

Embryology Course Materials

1947

EMBRYOLOGY COURSE 1947

Staff

Donald P. Costello, Professor of Zoölogy, University of North Carolina,
in charge of the course.

Jean Brachet, Professor of Experimental Morphology, University of
Brussels.

Howard L. Hamilton, Assistant Professor of Zoölogy, Iowa State College.

John A. Moore, Assistant Professor of Zoölogy, Barnard College.

Ray Watterson, Assistant Professor of Zoölogy, The University of Chicago.

Assistants

Catherine Henley, The University of North Carolina.

Alice Ferguson, Louisiana State University.

M. S. L. BIRYOLOGY COURSE, 1947

Bauer, Mark H.	Princeton University (B.S., M.S., St. Joseph's)
Elsir, Charles B.	University of North Carolina (A.B. Maryville)
Bolton, Ellis T.	Rutgers University (B.S. Rutgers)
Buchanan, Douglas	Bartmouth Medical School (A.B., St. John's)
Calvert, Julie Neil	Bryn Mawr College (A.B. Wilson College)
Chmielowski, Adam Anthony	Marquette University (B.S., University of Buffalo)
Cloud, Preston E., Jr.	Harvard University (Ph.D., Yale University)
Cortelyou, Rev. John R., C.M.	Northwestern University and De Paul University (A.B., M.A., St. Mary's; B.S., M.S., De Paul)
Dickason, Mary Elizabeth	Smith College (A.B.)
Edwards, John Parman	Drury College (A.B.)
Gomberg, Charles Lawrence	McGill University (A.B.)
Gregg, James H.	Princeton University (A.B. University of Alabama)
Hill, Henrietta Jane	Dickinson College
Minchey, M. Catherine	Temple University (A.B. Pembroke, A.M. Brown University)
Holtzer, Howard	University of Chicago (A.B.)
Hopkins, Amos Lawrence	Harvard College
Isaac, Isaac Baba	Wesleyan University
Klau, Helene Hope	University of Oklahoma (A.B. Hunter College)
Leone, Charles A.	Rutgers University (B.S., M.S. Rutgers)
Magdeburger, Alice Elvira	Goucher College
Maxon, Marion Gray	Claremont Graduate School (A.B. Pomona)
Nelson, Betty Geneva	Johns Hopkins University (A.B. Mount Holyoke)
Odum, Howard Thomas	University of North Carolina (A.B. Univ. of N. Carolina)
Padykula, Helen Ann	Mt. Holyoke College (B.S., Massachusetts State)
Riggs, Austen Fox II	Harvard College
Robbins, Marilyn Elizabeth	Stanford University (A.B.)
Rosenbloom, Libby	University of Michigan (A.B. Brooklyn)
Russo, Evelyn Esther	Rosemont (A.B.)
Shrader, Ruth Elizabeth	Yale University Medical School (B.A. Minnesota)
Stolack, Richard B.	University of North Carolina (A.B. High Point College)
Weinstein, H. G.	University of Illinois (B.S. Worcester Polytech. Inst.)
Wenger, Byron S.	Washington University (B.S., M.S. Wyoming)
Zuckermandl, Emil	University of Illinois (Sorbonne)

REAR

31 Blair

32 Gregg

33 Odum 34 Hopkins

36 Bolton

37 Leone

40 Wenger

41 Schrader

44 Maxon

45 Hinchey

48 Cloud

49 Robbins

52 Nelson

53 Calvert

56 Dickason

29 Stolack 30 Cortelyou

27 Bauer

26 Buchanan

23 Edwards

22 Padykula

19 Magdeburger

18 Riggs

15 Holtzer

14 Russo

11 Hill

Weinstein 10 Gomberg

7 Isaac

6 Klau

3 Rosenbloom

2 Chmielowski

1 Zuckerkandl

FRONT

EMBRYOLOGY COURSE WORKS HOLE

TENTATIVE SCHEDULE MAY 1947

		Lab.	Lecturer	Lecture
	Mon. June 16	Fertilization (?)	Costello	Introduction & Fertilization D.C.
	Tues. 17	Fertilization (?)		Fertilization D. C.
(new moon)	Wed. 18	Teleosts	Hamilton	Teleosts H. H.
	Thurs. 19	Teleosts		Teleosts
	Fri. 20	Teleosts		Teleosts
	Sat. 21	Teleosts		Teleosts
	Sun. 22			
	Mon. 23	Squid	Hamilton	Squid H. H.
	Tues. 24	Squid		Squid
	Wed. 25	Cell lineage	Costello	Cell Lineage D. C.
	Thurs. 26	Cell lineage		Cell lineage
	Fri. 27	Echinoderms	Watterson	Echinoderms H. W.
	Sat. 28	Class Picnic (?)		
	Sun. 29			
	Mon. 30	Echinoderms		Echinoderms
	Tues. July 1	Echinoderms		Echinoderms
	Wed. 2	Class Exper. Echinoderms		Guest lecture (?)
(full moon)	Thurs. 3	Class Exper. Echinoderms		no lecture (?)
	Fri. 4	Annelida	Costello	Annelida D. C.
	Sat. 5	Annelida		Exper. Embry. Ann. W. Holl. D. C.
	Sun. 6			
	Mon. 7	Mollusca	Patterson	Mollusca R. W.
	Tues. 8	Mollusca		Mollusca (?) or Guest lecture ?
	Wed. 9	Tunicates		Tunicates J. M.
	Thurs. 10	Tunicates		Tunicates J. M.
	Fri. 11	Coelenterates		Coelenterates J. M.
	Sat. 12	Coelenterates		Coelenterates J. M.
	Sun. 13			
	Mon. 14	Chemical Embryology	J. Bracht	(?)
	Tues. 15	Chemical Embryology		
	Wed. 16	Chemical Embryology		
(full moon)	Thurs. 17	Experimental Period	Staff	No Lectures.
	Fri. 18	Experimental Period		
	Sat. 19	Experimental Period		
	Sun. 20			
	Mon. 21	Experimental period		
	Tues. 22	Experimental period + Preparation of Reports.		
	Wed. 23	Presentation of Reports on Group Experiments.		

Wednesday Evening Embryology Seminars - 8:00 P. M.

Wed.	June 18	Fertilization (?)
Wed.	" 25	
Wed.	July 2	Cell division (?)
Wed.	" 9	
Wed.	" 16	

EMBRYOLOGY CLASS SCHEDULE, 1949

		Lab		Lecture
Mon.	June 16	Fertilization	Costello	Introd. & Fertilization D.C.
Tues.	17	Fertilization		Fertilization D.C.
noon) Wed.	18	Telcoasts	Hamilton	Telcoasts H.H.
Thurs.	19	Telcoasts		Telcoasts
Fri.	20	Telcoasts		Telcoasts
Sat.	21	Telcoasts		Telcoasts
Sun.	22			
Mon.	23	Squid	Hamilton	Squid H.H.
Tues.	24	Squid		Squid
Wed.	25	Cell lineage	Costello	Cell Lineage D.C.
Thurs.	26	Cell lineage		Guest lecture
Fri.	27	Echinoderms	Watterson	Echinoderms H.W.
Sat.	28	Echinoderms		Echinoderms
Sun.	29			
Mon.	30	Echinoderms		Echinoderms
Tues.	July 1	Class. exper. Echin.		
Wed.	2	Picnic (?)		No lecture
noon) Thurs.	3	Class exper. Echin.		No lecture
Fri.	4	Annelida	Costello	Annelida D.C.
Sat.	5	Annelida	Metz	Fertilization studies on Paramecium
Sun.	6			
Mon.	7	Mollusca	Watterson	Mollusca H.W.
Tues.	8	Mollusca	Costello	Exper. embryol. annelids & molluscs D.C.
Wed.	9	Tunicates (Picnic?)	Moore	Tunicates J.H.
Thurs.	10	Tunicates	Moore	Tunicates J.H.
Fri.	11	Coelenterates	Moore	Coelenterates J.H.
Sat.	12	Coelenterates	Moore	Coelenterates J.H.
Sun.	13			
Mon.	14	Chemical Embryology	Brachet	
Tues.	15	Chemical Embryology	Brachet	
Wed.	16	Chemical Embryology	Brachet	
noon) Thurs.	17	Experimental Period	Staff	No lectures
Fri.	18	Experimental Period		
Sat.	19	Experimental Period		
Sun.	20			
Mon.	21	Experimental Period; Preparation of reports		
Tues.	22	Presentation of reports on group experiments		

Wednesday Evening Embryology Seminars - 8:00 PM

Wed.	June 18	Specific interacting substances of eggs and sperm.	Dr. Watterson
Wed.	25	Genes and development.	Dr. Goodrick
Wed.	July 8	Cell division.	Dr. Jean Clark Dan crest in the mouse embryo.
Wed.	9	Origin of pigment cells from the neural	Dr. Mary E. Rawles
Wed.	16	Factors involved in gastrulation with special reference to the work of Holtfreter.	Dr. Hamilton.

EMBRYOLOGY COURSE 1947

Lectures

Monday, June 16, 9:00 A. M. Introductory Remarks Dr. Costello

 Fertilization I. Dr. Costello

Tuesday, June 17, 9:00 A. M. Some Experiments on the attainment of
 the fertilizable condition by the ovum.
 Dr. Costello.

EMBRYOLOGY COURSE, 1947

Lectures

Wednesday, June 18	9:00 AM	Teleost embryology: From earliest germ-cells to gastrulation. Dr. Hamilton
Thursday, June 19	9:00 A M	Gastrulation and organogenesis in teleosts. Dr. Hamilton
Friday, June 20	9:00 A M	Mechanics of teleostean development. Dr. Hamilton
Saturday, June 21	9:00 A M	Location of prospective organ-forming areas in the teleostean blastoderm with special reference to gastrulation and comparison with other forms. Dr. Hamilton
Monday, June 23	9:00 A M	Normal and experimental embryology of the squid. Dr. Hamilton
Tuesday, June 24	9:00 AM	Methods of spermatophore transfer and the structure of the spermatophore in the squid, <u>Loligo pealii</u> . Dr. Hamilton

EMBRYOLOGY LECTURES 1947

(To be held in the Embryology Class Room)

Wednesday, June 25,	9:00 A. M.	Cell-lineage and Spiral Cleavage Dr. Costello
Wednesday, June 25,	8:00 P. M.	Genes and Development - with special reference to the develop- ment of color patterns and mendelian characters in fish Dr. H. B. Goodrich
Thursday, June 26,	9:00 A. M.	No lecture - Laboratory work
Friday, June 27,	9:00 A. M.	Normal development of starfish and sea urchins prior to onset of metamorphosis. Dr. Watterson
Saturday, June 28,	9:00 A. M.	Experimental analysis of sea urchin development 1. Dr. Watterson
Monday, June 30,	9:00 A. M.	Experimental analysis of sea urchin development 11. Dr. Watterson
Monday, June 30,	8:00 P. M.	Moving picture in auditorium in brick building. "Development of the sea urchin through Metamorphosis" Dr. Watterson
Tuesday, July 1, Wednesday, July 2	9:00 A. M. 9:00 A. M.	No lecture - Entire period 9:00 a. m. on used for experi- mental work by class

EMBRYOLOGY LECTURES 1947

(To be held in the Embryology Laboratory)

- Wednesday, July 2 8:00 P. M. Seminar on Theories of Astral Cytokinesis.
Dr. Jean Clark Dan.
- Thursday, July 3 9:00 A. M. Embryology of Annelida.
Dr. Costello.
- Friday, July 4 9:00 A. M. Fertilization Studies on Paramoecium.
Dr. Charles Metz, Jr.
- Saturday, July 5 9:00 A. M. Embryology of Mollusca
Dr. Watterson.
- Monday, July 7 9:00 A. M. Experimental Embryology of Annelids and
Molluscs. Dr. Costello.
- Tuesday, July 8 9:00 A. M. ~~Normal and Experimental Embryology of the~~
Regeneration in Hydroids.
~~Tunicates.~~ Dr. Moore.
- Thursday, July 10 9:00 A. M. Development of Coelenterates.
Dr. Moore.
- Friday, July 11 9:00 A. M. Development of Tunicates.
Dr. Moore.
- Saturday, July 12 9:00 A. M. Asexual Reproduction in Tunicates.
Dr. Moore.

