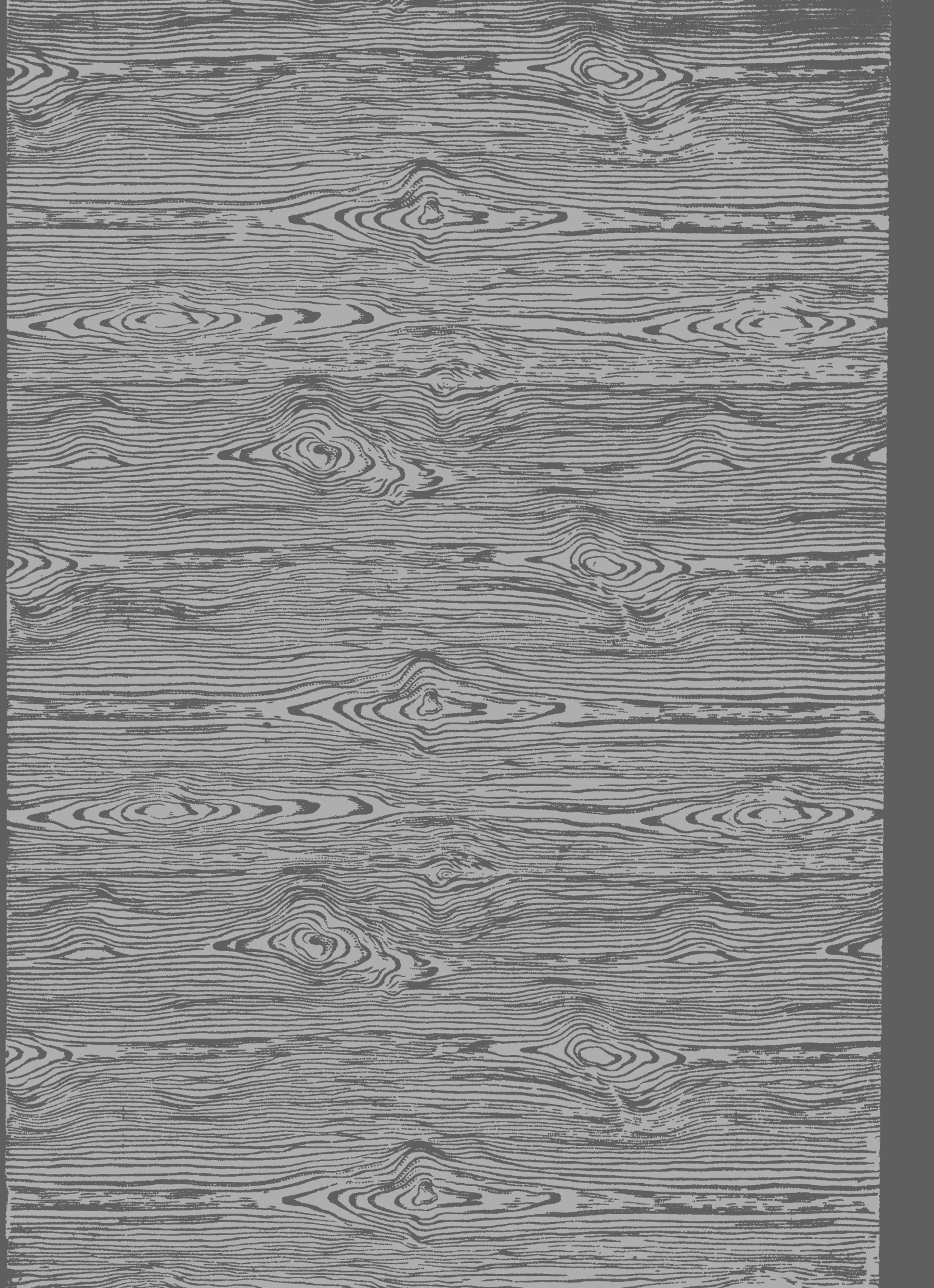
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