EMBRYOLOGY COURSE 1947

- Thursday, July 10 8:00 P. M. Embryology Seminar: Origin of Pigment
Cells from the Neural Crest in the Mouse
Embryo. Dr. Mary Rawles.
- Monday, July 14. 9:00 A. M. The Biochemistry of Fertilization.
Dr. Brachet.
- Tuesday, July 15. 9:00 A. M. The metabolism during cleavage of the egg.
Dr. Brachet.
- Wednesday, July 16. 9:00 A. M. The biochemistry of mosaic and regulatory eggs.
Dr. Brachet.
- Wednesday, July 16 8:00 P. M. Some factors involved in gastrulation with
special reference to the work of Holtfreter.
Dr. Hamilton.

EMBRYOLOGY COURSE, 1947

Experimental Period

- Group I: Cœlenterata: Axial gradients and some aspects of regeneration in Tubularia. Dr. Moore
 Magdeburger Chastelowski
 Maxon Weinstein
- II: Cœlenterata: Isolation of blastomeres in Hydraetina, vital staining, etc. Dr. Moore
 Hinchey Gosberg
 Bauer Edwards
- III: Mollusca: Polar lobe extirpation on Ilyanassa eggs. Experiments on Loligo. Dr. Hamilton
 Calvert Padykula
 Dickason Gregg
- IV: Teleosts: Production of cyclopia. Defect experiments. Dr. Hamilton
 Klau Russe
 Shrader Zuckerkandl
- V: Teleosts: Toadfish. Dr. Goodrich
 Isaac Odum
 Riggs
- VI: Teleosts: Physiological responses of embryos. Dr. Armstrong
 Buchanan Hopkins
- VII: Annelida: Isolation of blastomeres of Nereis. Dr. Costello
 Hill Cortelyou
 Nelson
- VIII: Annelida: Chaetopterus chemical embryology. Dr. Costello
 Stolack
- IX: Echinodermata: Fertilizin. Blastomere isolation, etc. Dr. Metz
 Wenger Robbins
 Blair Rosenblom
- X: Echinodermata: Fertilizin and Antifertilizin Dr. Metz
 Bolton Leone

Students having special reasons for wishing to be assigned to a particular group may see Dr. Costello before 4:30 PM Wednesday. Good binocular dissecting microscopes are essential for members of Groups II, III, VII. If materials are not available, re-assignments may be necessary.

EMBRYOLOGY CLASS SEMINAR

9:00 AM, Tuesday,
July 22

Each paper is limited to 15 minutes.

- 1) Factors for sperm agglutination by blood serum. Miss Robbins
- 2) Specificity of fertilizin and fertilization at high pH. Miss Rosenbloom
- 3) Precipitation in the fertilizin-antifertilizin reaction. Mr. Bolton
- 4) Experiments on Ilyanassa and the squid. Miss Calvert
- 5) Isolation of blastomeres of Nereis. Miss Nelson
- 6) Centrifuging the eggs of Mactra. Miss Padykula
- 7) Isolation of blastomeres of Hydractinia. Miss Hinchey
- 8) Constriction of uncleaved Arbacia eggs. Mr. Wenger
- 9) Methods for injecting teleost embryos. Mr. Hopkins
- 10) Heteroplastic grafts of Fundulus to yolk sac of toadfish, and the problem of pigment cell differentiation. Mr. Odum
- 11) a. Fertilization of Fundulus eggs in 30% sea water
b. Development after treatment with alcohol and $MgCl_2$
c. Puncturing blastomeres
Miss Shrader
- 12) Axial gradient and inhibitor substance in Tubularia. Mr. Chmielowski
- 13) Chemical embryology of the unfertilized Chaetopterus egg. Mr. Stolack

Papers not presented by 12:00 noon will be deferred until 1:30 PM.

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

Glassware, 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 H₂O (70% sea H₂O, 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 $\frac{1}{4}$ - $\frac{1}{2}$ ". 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. Somites. 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-brain 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 chlorotone. Consult paper by Oppenheim '37 for further details of developmental stages.

Bibliography
DEVELOPMENT OF THE TELEOST

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

I. General Reference Works

- Brachet, A. 1935 Traite d'embryologie des vertebres. 2nd ed.
Oppenheimer, J. M. 1936 Historical introduction to the study of teleostean development. Osiris, 2, 1936.

II. Special References on Descriptive Embryology

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Embedding masses, when they are made in such a way that they will set in the presence of a solvent, are very different from the ordinary, anhydrous masses, and the method of preparing them is also different. The masses are made in such a way that they will set in the presence of a solvent, and the method of preparing them is also different. The masses are made in such a way that they will set in the presence of a solvent, and the method of preparing them is also different.

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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
15°C													
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
20°C													
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	
25°C													
Sea water				1						2			
1/2 sea													
1/2 distil.			1					2				3	
Tap Water					1							2	
Dist. water						1						2	
30°C													
Sea water								1					2
1/2 sea													
1/2 distil.								1					2
Tap water									1				2
Dist. water										1			2

	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	7	8.5
			3		4	5	6	
		3		4	5	6	7	

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 Merriman, Osborn Zoological Laboratory and Bingham Oceanographic Laboratory, Yale University.

FERTILIZATION

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

Use the ordinary low power of the compound microscope (approximately 100 diameters magnification, i.e., 10X eye-piece and 10X objective) for observation and study. This permits maintaining the eggs in a considerable volume of water in a Syracuse watch glass so that concentration of sea water by evaporation is not rapid. Most phenomena can be seen readily with this magnification, which affords excellent definition. Higher powers may be used if one desires to observe spermatozoa in detail under a coverslip. After sperm penetration in Nereis has been followed by observing the eggs in the watch glass for at least 95 minutes, it will be instructive to inseminate a second batch, and periodically mount some of these under a 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 super-numerary spermatozoa and other particles at the edge of the jelly. 5 or 6 minutes after fertilization the vitelline (fertilization) membrane will be noticeable due to the formation of the narrow perivitelline space upon jelly extrusion. At 7 or 8 minutes, the entrance cone begins visibly to form. Find an egg showing a profile view of the entrance cone and the sperm which is to enter. From 8 to 12 minutes or longer, the sperm is clearly visible outside the vitelline (fertilization) membrane at the tip of the conspicuous entrance cone. In the course of the next 8 to 10 minutes, the vitelline membrane is indented slightly at its point of contact with the entrance cone. This tends to obscure the sperm from view to some extent, and at about 20 minutes after fertilization the egg wrinkles, becoming distorted and quite irregular in somewhat amoeboid fashion. The entrance cone has already flattened considerably, but is still present, and although the sperm is partially obscured from view, the entrance of its head into the egg is not completed until some time later (ref. #12,15,16). The final penetration of the sperm head thru the membrane, leaving the middle piece and tail outside, may also be observed. At about 30 minutes, the egg rounds up again, but as the time approaches for 1st polar body formation, the egg elongates in a direction perpendicular to the polar axis. If no eggs lie so that the forming polar body is on the horizon, the dish should be shaken. The 1st polar body may form at about 36-40 minutes and it lies in the space between the egg and the vitelline membrane. This space is wider in the region of the animal pole than elsewhere. The second polar body often forms at about 50 minutes and commonly does so immediately under the first polar body, which is thus lifted into 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 insomated 5 to 8 minutes earlier in a Syracuse watch glass containing alkaline NaCl (pH 10.3-10.5. Observe immediately. The vitelline membranes will elevate due to a sudden inhibition of jelly release through the membrane and a subsequent accumulation of the jelly in the perivitelline space (ref.#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 (indistilled 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).

Breeding habits of *Nereis limbata*

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

CHAETOPTERUS:

The chaetopterus egg is rather dark and granular. It is 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 the eggs are taken from the female, the large immature nucleus is in the center of the egg. After about 15 minutes in sea water, maturation will have proceeded to the metaphase of the first polar spindle at which stage development is arrested unless the egg is inseminated or artificially activated. The spindle cannot be distinguished as much 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 1st polar body forms, the egg again rounds up (16-19 minutes), but it elongates again in the same manner to form the 2nd polar body at perhaps 23 minutes. The 2nd polar body often forms under the first, which is thus pushed away from the surface of the egg. The egg again rounds up (26-30 minutes); The egg pronucleus may now sometimes be seen migrating toward the center of the egg, and occasionally the sperm pronucleus may also be detected. By 35 minutes, the clear zone has extended from the polar region toward the equator of the egg, and at 37-40 minutes a typical "pear shaped" stage is reached. The polar bodies lie at a position corresponding to where the stem attaches to a pear, and the bulge which forms the polar lobe begins quite suddenly at the anti-polar end of the egg (40-43 minutes). When the polar lobe is fully developed, however, it corresponds to the stem end of the "pear", and the polar bodies are opposite.

Observe the extrusion of the polar lobe which contains coarse globular material. By 47-48 minutes the cleavage furrow begins at the animal pole and passes to one side of the polar lobe, which thus comes to be attached to one of the two blastomeres. The resorption of the polar lobe into this blastomere causes it to become larger than its mate, and at about 51 minutes two smooth unequal blastomeres lie against each other. Polyspermic eggs will now often be in an abnormal 3 cell stage. By 60 minutes the two blastomeres are quite fused together. At 67-70 minutes the 2nd cleavage takes place. The large blastomere again forms a polar lobe, and a 4 cell stage results with one blastomere larger than the other three. By 90 minutes, or

earlier, the clear nuclei in the 4 cells may readily be made out. At 93-97 minutes the 3rd division takes place, forming 4 relatively large micromeres. A profile view will reveal the macromeres, micromeres, and polar bodies. A polar view will show the rotated displacement of the micromeres, resulting from spiral cleavage, although the displacement is not great or conspicuous in Chaetopterus.

CONCENTRATION AND ACTIVITY OF SPERMATOCYTES:

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 Cayadetta 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 Horstadius 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.

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ARBACIA PUNCTULATA

Fertilization

Gametes of Arbacia are best obtained by cutting around the peristome (on oral surface) and removing Aristotle's lantern, taking care not to injure the gonads. The perivisceral fluid is then drained from the body and the animal, aboral surface down, placed to shed on a Syracuse watch glass slightly moistened with sea water. Wash the hands and scissors with running fresh water after each animal is opened to avoid contamination of one sex with gametes or body fluids of the other. There is no convenient method of ascertaining the sex of sea urchins without cutting into their perivisceral cavities, but when this is done, the ripe ovaries will be observed to be deep reddish brown in color, and the ripe testes a grayish white, with flecks of brown. If the eggs are shed through the gonopores of the female into the Syracuse dish, they should be removed to 200 cc. of sea water in a finger bowl within ten minutes, to avoid the injurious effects of crowding. The sperm are best kept "dry" just as they exude from the testes. A drop of "dry" sperm may be diluted with 10 cc. sea water in a watch glass just before insemination. Do not use sperm that have been diluted more than 20 minutes and avoid too high a sperm concentration. The latter leads to polyspermy and abnormal cleavage. Two drops of diluted sperm should be sufficient for a finger bowl of eggs. Stir the egg dish immediately after adding sperm for insemination.

If shedding of the animals does not take place within ten minutes after removal of the lantern, make a second cut around the shell near the equator, just above the oral ends of the ovaries or testes. If, after this second cut, gametes do not stream from the gonopores, ovaries may be removed with curved forceps to 200 cc. of sea water in a finger bowl and the ripe eggs will be extruded through the ruptured ovarian walls. The egg suspension is then filtered through cheese cloth (which has been soaked in sea water) to remove ovarian debris, and after the eggs settle, the sea water is twice (at least) decanted and replaced with fresh sea water to remove tissue extracts. Washing of the eggs shed after cutting around the peristome may also be necessary if an ovary has been injured or the eggs otherwise contaminated with perivisceral fluid. Testes may be similarly removed to a dry watch glass with forceps, and exuded "dry" sperm used as indicated above.

A good batch of eggs from a ripe female should show uniformity of size, perfectly spherical form, and complete absence of immature eggs (in the germinal vesicle stage). The egg of Arbacia is shed in the fully mature state, both polar bodies having been given off in the ovary. Very rarely, the polar bodies remain on the egg surface. Usually they are not present, and the only good index of polarity of the egg is the funnel-shaped space in the jelly which marks the previous point of attachment of the egg to the ovarian wall.

The unfertilized eggs should be examined in a drop of sea water under a cover glass and the following parts noted: jelly layer (rendered readily visible with a little Janus green solution or by a suspension of Chinese ink); vitelline membrane (very

thin); mature egg nucleus (a small, clear sphere); protoplasmic granules of two kinds, (1) red pigment granules containing echinochrome) (2) smaller, colorless yolk granules. Two other types of granules, i.e. the mitochondria and the oil spheres are present but usually distinguishable only after stratification by centrifuging.

Inseminate some of the eggs as indicated above and examine immediately under low power. Sperm penetration occurs too rapidly to be easily detected. Within a few seconds after insemination the cortical responses of the egg begin. The vitelline membrane elevates rapidly from the egg surface, leaving a perivitelline space. This membrane thickens and hardens during the next five minutes and after alteration is called the fertilization membrane. At the protoplasmic egg surface (which is at first slightly disrupted by the elevation of the vitelline membrane) a new, clear layer is secreted - the hyaline plasma membrane which is presumably a calcium-proteinate acting as a cement to hold the blastomeres together after cleavage. It disappears in calcium-free sea water. After insemination, the jelly layer can often be clearly made out by the ring of sperm entrapped near its surface. Moser (1939) has correlated the elevation of the vitelline membrane with the breakdown of certain cortical granules. These granules are embedded in the cortex and are not easily displaced by centrifuging as are the granules of the underlying fluid endoplasm. In the centrifuged eggs these cortical granules show up clearly at the hyaline zone.

Examine eggs centrifuged in an electric centrifuge at 6,000 to 10,000 times gravity and identify the following layers in order from centripetal to centrifugal pole: (1) oil cap, (2) hyaline zone containing nucleus, with cortical granules visible at periphery in optical section, (3) "Fifth layer", or so-called "mitochondria", (4) zone of yolk spheres, (5) zone of pigment granules.

Mount centrifuged unfertilized eggs under a coverglass on a slide, and focus on cortical granules at hyaline zone under high power. Then carefully add a drop of dilute sperm at the edge of the coverglass. As the spermatozoon penetrates the egg there is a wave of breakdown of the cortical granules, which is followed by rapid membrane elevation.

The cortical responses of the normal and centrifuged eggs to insemination may be slowed down by decreasing the temperature. This can be done by placing small dishes of eggs and sperm in an ice bath and examining them on a chilled slide after insemination.

It is also of interest to examine fertilized and unfertilized eggs by dark-field illumination (even a dark-field stop disc placed in an ordinary condenser is often effective) and note the change in the egg cortex upon insemination. This disappearance at fertilization of a cortical layer which is bright under dark-field illumination was first described by Runnström (1928).

Make sketches of all distinctive features of the Arbacia egg mentioned above.

Inauguration of Cleavage

The time between fertilization and first cleavage varies slightly for different lots of eggs at a given temperature. Some details of the mitotic process (including sperm aster, diaster and spindle) can be observed in the living eggs, particularly if they are slightly flattened by compression under a coverglass. Remember that eggs under a coverglass develop normally for only a short time and under compression this period may be markedly decreased.

One of the first internal phenomena clearly visible is the formation of the sperm aster, which appears as a round, somewhat clear region at the time the pronuclei come into contact. The sperm aster then elongates, to give the "streak" stage, with a somewhat clear crescentic streak extending across most of the diameter of the egg. The streak then fades, and is replaced by two clear regions, marking the two asters of the cleavage spindle. The following time schedule applies to 50 per cent of the eggs at 20° C. (Fry, 1936): Round sperm aster, 11 min.; "streak" stage, 30 min.; fully formed diaster, 45-50 min.; first cleavage, 65 min.; second cleavage, 106 min.; third cleavage, 145 min. It is important to note that first cleavage of some eggs will occur as early as 56 minutes at 20° C., and at 45 to 47 minutes at 22° C. **Observe** and sketch cleavages through the 8 cell stage, keeping a time-schedule of events at the temperature of the laboratory. Note the behavior of the hyaline plasma membrane during the cleavage process, the relation of the first three cleavage planes to each other and to the egg polarity, and from time to time, examine a fresh sample of eggs under compression to observe such internal phenomena of mitosis as are visible in these living eggs.

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(Complete series of figures of development of *Loligo* on plates 1-VII; seriation of stages. Consult particularly plate 7, figs. 4 and 4a for newly hatched squid. Note that Naef describes the Mediterranean species, *L. vulgaris*, which develops more slowly than *L. P.*)
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- 1) Observe growth of mantle and fins. (The shell gland is meanwhile invaginated and not visible.)
- 2) The eye-stalks are prominent.

On the posterior side

- 3) The formation of the siphon by conrescence of the anterior siphonal folds. The posterior siphonal folds continue as ridges to the anterior side.
- 4) The anus, between the gill primordia.
Note the further growth of other primordia; contractions of the yolk sac, etc.

C. Old Stages. (Before hatching - Chart figs. 5 and 6)

- 1) Eye-stalks are very prominent. They contain the primordia of the optic and cerebral ganglia, the so-called "white bodies", also a separate mass of yolk.
- 2) The lens. The inner sector which is formed by the outer part of the optic vesicle (not like Vertebrates) is clearly visible as a club-shaped rod extending into the eye vesicle.
- 3) The mantle has overgrown the anus and gills. It is contractile. In the older stages it is beset with
- 4) Chromatophores. Note different types and colors: observe their contraction and expansion. They are equipped with muscle and are innervated.
- 5) The statocysts lie close together.
- 6) The feather-like gills can be observed through the mantle.
- 7) The branchial hearts will be found at the bases of the gills and the systemic heart between them. All three pulsate.
- 8) The rectum and the ink-bag
- 9) Trace the outline of the internal yolk mass and note the gradual decrease in size of the external yolk sac.
- 10) Observe the locomotion of an old embryo after it has hatched.

<u>Time after Fertilization</u>	<u>Stage</u>
20 min.	1st polar body
1 hour	2nd polar body
3 hours	1st cleavage
12 hours	Blastoderm over top of egg
24 hours	"Gastrula", thickened peripheral ring
2 days	Blastoderm half way over the egg
3 days	Blastoderm nearly covering the egg
3 1/2 days	Appearance of shell gland and eye stalks
5 1/2 days	Siphonal folds and arms appear, eyes project
6 1/2 days	Siphonal folds fused into a tube, eye stalks prominent
11-12 days	Hatching

6. Spreading of the Blastoderm. Study eggs about 24 hours after insemination. Later blastodermal stages may be obtained from egg strings. Note the gradual extension of the blastoderm about the yolk. The "blastocones" which are supposed to give rise to the yolk epithelium are not very distinct in Loligo. Note the thickening of the margin of the blastoderm - the formation of the antomesoderm (gastrulation).
7. Organ Formation. It is convenient to call the pole where the shell gland and mantle appear "dorsal" and the yolk pole "ventral". The siphon side "posterior" and the mouth side "anterior", although these designations are not correct from a comparative anatomical point of view. Study a sequence of at least 6 stages as represented on chart... Study embryos from all sides. Make drawings of different stages. Note:
- A) Early Stages (Chart figs. 1 and 2). cf. also text books of MacBride and Korschelt.
- 1) Shell Gland at dorsal pole
 - 2) Mantle Primordium, an ectodermal concentric fold beneath the shell gland. (fig. 2)
- On the anterior side:
- 3) Mouth
 - 4) Eye primordia - ectodermal invaginations.
- On the posterior side
- 5) Anterior and posterior siphonal folds. The former are the primordia of the siphon; the latter will form its retractor muscles.
 - 6) Statocysts.
 - 7) Gill primordia
- At the boundary of blastoderm and yolk
- 8) Primordium of the anus
 - 9) Note the rhythmical contractions of the yolk epithelium. They serve the purpose of circulating the liquefied yolk material in the yolk-sac vessels. The material is carried into the embryonic tissues in this way. (See Portmann, 192 6).
- B) Medium Stages (Chart figs. 3 and 4). Note the gradual constriction of the yolk sac. The latter continues into the embryo which is thus formed around a core of yolk-mass.

5. The cap and cap thread at the smaller tip end.
6. The middle tunic which may be slightly swollen.
7. A liquid space around the sperm mass, lined by middle and inner tunics.
8. Of the three "membranes" which are formed around the ejaculatory apparatus and inside the "tunics", the middle membrane can be most easily identified. It is relatively thick and extends from the cement body to the cap. It is fastened to the outer tunic at the cap end. This fusion will never break during the process of explosion; but the entire contents of the capsule will evaginate at this point. (cf. diagrams of Drew, 1919).
9. The outer membrane begins also at the cement body. It is so closely applied to the inner tunic that it is difficult to distinguish between them. The oral end of the inner tunic and outer membrane can be easily identified as a thickened ring around the middle membrane, at a short distance from the cap.

The evaginated inner tunic and outer membrane will form the sperm reservoir after the explosion. The sperm reservoir is closed at one end by cement from the cement body and open at the other end (see under 9). The sperm, mixed with a gelatinous mass, will ooze out slowly in a cloud; this will continue for hours or days. All other structures are left behind after explosion.