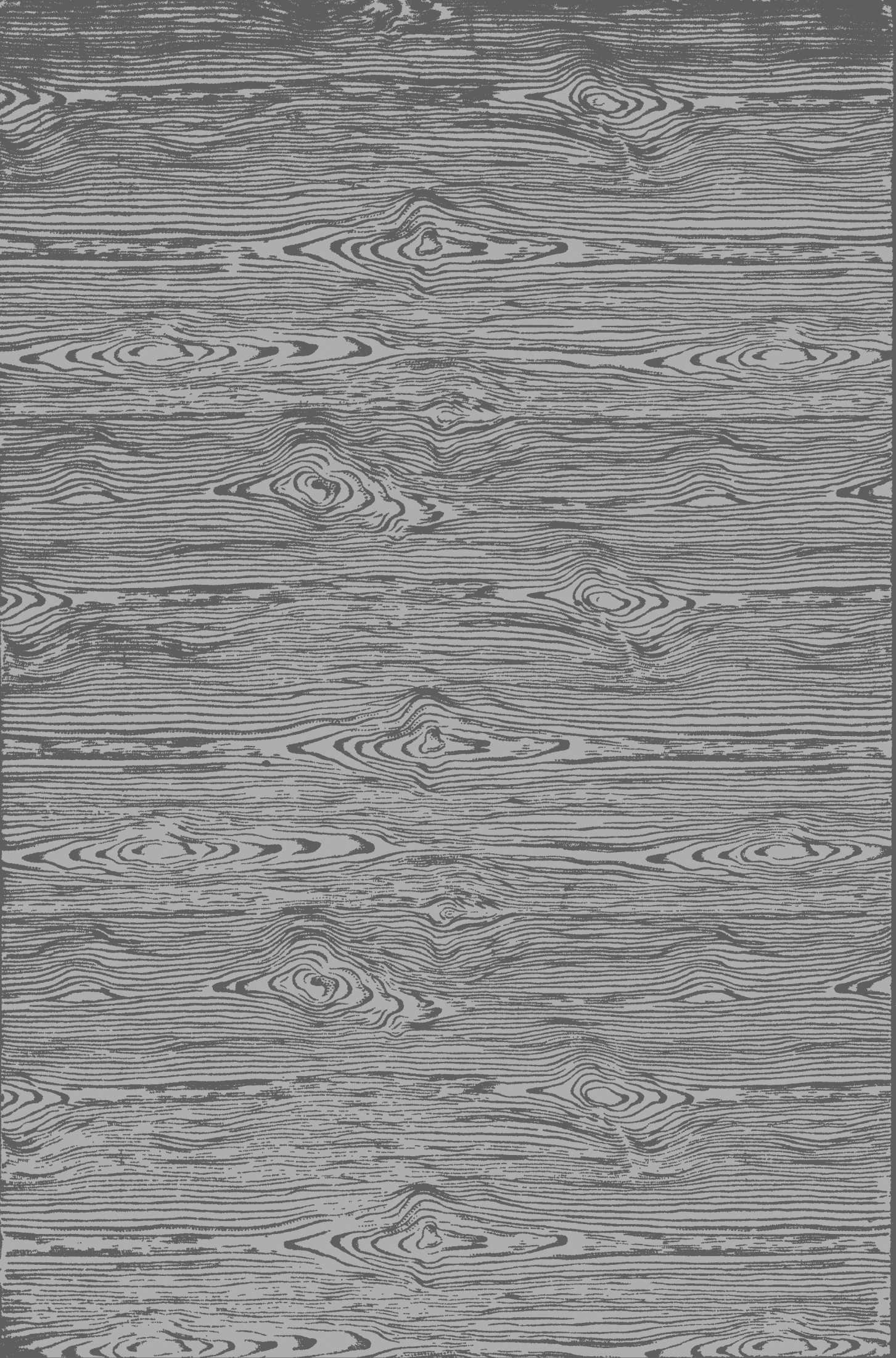
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