3. Fertilization and Maturation. Artificially fertilized eggs are more favorable for the study of the first phases of development than are those laid by the female because they lack the jelly envelopes. Fertilization and cleavage can be readily observed in this way. However, these eggs are very sensitive and must be kept in a large volume of water. Inseminate a watch glass of eggs and transfer a few eggs immediately into a depression slide (see sections C, D, and F of Technique). Observe the fertilization under high power. Note the penetration of a sperm through the micropyle. After a short time, the cytoplasmic cap will withdraw from the chorion, and a clear perivitelline space will appear, which indicates that fertilization is taking place.
The first polar body appears about 20 minutes after fertilization. Observe and time the appearance of the second polar body and the further divisions of the polar bodies. (See Hoadley, '30).
4. Cleavage. Mount 6-12 eggs in an upright position in a depression slide (See section F of Technique). Use eggs which have both polar bodies formed, and observe them at short intervals.
Observe the first cleavages and note their relation to the axes of symmetry of the egg. The first cleavage plane coincides with the median plane of the future embryo. (Consult the figs. of Watase, 1, 1891). The cleavage is meroblastic, and not spiral in contrast to other molluscan eggs.
5. Time Table of Development. There is considerable variation due to temperature differences, and the following table gives only a rough approximation of the times at which certain stages are reached.

- 2) Rinse with distilled water several minutes. The transfer from formaldehyde to distilled water must be gradual.
- 3) Stain with Ehrlich's Triacid for 5-10 minutes. The stain is made by diluting the stock solution: 6 drops to 8 cc of distilled water (8 cc. fills a syracuse dish about half full)
- 4) Rinse stain off with distilled water and put the spermatophores on slide under cover slip.

H. Preparation of Whole Mounts and Sections

Because of the large amount of yolk which they contain, squid embryos have a tendency to be friable and difficult to section especially in younger stages. The amyl acetate technique may be used (see Teleosts) or the dioxan technique as outlined below:

1. Fix the embryos in Bouin's solution. (If the embryo is highly motile it should first be anesthetized in sea water containing chloretone before being dropped in the fixative).
2. Transfer the embryos from the fixative into pure dioxan. Change to fresh dioxan at hourly intervals (2 changes) until they have been in dioxan for 3 hours.
3. Transfer to pure paraffin for 1 hour; change to a fresh paraffin bath for a second hour, and then to a mixture of paraffin containing 8 to 10% bayberry wax for a third hour.
4. Embed in paraffin-bayberry wax.
5. Section at 5 or 6 microns and stain with Heidenhain's haematoxylin or with Pronant's triple stain.

Observations of Normal Development

1. The Unfertilized Egg. Study mature eggs taken from the ovary of the squid. The mature egg is surrounded by a transparent chorion which is closely applied to it. At the pointed end, find a depression and a minute canal extending entirely through it. This is the micropyle. Note the polarity (blunt and pointed poles) and the bilaterality of the egg by turning it over. The more convex side of the egg is the future "anterior" or mouth side of the embryo. Note the thin cytoplasmic cap covering the yolk at the pointed pole. Study the extent of the cytoplasmic cap by rolling the egg. This cytoplasm will give rise to the embryonic structures.
Make a drawing of the egg and the chorion.
Study a normally laid egg string. The eggs are embedded in a gelatinous matrix which is produced by glands of the oviduct, and covered by a jelly membrane produced by the nidamental glands. Are the eggs wound spirally around a central core? Compare with the structure of the sperm mass below (2).
2. The Spermatophore. The excellent papers of Drew (1911 and 1919) and their illustrations should be consulted for all details.
Obtain spermatophores (see section C of Technique) and watch their explosion and the ejaculation of sperm.
Prepare some intact spermatophores for study (Section G of Technique). Observe the following structures:
 1. The opaque sperm mass in the center, surrounded by the inner tunic.
 2. The flask-shaped cement body in front of it.
 3. The spiral filament in front of the cement body.
 4. The outer tunic is the outermost layer of the entire envelope.

collected at low tide along the sandy beaches of Nonamessett Island. The clumps of strings are found attached to submerged objects in the shallow water. They can be kept in aquaria with running sea-water. The egg strings containing the older stages are usually darker and more weathered in appearance.

C. Procuring Gametes

Open male and female by making a longitudinal section (use large scissors) through the mantle from the siphon to the tip, cutting along the posterior (funnel) side. Remove the ink sac.

In the female - tear the thin wall of ovary with forceps and shake all transparent eggs into a finger bowl of water. If eggs are fully mature they separate readily from the ovary and appear as beautifully transparent as glass. Immature eggs are not transparent and will not develop.

In the male - pick up the bundles of spermatophores at the opening of the sperm duct, transfer them to a watch glass. The spermatophores will explode when placed into sea water; a concentrated sperm suspension will thus be obtained.

Note - If males are not available the sperm in the sperm receptacle of the female may be used.

D. Preparation of Cultures

Obtain gametes as directed in section above. Place eggs in a finger bowl and add several drops of sperm suspension. After 20-30 minutes transfer to a large dish filled with clean sea water and do not disturb for 2 1/2 - 3 hours. Keep in sea water table and change water at least twice a day.

E. Removal of Embryos from strings

Remove an egg string to a syracuse dish. Using the beading needles in the manner of knives cutting against each other, cut it in half. Place left hand needle so that the pressure forces several embryos clear of the jelly at the open end of one of the halves. Keeping this needle in place, puncture the chorion of one of the eggs with the tip of the right hand needle. Tear the chorion with a sharp jerk. The pressure of the enclosed fluid will pop the embryo from the membrane. When the exposed row of embryos has been removed, cut off the empty jelly and repeat the process. If the eggs are not first forced clear of the jelly they are difficult to remove without injury. This method can be used on all embryos, though the younger stages are more difficult to remove.

F. Methods of Studying Artificially Fertilized Eggs

For short observational periods eggs may be studied in depression slides and manipulated with the hair-loop. To obtain a polar view of the cytoplasmic cap which alone will undergo cleavage it is necessary to mount the eggs in an upright position. Place a small amount of vaseline in a depression slide, fill the latter with water and mount the eggs with a hair-loop so that they stand up.

G. Preparation of Intact Spermatophores for Study

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

1947

Development of the Cephalopod Egg

Type - Loligo pealii, the
common squid

Breeding Season: Females with mature eggs are available at Woods Hole from June through September, although the majority are spent by September.

Living Material - Loligo pealii, mature males and females.
Egg strings of Loligo pealii containing all the developmental stages

Demonstration Specimens- Dissected specimens of adult male and female squid which show the adult structures.

Equipment:

General Class Equipment - Aquaria in which to keep adults and egg strings.

Individual Equipment -
clean finger bowl
2 watch glasses
1 large glass dish or finger bowl
several syracuse dishes
pair of coarse scissors
2 pair forceps
hair loop
2 fine beading needles annealed into
glass handles
pipettes and bulbs, fine-tipped and
wide-mouthed
several depression slides
ordinary microscopic slides and coverslips
Solutions, etc. - concentrated (40%) formaldehyde
Ehrlich's Triacid Stain
distilled water
vaseline

Technique of Preparing and Handling Material:

A. Care of Adults

If adults are transported without undue disturbance from fish traps in live cars with large amounts of water and transferred to large aquaria with a good supply of running water they will live for a few days in the laboratory.

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

Adult squid are used for obtaining gametes for studies of artificial fertilization, the formation of polar bodies, and cleavage. The male also provides spermatophores for subsequent study.

B. Procuring egg strings

Stages showing organ formation are more readily obtained from naturally laid egg strings. These strings can be

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" (i.e. as it exudes from the testes). b) Cut around the test 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 bolting silk. d) Inject about 0.2 ml of 0.5M 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 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 1918; Hayashi 1945). When kept in the cold (2°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 midpiece that are roughly 0.3 μ 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 rod granules containing a pigment called ochinochrome which is a substituted naphthoquinone related to the K vitamins. (Ball, 1936, Hartmann et al. 1939, Tyler 1939). Upon centrifugation mitochondria and oil spherules 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. *Between 5000 and 50000*. Centrifuge a sample of unfertilized eggs at ~~about~~ 10,000 g for ~~1~~ *5 min.* 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 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 vaselino-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 Runnstrom, 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 to 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:-

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

Temperatures above 30-32° are lethal for Arbacia eggs. The first 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 an_1 (at the animal pole) and an_2 (see Hörstadius, 1939) while the macromeres and micromeres divide meridionally. At the sixth cleavage the an_1 and an_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 the 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 Hörstadius 1939) that the an_1 and an_2 and veg_1 cells form the ectoderm (gut) and part of the mesoderm (coelom) and the micromeres form mesoderm (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, 1941) 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 stomadeum. 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 stomadeum and anterior part of the oesophagus. Two short outgrowths, the oral (antero-lateral) arms are formed on the oral lobe, and, at the anal side, two longer anal (aboral or postoral) arms grow out in the same general direction. The original triradiate spicules form skeletal rods which extend into the oral arms (oral rods) the anal arms (anal rods), dorsally through the body (body rods) and laterally (ventral transverse rods). The rods are each made up of three or four parallel parts joined by cross bars. Different species of sea-urchins differ in this regard, so the structure of the skeletal rods is a useful characteristic in hybridization studies. The embryo continues to elongate in the dorso-ventral direction and becomes pointed at the postero-dorsal end where the body rods meet.

The axis running thru oesophagus, stomach and 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 posterolaterally from the oesophagus. That on the left side becomes larger and later acquires a dorsal opening called the pore canal. The right coelom buds off cells to form the madreporic vesicle but otherwise remains rudimentary. The left coelom undergoes extensive later development in the formation of the structures of the adult sea-urchin. These changes do not occur until the second week when metamorphosis begins in properly fed larvae and will not be studied here. It should be mentioned, however, that the adult organs are built up in and around a structure termed the Echinus rudiment which is formed by the fusion of an invagination (amniotic invagination) of the ectoderm on the left side with the mid-portion (hydrocoel) of the left coelom. The left side of the pluteus becomes, then, the future oral face of the adult. Draw a 3-day old pluteus in postero-ventral and side view

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Asterias forbesii (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 jolly-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 90% 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 uninseminated 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 tho 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 (1879) 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 poro-canal.
7. Fully formed Dipleurula (early Bipinnaria) larva. This larva represents an early larval type common to Asterozoa, Echinozoa, Ophiurozoa and Echinozoa (see Korschelt, vol. 1 p. 499). 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 Asteroid 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 madrepore 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. (Gemmill, 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 Gemmill, MacBride, and Korschelt.

Echinarachnius parma

Obtaining gametes

The sexes are separate in *Echinarachinus* (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 *Echinarachinus* 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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Artificial Parthenogenesis in *Arbacia* and *Echinarachnius*

In 1896-1899 Morgan, Mead, Hertwig and Loeb demonstrated that development could be initiated and normal larvae obtained by treatment of unfertilized eggs with salt solutions. Since that time artificial activation of eggs has been the subject of a great many investigations. (See review by Tyler, 1941 and your experimental embryology texts for discussion and references). The present exercise is essentially a repetition of Loeb's classical double treatment method (modified after Just, 1939).

Equipment:- Scissors, blunt forceps, 6" square of cheesecloth, 10 finger bowls, 6 syracuse dishes, 4 stender dishes with lids, 3 slides and coverslips, 1 graduated pipette (5 ml, wide tip), 1 graduate (100 ml), 3 ordinary pipettes, 1 narrow long-tipped pipette.

Solutions:- 2 liters filtered sea-water
20 ml butyric acid sea water (8 ml N/10 butyric acid + 100 ml sea water)
200 ml hypertonic sea water (32 ml 2½M NaCl + 100 ml sea water).

Treatment:- Obtain eggs of *Arbacia* or of *Echinarachnius* as previously described. Guard against accidental introduction of sperm into the dishes of eggs. To 3 ml of a dense suspension of the eggs add 3 ml of the butyric acid sea water. After 90, 105, 120 and 135 seconds remove samples with not more than 1 ml of the solution to finger bowls containing 200 ml of sea water. Determine in which of the four bowls the eggs show the highest percentage of membrane elevation. Pour off about half of the water from the bowl to facilitate concentrating the eggs, then transfer the eggs, with not more than 3 ml of the solution, to a graduate and make the total volume up to 50 ml with sea water. At 10 minutes after the start of the butyric acid treatment pour the eggs gently into a finger bowl containing 66 ml of the hypertonic solution. After 20, 25, 30 and 35 minutes in this solution transfer samples, with not more than 1 ml of solution, to 100 ml of sea water, wash once and culture in covered finger bowls or stender dishes.

Development:- Examine, at intervals of about ½ hour for 3 hours, some of the eggs that have received only the butyric acid treatment and note the repeated formation and disappearance of a monaster. In the doubly treated eggs look for accessory cytasters and for amphiasters. Note the time and pattern of cleavage and sketch some of the early cleavage stages. At about 3 hours estimate the percentage cleavage and the following day the percentage of "top swimmers" in the four dishes. Remove the top swimmers to another bowl, culture for two or three more days and estimate the percentage of normal plutei obtained.

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Specific Interacting Substances of Eggs and Sperm

F. R. Lillie (1912) demonstrated that ripe eggs of *Arbacia* and of *Nereis* give off a substance, called fertilizin, which activates and agglutinates the species sperm. Similar fertilizins have since been reported in many species of invertebrates and they, together with the antifertilizins from sperm with which they react, have been the subject of many investigations, of which most of the more recent are listed below along with some of the older ones. The present exercise includes more tests with this material than can be completed in the time allotted. Only the simpler tests in the first part of each of the following sections should be undertaken during the class period assigned to the work. The additional material is presented as a guide to further work for those who may elect such investigations at the end of the course.

Equipment:- 4 finger bowls, 50 syracuse dishes, 1 graduate (100 ml), 1 graduated pipette (5 ml), 4 ordinary pipettes (droppers), 1 large pipette (25-50 ml cap. + 25-50 ml rubber bulb), 2 centrifuge tubes (15 ml).

Solutions:- 1 liter filtered sea water; 10 ml 1N HCl; 10 ml 1N NaOH

Fertilizin:- Obtain eggs and sperm of *Arbacia* by one of the usual methods. Wash the eggs once and concentrate the suspension to about 25% by volume. After about 15 minutes mix 2 drops of the supernatant egg water with 2 drops of a 1% sperm suspension (one drop of "dry" sperm in 5 ml of sea water) and examine with the microscope. Note the agglutination of the sperm and, a few minutes later, the reversal of the clumping. Are the sperm still motile after reversal? To 2 drops of a strong egg-water (in which eggs have stood several hours, or obtained by acidification-see below) and to 2 drops of sea water in a control dish add 2 drops of 1% sperm. After reversal of the agglutination add 2 drops of egg-water to each dish. Do the reversed sperm re-agglutinate?

To 5 ml of a strong egg-water and to 5 ml of a control dish of sea water add 1 drop of 'dry' sperm. Shake the dishes. What difference in behaviour of the drops of sperm do you observe and how do you account for it?

To 1 ml of a strong egg-water and to 1 ml of a control dish of sea water add 2 drops of 1% sperm. After agglutination has reversed add 1, 2, 4 and 8 drops from each to dishes containing 5 ml of a dilute suspension of eggs (about 100 eggs per ml). Determine the percentage fertilization in each of the 8 dishes. Has the egg-water treatment had any effect on the fertilizing power of the sperm? Titration of fertilizin solutions may be done by testing serial dilutions of the solution with a standard sperm suspension. The dilutions may be prepared with an ordinary pipette (dropper) as follows:-Place 2 drops of sea water in each of a set of dry dishes. Add 2 drops of egg-water to the first dish, rinse the pipette with sea water, mix the drops, draw up most of the mixture, expel 2 drops into the second dish and return remainder to the first dish. Repeat this procedure with the succeeding dishes. Then add 2 drops of 1% sperm to each dish and examine at once. The first dish contains a four-fold dilution of the egg water, the second eight fold, etc. The fertilizin titer can be expressed as the greatest dilution of egg-water that gives a microscopically perceptible agglutination reaction. Titrate your egg water using eight 2-fold dilutions.

Evidence concerning the source of the fertilizin may be obtained from the following tests. Divide about 200 ml of a freshly prepared 10% egg suspension in two equal parts and acidify one part to about pH 3 to 3.5 (requires about 1 ml of 1N HCl per 100 ml). A few minutes later draw off 50 ml of supernatant from each dish, neutralize the acid egg-water with 0.5 ml of 1N NaOH and determine the fertilizin titers. Examine the acid-treated eggs with the microscope and note the absence of the gelatinous coat. Neutralize and wash the acid-treated eggs. After several hours determine fertilizin-titer along with that of the similarly aged control. Acidify the control to pH 3 to 3.5, draw off the supernatant, neutralize and compare its fertilizin titer with that of the first acid-egg-water. Is there evidence of secretion of fertilizin by the eggs? What is the apparent source of the fertilizin?

To test for activating action of fertilizin allow a dilute (1%) sperm suspension to stand for 1 hour or until motility has decreased considerably; then add 2 drops to 2 drops of a strong egg water and to 2 drops of sea water. Examine the two dishes microscopically and note roughly the activity exhibited by the spermatozoa. Absorption tests may be made by adding a concentrated sperm suspension (10% or greater) to an equal volume of moderately strong egg-water, centrifuging after 1 hour and testing the supernatant as well as a similarly diluted sample of the egg-water for agglutinating action on dilute (1%) sperm. Does absence of agglutinating action necessarily mean binding of fertilizin by the sperm? What other tests would be necessary?

Specificity may be examined by testing Arbacia fertilizin on sperm of closely related and distantly related animals, that are available in the laboratory. Where reactions are obtained absorb the Arbacia egg-water with the foreign sperm, as described above, and test the supernatant on both species and foreign sperm.

Antifertilizin:— This material may be prepared from a concentrated (10% or greater) sperm suspension by (a) freezing and thawing the suspension, (b) heating to 100°C. for one minute or (c) acidifying to pH 3. The treated suspension is then centrifuged or filtered and the supernatant or filtrate will be found to contain the active material.

To demonstrate the agglutinating action of antifertilizin add 2 drops of the solution prepared by methods, a, b or c to 2 drops of a 1% suspension of freshly washed eggs. Shake the dish several times and examine macroscopically and microscopically after 1 to 5 minutes. Note the formation of a precipitation membrane on the surface of the gelatinous coat of the egg.

Titration of the antifertilizin is performed in the manner described above for fertilizin. Prepare a set of 8 two-fold serial dilutions of 2 drops of the antifertilizin solution and add to each 2 drops of a dilute (ca. 100 eggs per drop) freshly washed, egg suspension. Examine at once and again after 1 hour. Note differences in width of the gelatinous coat and in the precipitation membrane in the different dilutions and at different times. The end point may be taken as that dilution beyond which a precipitation membrane is no longer visible. Determine the titer of your preparation. Does the egg-agglutination reaction reverse spontaneously?

To demonstrate neutralization of the fertilizin add 2 drops of strong antifertilizin solution to 2 drops of a moderately strong egg-water. Prepare a control of 2 drops of egg water plus 2 drops of sea water. After $\frac{1}{2}$ hour add 2 drops of a 1% sperm suspension

to both dishes. Note the degree and duration of the agglutination reaction. Titrations may be performed with duplicate serial dilutions of the egg water to one set of which is added a constant amount of the antifertilizin solution while the other gets an equal volume of sea water then sperm added after $\frac{1}{2}$ hour; or with duplicate serial dilutions of the antifertilizin plus constant egg-water to one set and sea water to the other, then eggs added after $\frac{1}{2}$ hour.

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Production of Exogastrulae in Arbacia and Echinarachnius.

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
100 ml Li-sea-water (20 ml of m/2 LiCl + 80 ml sea water).

Treatment:- Obtain eggs and sperm of Arbacia or of Echinarachnius 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 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, another sample to a control dish of sea water. After 2, 4, 8 and 12 hours transfer samples (ca. 0.05 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 "Dauerblastulae". 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.

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Development of Isolated Blastomeres of Arbacia and Echinarachnius

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"x6"), 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 (30 grams NaCl in 1 liter of sea-water)
100 ml of Ca-free sea-water (1000 ml M/2 + 22 ml NaCl
M/2 KCl + 195 ml m/3 MgCl₂.6H₂O + 103 ml M/3 Na₂SO₄ +
6 ml M/2 NaHCO₃, 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 Echinarachnius in the usual way. Inseminate (noting time) a sample to test for fertilizability and to determine first cleavage time. Ten minutes later 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 fore-arm 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) listed above. The time of first cleavage is determined on the control sample that was fertilized 10 minutes before the experimental set.

2. ^{Ca-}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 once 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 sister. 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 a pair 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 ~~one~~ 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 ^{each} 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-thickness?

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, 1946; Tyler, 1942 and experimental embryology texts for further analysis).

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PREPARATION OF FIXED AND STAINED MATERIAL BY

THE FLATTENING METHOD

The following is a brief account of a rapid slide-making that is useful for such purposes as making chromosome counts, determining stages of mitosis, fertilization, etc., on developing eggs and other material. It involves flattening the eggs or embryos between two coverslips and is, therefore, unsuitable for any work in which it is desired to retain the normal shape of the cells.

Equipment: - 6 Syracuse dishes, 2 or more clean No. 1 coverslips, 6 Columbia staining dishes (coverslip-size), 1 forceps, 1 dissecting needle, 2 or more slides.

Solutions:- Bouin's fixing fluid, Delafield's hematoxylin, Alcohol series (30%, 50%, 70%, 85%, 95%, and 100%), Acid-alcohol (1% HCl in 30% alcohol), Xylol, Canada Balsam or Euparal.

Procedure:- The method consists simply in joining and later separating two coverslips, one of which contains a drop of the fixing fluid, the other a drop of the egg suspension. The coverslips with adhering flattened eggs are subsequently handled in the same manner as slides of sectioned material. To facilitate later separation the coverslips should be joined crosswise. The following illustrates the procedure. Support one coverslip on the edges of two Syracuse dishes placed next to each other (or some other convenient support) and add a small drop of fixing fluid (Bouin's). Hold the other coverslip in one hand and add a small drop of the egg suspension at the desired stage. Invert the second coverslip over the first in crosswise position (so that the corners do not coincide) and release it as soon as the drops touch. The drops should not be allowed to undergo any appreciable evaporation before joining. The size of the drops should be such that when joined the fluid does not quite fill the space between the two coverslips. The eggs are thus flattened and the degree of this flattening can be regulated to some extent by the size of the drops. The fixing fluid acts rapidly on cells of small diameter, especially when they are flattened. With most marine eggs of 0.05 to 0.2 mm., two to ten minutes usually suffices for good fixation in Bouin's fluid. The joined coverslips are then carefully placed in a Syracuse dish and 70% alcohol is added whereupon the coverslips tend to separate. Lift the upper coverslip off by means of a fine forceps using a needle placed at the opposite edge of the upper coverslip to prevent it from sliding while being removed, and place it egg-side up in another dish of 70% alcohol. Sliding of one coverslip over the other will cause distortion and loss of eggs or fragments thereof. Ordinarily about half of the eggs will adhere to each coverslip. If the fixing fluid had been allowed to act for too long a time before the eggs were actually flattened, many of the eggs would fail to adhere to the coverslips when they are separated. Allow the coverslip to remain in two or three changes of 70% alcohol for a sufficient length of time (usually at least an hour) to remove the yellow color of picric acid (of the Bouin's fluid) from the eggs.

Transfer the coverslips through 50% and 30% alcohol (about one minute in each) to Delafield's hematoxylin for ten minutes or

longer. The Columbia staining dishes are convenient for this and subsequent handling. Then wash once in tap water and place the coverslips in the acid alcohol. The latter should be in a Syracuse dish and the progress of the destaining followed under the microscope. When the stain no longer comes out of the eggs in visible clouds (about one-half to one minute after 10 - 15 minutes staining) immerse the coverslip in tap water. After at least three changes of tap water during 5 to 10 minutes run the coverslips up through the alcohols and Xylol and mount on a slide with balsam or omit the xylol and mount in euparal. Counterstaining with eosin or other dyes may be used in the usual way if desired.

References:- Tyler, A., 1946. Rapid slide-making method for preparations of eggs, protozoa, etc. The Collecting Net, vol 19, pp. 40-41.

CULTURING EMBRYOS ON VASELINE-SEALED SLIDES

AND COVERSLIPS AND IN CELLOPHANE TUBING

It is often desirable to culture single embryos or small numbers of them in a small volume of medium so that they can be readily observed from time to time and so that their swimming activities do not make searching for them too time consuming. For this purpose they may be kept in a small drop in a chamber formed by a slide and coverslip sealed together with vaseline. It is also often desirable to culture large numbers of embryos in rather concentrated suspension. The use of cellophane tubing helps overcome the difficulty of attempting to transfer large numbers of swimming embryos to fresh sea water and permits satisfactory development of rather concentrated suspensions.

Equipment: Vaseline (fairly pure petroleum), slides, coverslips, ordinary droppers, fine-tipped droppers, cellophane tubing (Visking cellulose sausage casings ca. 1" diam.), funnel (6"), Syracuse dishes, finger bowls, and scissors.

Solutions: Filtered sea water, Diatom culture.

Vaseline-Slide Method: Warm a small amount of vaseline in a beaker to just above the melting point and with a warm dropper make a hollow square of vaseline on a clean, dry slide. A bent metal rod can also be used for this purpose in place of the dropper. The outside dimensions of the square of vaseline should be roughly the same as that of the coverslip that is to be used and the height should be about 1 mm. Place a small drop containing the eggs or embryos within the square on the slide. This drop should be of such size that when the coverslip is added, completely sealed to the slide and contact made with the drop, the diameter of the drop will be about 2 to 3 mm. The coverslip should be pressed down sufficiently so that a completely sealed chamber is formed, with no air channels running through the vaseline. Avoid having the drop come in contact with the vaseline. The slide should be kept in a cool place and the embryos transferred to a fresh preparation at intervals of one to four days depending upon the amount of living material present.

Cellophane Tubing Method: The seamless cellophane tubing is usually supplied in flat rolls of about 100 feet. Cut off a piece about one foot in length and tie a knot in one end of it. Soak the piece in sea water for a few minutes, then open the other end by sliding the two sides of the flattened tube against one another. Introduce a funnel into the open end of the tube and pour in about 100 cc. of the suspension of eggs or embryos. Tie off the tube in such a way that an air pocket of about one-fifth of the volume of fluid is left. Then place the tube in a dish or aquarium of running sea water in such a way that the water tends to flow past it. With small embryos of annelids, mollusks, or echinoderms, several thousand embryos per 100 cc. can be kept alive in this manner provided they are changed to fresh tubes once or twice a week. To obtain later developmental stages and growth food materials must be added. For this purpose a small amount of a dense suspension of diatoms (*Nitzschia*) should be added at each transfer.

CHEMICAL EMBRYOLOGY OF MARINE INVERTEBRATE EGGS

A. Cytochemical methods:

These methods allow the detection of some of the chemical constituents of the cell (proteins, lipids, glycogen, nucleic acids, enzymes, etc.). They have not been used on marine eggs very extensively so far, with the exception of Ries' papers on respiratory enzymes in mosaic and regulatory eggs. The results obtained with these methods should be examined critically. For such a critical discussion of cytochemical methods see Lison's and Brachet's books.

The experiments essentially consist in the application of a series of these cytochemical tests to unfertilized and fertilized eggs of both the regulatory (Arbacia, Asterias, Echinarachnius) and the mosaic (Chaetopterus, Nereis, Ciona) types, as well as cleavage stages and gastrules. Arbacia is not a very good material for this sort of work because of the presence of the red pigment echinochrome. The same tests should also be made on unfertilized and fertilized eggs which have been centrifuged for 10 minutes. Such eggs should be fixed immediately after centrifugation.

Do all tests on a large number of eggs (several hundred). Observe the eggs carefully and make accurate drawings.

1. Detection of Amino Acids present in the protein: (cf. Serra: Stain Technol. 21: 5. 1946)

a. Test for sulfhydryl group:

(Method of Giroud and Bulliard - Lison, L. Histo Chimie Animale 1936)

- a) Fix with 10% trichloroacetic acid for 10 minutes.
- b) Immerse in 5% aqueous solution of zinc acetate for some seconds (excess does no harm).
- c) Then immediately treat with a 10% aqueous solution of sodium nitroprusside, containing about 2% concentrated ammonia. The pieces (eggs) acquire a bright red coloration, which attains its maximum in 3-5 minutes.
- d) Mount in pure glycerine for microscopic observations, if necessary, with a preliminary washing in distilled water.

b. Test for tyrosine (Millon's modified)

(Method of Serra, J. A., and Queiroz Lopes, A., Nature 155 page 792, 1945)

- a) Immerse the egg for 30 minutes in a few cc of mercuric solution (composition: HgSO_4 7.5 g; HgCl_2 5.5 g; Na_2SO_4 7.0 g; - dissolved in 85 ml. of distilled water to which 12.5 g. of concentrated H_2SO_4 is added; after dissolving diluted to 100 ml. with distilled water). Perform the treatment in a small glass stoppered bottle placed in a water bath which is maintained at 60° C.
- b) After the 30-minute treatment, cool the bottle in running water and allow to stand at room temperature for 10 minutes.
- c) Dilute the mercuric solution in a bottle by addition of an equal volume of distilled water.
- d) Develop the color, adding now some drops of a freshly prepared 1 M solution of sodium nitrite (6.9 g NaNO_2 in 100 ml. of water). The coloration attains its maximum in three minutes, and lasts for some months. The eggs are mounted and observed in pure glycerine where they can be squeezed or squashed if necessary.

c. Test for Arginine:

(Thomas, Lloyd E., J. C. C. P. 28, #1, p. 145, 1946)

- a) Fix in Bouin's fixative for five minutes.
- b) Wash in running water
- c) Add alpha naphthol for at least three minutes, at room temperature.
- d) Sodium hypochloride - 20 seconds
- e) Urea - 5 seconds
- f) Mount and observe, in glycerine
- g) Red color will appear if arginine is present

Reagents: 10% alpha naphthol in 95% alcohol. 0.15 N sodium hypochlorite in 0.05 N NaOH. 20% urea in 0.05 N NaOH.

2. Detection of lipids (plasmalogen)

Technique: Fix (eggs) in saturated corrosive sublimate (HgCl₂) (poison!) for 5 minutes. Then wash with distilled water and put into Feulgen reagent for 15 minutes. Wash three times in water saturated with SO₂, then rinse in water. Observe under the binocular. Then make a slide with glycerine and observe under the 'scope.

Controls:

- 1. Same as above, but omit the sublimate.
- 2. Fix the eggs in Carnoy's fluid for 30 minutes, and wash in alcohol twice for 15 minutes. (This extracts most of the lipids). Then same as above.

(Carnoy's fluid is: alcohol 6 p.; chloroform 3 p.; acetic acid 1p)

3. Detection of glycogen (Bauer)

Place a few of the eggs which were fixed in Carnoy and treated with alcohol into 4% chromic acid for one hour. (This treatment liberates aldehyde groups in polysaccharides). Wash in distilled water, then stain with Foulgen's reagent as above.

4. Detection of respiratory enzymes.

a. Peroxidase (Propan's method)

Fix one group of eggs in 10% formol for 10 minutes (this treatment destroys the catalase) and wash thoroughly in distilled water. Immerse the eggs in a saturated solution of benzidine in water containing one drop of acetic acid per 10 cc.. Then immerse in H₂O₂ (perhydrol diluted 1%). Note the oxygen bubbles (catalase) and the blue, then brown color. Repeat the test on fresh eggs.

b. Indophenoloxidase (Child's method in Physiological Zoology 17. 129, 1944).

a) 1 drop of dimethylparaphenylenediamine in 10 cc H₂O is 1/40 of a cc)

b) 1 mgm alpha naphthol in 10 cc H₂O. Add a drop of each to 1 cc of the solution containing the fresh eggs to be tested.

Do you observe the gradient pattern described by Child?

5. Detection of nucleic acids. This is best done on sections of eggs which were fixed and embedded in paraffin.

Material: Arbacia: fertilized eggs and plutei (or gastrulae)
Chaetopterus: unfertilized, fertilized eggs and trochophores

Fixation: Fix for 15-30 minutes in Carnoy or Sorra (9 parts alcohol, 1 part formol, 0.5 part acetic acid)

Embed as usual.

a. Thymonucleic acid (Feulgen reaction): Paraffin is removed as usual and sections are rehydrated. The slides are hydrolyzed for six minutes in N HCl at 60° C., then rinsed in H₂O. They

are left for one hour in Feulgen's fuchsin sulphurous reagent, then washed three times in SO_2 water (1 gm NaHSO_3 in 100 cc H_2O plus 1 cc concentrated HCl). Wash in water, dehydrate in alcohols as usual and mount in Canada balsam.

As a control, treat another section the same way, but omitting the HCl hydrolysis.

b. Ribonucleic acid: Stain the sections in:

1. A saturated aqueous solution of toluidine blue for twenty minutes. Wash in running water. Leave the slides in 95% alcohol for five minutes. Mount as usual.

2. Unna's methyl green - pyronin mixture (0.15 gm. methyl green; 0.25 gm. pyronin P; 2.5 cc. 95% alcohol; 20 cc. glycerine; 0.5 gm. carboic acid; 100 cc. H_2O). Stain for twenty minutes, rinse in water, differentiate in 95% alcohol, then into xylol and balsam.

Both nucleic acids stain with all basic dyes. However, when the Unna mixture is used there is a competition between the two dyes and the nucleic acids, so that ribonucleic acid stains red and thymonucleic acid stains green. But the reaction is specific for ribonucleic only when it is shown that there is no staining when this acid has been previously digested away with ribonuclease as follows:

Incubate one slide for one hour at 56° in a McIlwain citric acid buffer at pH 6.75. This is used as a control. Treat a second slide the same way but add 0.1 mgm of ribonuclease per cc. of buffer. Then stain both slides with methyl green-pyronin.

B. Properties of the nucleic acids and nucleoproteins.

Ribonucleic acid is mostly present in the form of cytoplasmic ultramicroscopic granules (microsomes) which also contain lipids (including plasmalogen) proteins and respiratory enzymes. The thymonucleic acid is bound to histone in the nucleus. This thymonucleohistone forms very long, thread-like macromolecules which have considerable viscosity (Mirsky and Polkister: J. Gen. Physiol 1946)

a. Isolation of microsomes from centrifuged Arbacia eggs:

Collect and wash the eggs from two ripe females. Remove the seawater after centrifugation. Then, crush the eggs in a mortar (in an ice-bath) with sand and add 10 volumes of cold phosphate buffer (M/200, pH7). Mix well and centrifuge for 10 minutes at 3,000 r.p.m. Observe the different layers under the microscope - yolk platelets, pigment, fats. The microscope layer can be seen as a cloudy layer between the fats and the pigment;

Remove this layer with a thin medicine dropper, and centrifuge for 20 minutes at 12,500 r.p.m. in the ultracentrifuge. Observe the supernatant fluids and the pellets. Do the indophenoloxidase, peroxidase, -SH and plasmal tests on these pellets.

Add 0.1 cc. of 10% trichloroacetic acid for each pellet, and mix well. Precipitate the protein from the supernatant fluids (see above) with 0.1 cc of 20% trichloroacetic acid for each 0.1 cc of fluid. Then centrifuge both mixtures and repeat the extraction with 0.1 cc trichloroacetic on the supernatant fluids. Add 0.1 cc of water and 0.1 cc of Bial's pentose reagent to each. Immerse in a boiling water bath for 10 minutes. (Bial's reagent: Dissolve 10 mgs. orcinol per cc of a solution of concentrated HCl containing 0.1% ferric chloride.)

b. Isolation of thymonucleohistone from Arbacia testis:

Crush and extract a sea-urchin testis with aM NaCl . Observe the formation of a viscous solution of thymonucleohistones. Centrifuge

at ordinary speeds for 10 minutes. Dilute the supernatant with 10 volumes of distilled water. A fibrous precipitate of thymonucleohistone settles out.

Demonstrate the presence of large amounts of arginine in the precipitate with the cytochemical reaction used above.

Demonstrate the presence of thymonucleic acid in the precipitate with the Dische diphenylamine test. Try the same reaction on the microsomes which have been isolated in the previous experiment.

Dische's test: Heat on the water bath during 10 minutes 1 vol. of the solution containing nucleic acid and 2 volumes of the reagent: 2% diphenylamine in a mixture of 39 parts acetic acid and 1 part of sulfuric acid.

The presence of thymonucleohistone in the sperm heads (vesicles) can be demonstrated by doing the arginine and Feulgen cytochemical tests on smears of sea-urchin testis. (for the Feulgen test it is necessary to get rid of the interfering plasmalogen by leaving the slides in 95% alcohol for 20 minutes.)

Repeat the arginine and Feulgen tests on smears which have been treated for 10 minutes in 2 M NaCl and then washed with water.

See Schneider's papers for a quantitative method of estimation of both types of nucleic acids with the Bial and Dische tests, in J. Biol. Chem. 161 : 293, 1945, and 164: 747, 1946.

Remove the fixative using a pipette of small diameter equipped with a syringe bulb, and fill the vial with 70% alcohol. Wash in 70% until the eggs are white. It is advisable to avoid washing too long in 70%, since the stain employed is best when it does not penetrate the macromeres. These latter should therefore be left slightly acid. Thus the eggs are removed from 70% immediately after the last wash which removes no picric from them, hydrated in 50%, 35%, and washed thoroughly in 2-3 changes of water.

II. Staining:

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

III. Mounting:

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

Alternative Method.

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

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

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Embryology of Annelida

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

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

Cultures of advanced stages will be prepared. If you wish to prepare your own cultures proceed as follows: Hydroides both males and females will spawn immediately after being removed from their calcareous tubes. Remove several and place them in finger bowls (one worm per dish to keep the sexes separate). Remove the animals after they have spawned. Let sperm stand for about $\frac{1}{2}$ 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 hrs, gastrulation after 8-12 hrs; the trochophore stage lasts from 20 hrs. to 2 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 Hatschek ('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 chlorotone 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 (prooral). 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 pretrochal hemisphere. Note the red eye pigment.
- 8) Two statocysts on the ventral side.
- 9) The digestive tract, consisting of: mouth opening, stomadaeum (oesophagus; ectodermal), enlarged stomach (entodermal), narrow intestine (entodermal except for the end portion which is invaginated ectoderm = proctodaeum), and the anus, an opening behind the vegetal pole. All parts are beset with cilia. Feed india ink and study the mechanism of food intake.
- 10) The anal vesicle, a large vacuolated cell at the posterior end, not found in other trochophores.
- 11) The cavity between intestine and outer body wall is not a true coelom but a primary body cavity, the persisting blastocoele.
- 12) The larval kidneys (paired) are typical protonephridia with flame cells; they open near the anus. They appear as slender cords near the statocysts, extending between oesophagus and anus. They are best identified in animals with 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 octomesoderm cells, single or in small groups, will be seen attached to the stomach, to the inner body wall, near the apical organ, etc.
- 15) The important entomesodermal cells (derivatives of 4d Teloblasts) which will give rise to the mesodermal structures of the worm body are difficult to distinguish. They are small groups of cells near the lower end of the head kidney.

Draw lateral and polar views.

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 postrocha hemisphere. The setae (= chaetae) will be differentiated inside of them. These sacs are the first indication of the first two segments of the worm.
- 8) Observe the trochophore in locomotion.

B. Advanced 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. 2½-3 days (Wilson, figs. 90-91)

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

Observe the following new features:

- 1) Sense hairs at the apical pole.
- 2) Additional eye spots; number variable
- 3) Mesotrochal ciliary bands at the boundaries of the segments.
- 4) Parapodia with parapodial cirrhi appearing on segments. 2 and 3.
- 5) Tentacular cirrhi on the head.
- 6) Stomadaeum, stomach, intestine become distinct.

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

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

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

Observe:

- 1) Frontal antennae, tentacular cirrhi, palpi, all on the head.
- 2) Anal cirrhi.
- 3) Two large and several small eyes.
- 4) Jaws inside of the oesophagus; they can be protruded. The animal is carnivorous and rapacious.
- 5) Study the finer structure of the setae, and their motility. (muscles at their bases).

III.

The Trochophore of Sabellaria.

This larva shows very long bristles which have probably both a suspensory and a protective function. Study trochophores, 2 days and older (consult D. P. Wilson '29, and Novikoff '38).

Observe:

- 1) Stiff apical cilia in the place of the apical tuft.
- 2) The prototroch consists of 3 rows of cilia, and shows a gap on the dorsal side.
- 3) The neurotroch in the midventral line.
- 4) One eye on the left side. More eye spots develop later.

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

1V.

Gastrulation and Formation of the Trochophore in Hydroids

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

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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 ('97) figs. 80-82 and the textbooks of Parker-Haswell vol. 1 (1928) fig. 657 on p. 734, or MacBride figs. 263 ff. on pages 301 ff, or Korschelt, 1936, vol. 2, pp. 881-891. Study dorsal, ventral and lateral views. Mouth and foot are on the ventral side, the anus is on the right side.

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

II. EARLY STAGES OF THE VELIGER LARVA.

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

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Lamellibranchia (Pelecypoda)

III. The Veliger Stage.

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

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

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

IV. The Trochophore Stage.

Study the Trochophore stage of Cumingia (12-17 hrs. after fertilization) and its transformation into the Veliger larva. (18-24 hrs. after fertilization).

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

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

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

Draw side and polar views in outline, showing ciliation.

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COELENTERATES

Introduction to Hydrozoa

a) Sensitiveness of the material

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

b) Types of Life History Illustrated

There are two phases of embryology in the Hydrozoa. Characteristically, a long series of asexual reproductions (by budding and other methods) is interrupted at irregular intervals by isolated examples of sexual reproduction. This alternation of sexual and asexual generations is closely paralleled by an alternation of structure, since zygote production is usually accomplished by 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 *Gonionemus*, but it is unfortunately now nearly extinct at Woods Hole. The genera picked for study are arranged in the order of diminishing completeness of the medusa form, the first having free-swimming medusae and the last mere sporosacs. The utter degeneration of the medusa-form is illustrated by the familiar *Hydra*.

c) Order of Study

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

Laboratory Procedure

A. Study of Forms with Perfect Medusa

Examples: *Bougainvillia*, *Obelia*, *Podocoryne*.

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

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 cover slip with absorbent paper to produce a slight pressure on the buds. Sketch three stages in medusa development.

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

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

Obelia (June, July, August)

The gibbines 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 colonics 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 membrand near the bases of the tentacles. This makes possible an aversion of the bell when the medusa comes to rest, so that the manubrium sticks out from the center of the convex side, like the handle of a post-hurricane umbrella. Watch the swimming movements, and see how this

happens. In the everted condition, the manubrium is still morphologically sub-umbrellar, though this term has lost its appropriateness.

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

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

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

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

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

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

B. Study of Forms with Imperfect Medusae.

Examples: *Pennaria*, *Tubularia*.

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

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

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

Mature *Pennaria* colonies festooned with shedding medusae 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 females eject with greater travail the three to six opaque pink eggs. In the south, Pennaria medusae generally break loose from the colony and swim about during this discharge, but at Woods Hole they generally remain attached, and the eggs may not be ejected until long after fertilization. The medusae finally drop off, swim very feebly if at all, shrivel rapidly and die in a few hours.

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

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

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

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

TUFULARIA (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 awhile, metamorphoses into a polyp; Asexual multiplication by buds; Colony formation; Degenerate medusae (gonophores) formed on a blastostyle; Gonophores mature in situ; Sperm are shed, eggs fertilized in situ.
- b) Hydractinia, Eudendrium; Zygote develops into planula larva either inside gonosome (Eudendrium) or after being shed from gonosome (Hydractinia); Planula metamorphoses into polyp; Asexual multiplication by buds; Colony formation: Gonosomes formed 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 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 Goette '07. Cf. also texts of Hyman '40 and Korschelt '36.

HYDRACTINIA. (June, July, August)

Colonies of this form are fairly common on Littorina snail shells inhabited by the small hermit crab, Pagurus. There are three types of individuals in the 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 prolocus; each bears a number of gonophores, which are medusa-buds reduced to the status of sporosacs. Ripe "male" and "female" colonies can be told apart with the naked eye since the eggs within the sporosacs are dull green against the red hydrorhiza, and the sperm when mature are a white mass.

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

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

b) Cleavage and Development.

If a number of "male" and "female" colonies of Hydractinia are put together in a large dish of sea water (or a pair of prime colonies in a fingerbowl) and left overnight, eggs should be shed and fertilized between 7 and 9 a. m. WST. The shedding can be controlled by light. If fertilization and cleavage stages are needed later in the day or in the evening, the colonies may be kept illuminated during the preceding night, put in the dark for a couple of hours and 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 meanwhile actively sending out a number of anastomosing and encrusting hydrorhiza processes from which sprout new polyps.

Illustrations of cleavage in Bunting '94, Beckwith '14; of later planula development in Teissier '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 ripe sporosac being bright orange in color. "Male" colonies bear light pink sporosacs arranged in groups of two to four or more in a line, the lines radiating from a common point on the base of the degenerated hydranth. The ripest male sporosacs occur at the periphery of the cluster and are white with sperm. Sketch both male and female sporosacs.

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

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

Development of Scyphozoa

AURELIA OR CYANEA (April - June)

Both these jellyfishes have oral lobes extending downward. In mature specimens granular material will be found entangled on the lobes or contained in small brood sacs in the lobes. 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. '09) (Hein '00). Is cleavage regular? Sketch the embryonic stages that are available.

Select a number of active planulae and place them in clean watch glasses of sea water for further study on later days. Their gradual change in form, attachment to the bottom, acquisition of tentacles and elongation into the sessile scyphula stage can be easily followed. The resemblance of the scyphula to a simple polyp is obvious. Attachment plus the formation of 2 to 4 tentacles occurs on the second day, as does the development of an open mouth. There are 8 tentacles at 4 days, 16 at 2 weeks, 24 at 1 month. Sketch the scyphula in side view and in top view.

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

Aurelia life history illustrated in the texts of MacPride '14, Korschelt '36 and Hyman '40.

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REGENERATION IN TUBULARIA

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

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

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

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

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

COLLECTION AND CARE OF TUBULARIA

Theoretically Tubularia is a solitary form but practically so many

individuals grow together that a dense tangled mass usually results in the older forms. Young short stems are the best for experimental work and can be obtained from floats and rocks in swift current. In general it is best to collect your own stems. Since the stems need running water and a low temperature 12C they do not keep well in the laboratory. In nature the hydranths drop off about the end of July and the stems remain dormant until the water cools down in the fall. At Woods Hole the stems appear in mid June and can be used until August. However, since the waters of Cape Cod bay on the North Shore are much colder, Tubularia may be obtained from the North end of the canal throughout August.

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

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

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

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

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

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

DOMINANCE IN TUBULARIA REGENERATION

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

remove some of the stem when cutting off the hydranth.

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

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

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

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

EXPERIMENTAL CONTROL OF POLARITY

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

GRADIENTS IN TUBULARIA

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

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

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EMBRYOLOGY OF THE COELENTERATES

1. LIFE CYCLE

The "typical" hydrozoan exhibits an alternation of generations- the succession of two morphologically different adult types, each characterized by a different manner of reproduction. These generations are-

1. The HYDROID GENERATION. The hydroid is usually attached and colonial. The individual members of the colony may be alike, or the colony may exhibit polymorphism. In the latter case some, the TROPHOCYTES, are specialized for securing food; others, the GONOCYTES, are specialized for reproduction. In some forms additional types of specialized individuals occur. ASEXUAL reproduction, by budding, forms the medusa. In general a colony produces either male or female medusae.
2. The MEDUSA GENERATION. The medusa originates as a bud from the hydroid colony. This individual leaves the colony and carries on a free living existence. Gonads develop on the radial canals or on the sides of the manubrium. Sperm or eggs are produced in these gonads, reproduction is therefore of the SEXUAL type. The gametes are liberated and fertilization occurs. The fertilized egg develops into a PLANULA larva. The planula attaches and develops a hydranth. Lateral budding occurs and in this manner a colony is formed.

An alternation of generations, with well developed hydroid and medusa generations occurs only in the CLASS HYDROZOA. In the CLASS SCYPHOZOA the medusa generation is well developed but the hydroid generation is reduced or wanting. The medusa generation is entirely absent in the CLASS ANTHOZOA. Even within the Class Hydrozoa there is considerable variation in relative development of the two generations.

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DEVELOPMENT OF THE HYDROZOAN EGG WITH SPECIAL REFERENCE TO PENNARIA TIARELLA.

Female medusae are produced on the hydranths, immediately above the basal whorl of tentacles. Male medusae are produced in a similar position, but on different colonies.

The medusae of Pennaria are liberated from the parent colonies in the early hours of the evening. They contain mature gametes at this time. According to Beckwith maturation occurs between 4 and 6 A.M. in the morning preceding the shedding of the eggs. In some cases the medusae remain attached and liberate their gametes from the parent colony.

Fertilization occurs in the ocean. Cleavage is very irregular- both in location of the cleavage planes and in tempo. Nuclear division, with delayed cytoplasmic division, is frequent. Irregular cleavage is frequent in the hydrozoan egg.

A typical blastula, with central blastocoel, is formed in some hydrozoan eggs. This cavity subsequently becomes filled with cells which originate by proliferation or immigration from the cells of the blastula wall. In some species this proliferation appears to occur all around the blastocoel while in others an immigration occurs from the end which will be posterior in the larva and the distal end of the hydroid. The solid embryo formed by these movements is known as a morula. The outer cells of the morula will form the ectoderm and the inner cells the endoderm.

The morula elongates, becomes ciliated, and is then known as a planula. The planula is blunt at one end. This end is forward when the animal moves through the water and it is the pole of attachment. The planula carries on a free living existence for a variable period of time and then becomes attached at the blunt anterior end. A central cavity appears in the endoderm. This will become the gastro-vascular cavity of the adult. The body is now composed of two layers- an outer ectoderm and an inner endoderm. The free end differentiates into the first hydranth. Other hydranths appear by lateral budding.

TUBULARIA.

In this genus there is a further degeneration of the medusa (in comparison with Pennaria). The medusa never becomes free but remains attached to the hydranth from which it arose as a bud. Egg production, fertilization, and early development take place within the modified medusa.

The egg develops in the cavity between the modified manubrium and the inner surface of the bell. As is frequent in the coelenterates the eggs grow by engulfing neighboring cells. The fully formed egg is concave on the surface adjacent to the manubrium and convex on the surface adjacent to the bell. The polar bodies arise near the middle of the convex surface. This will be the basal end of the future hydroid colony. Nuclear division is more rapid than cell division in the early stages. A blastocoel may or may not be formed. If it does form multipolar budding fills the cavity. The ectoderm differentiates first and later a cavity forms in the endoderm. Two sets of tentacles appear: a circle of long aboral tentacles at the margin of the disc-shaped embryo; and four small oral tentacles on the convex side. The larva is now known as an actinula. It leaves the parent medusa and attaches. The tentacles of the first hydranth have already formed. A stolon grows out from the basal end and new individuals arise by budding.

GONOTHYREA.

In this genus the medusae are also degenerate. They are produced by certain modified individuals known as gonangia. The medusae buds form on the stalk of the blastostyle. Within each medusa 2-3 eggs are formed. The fully formed medusae have tiny tentacles and traces of radial canals. They push out from the gonotheca but remain attached to the top of the blastostyle. The embryos develop to the planula stage within the medusa. They are liberated as planulae and after a short free living existence attach by the blunt end. The small end develops the first hydranth.

CAMPANULARIA.

The degeneration of the medusa is carried one stage further in this genus. All that remains of the medusa is a small bud on the blastostyle. Each of these produces an egg which remains within the gonotheca until the planula stage.

The degeneration of the medusa stage reaches its completion in some fresh water forms such as Hydra.

Experimental EMBRYOLOGY.

The egg of the coelenterate, especially of the hydrozoa, is thought to be of the extreme regulative type. Zoja isolated the blastomeres of Clytia and found-

- 1/2 blastomeres formed planulae and hydroids
- 1/4 blastomeres formed planulae and hydroids
- 1/8 blastomeres formed planulae only (Mass got hydroids)
- 1/16 blastomeres formed planulae.

In Liriope a 32 celled blastula is formed which consists of a single layer of cells. At next division 32 inner cells are formed.

1/2 blastomeres produce endoderm at 4 cleavages then later form medusae. 1/4 blastomeres form two layered embryo. 1/8 blastomeres ~~form~~ do not form ectoderm and endoderm- merely a sphere.

In Amphibetia the unattached pole of the egg is yellow. At this pole the maturation divisions occur, 1st. cleavage begins, gastrulation begins, it is the posterior pole of the planula and the oral pole of the hydroid. Fragments of the egg- down to 1/10 the normal size form planulae- but always with the original polarity.

TUNICATES

A comprehensive survey of developmental processes in the tunicates would include examination not only of eggs and embryos but also of metamorphosing larvae, various types of vegetative reproduction, and regenerating forms. Because these developmental forms must be constantly related to the structure of larvae and adults, it is necessary that the student should first of all review in a standard text the anatomy and life history of the common types of tunicates.

A. SIMPLE ASCIDIANS

DEVELOPMENT TO THE TADPOLE STAGE.

Styela (Old name Cynthia)

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

a. Methods

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

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

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

b. Mature Unfertilized Egg.

Sketch the mature unfertilized egg, which should show the following

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

Tunicates

- (2) Small spherical inner follicle cells ("nurse cells") between chorion and egg itself. They contain yellow granules.
- (3) Peripheral layer of egg, a clear layer containing minute yellow granules.
- (4) Central part, consisting of gray yolk platelets.
- (5) Germinal vesicle, a very large eccentrically placed mass.

C. Post-Fertilization Rearrangements

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

Carefully watch for the rearrangement of egg substances starting within 2-8 minutes after fertilization. The clear yellowish peripheral matter streams to the lower pole over the yolk, followed by the clear protoplasm from the animal pole. If the yellow pigment is not visible, try a more brightly colored egg. Use only daylight for illumination, and have the diaphragm on the microscope wide open. Roll the egg around by moving the cover slip with a needle. The yellow inner follicle cells may also be migrating; watch the egg cortex.

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

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

D. Cleavage

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

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

7th cleavage to 112 cells after 20 minutes

8th " " 218 " " 20 "

To neural plate stage, 2 more hours.

Fully formed tadpole 12 hours after fertilization

- (1) First cleavage. Equal, separating the two horns of the yellow crescent from each other, likewise bisecting the clear protoplasm anterior to the yellow.
- (2) Second cleavage. Nearly equal, vertical, at right angles to the first. The two posterior cells (B3 on the left and B3 on the right) contain little yolk and practically all the yellow crescent substance. The two anterior cells (A3 on the left and A3 on the right) contain much yolk, and practically none of the yellow crescent substance. The clear protoplasm goes equally to the four cells.
- (3) Third cleavage. Horizontal, the upper quadrant (cells a4, a4, b4 and b4) somewhat smaller than the lower quadrant (cells A4, A4, B4 and B4.) The yellow crescent substance is almost entirely confined to the two posterior dorsal cells (B4, B4).
- (4) Fourth cleavage. The planes of cleavage vary in different quadrants, but the new cells do not overlap the sagittal plane of the embryo. Two of the antero-dorsal cells and two of the postero-ventral cells of the 16-cell embryo are crowded away from this sagittal plane, but all other cells touch it. The dorsal and ventral hemispheres at this stage are mirror images. The yellow pigment lies in four posterior cells (B5.1, B5.2, B5.1 and B5.2).
- (5) Fifth cleavage. Cleavage in the dorsal (vegetal) hemisphere precedes that in the ventral (animal) hemisphere, and cleavage in the anterior part of each hemisphere precedes that in the posterior part.

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

Six yolk-filled cells at the vegetal (definitive dorso-posterior) pole anterior to the yellow mesoderm cells, will give rise to endoderm: A6.1, A6.3, B6.1, A6.1, A6.3, B6.1.

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

All the rest of the cells are ectodermal.

(6) Later cleavage, gastrulation, neurulation. The gastrula passes through disc-shaped, saucer-shaped and cup-shaped stages, starting with the 7th cleavage. As it finally becomes egg-shaped, the gastrula's blastopore, found at the small hind end,

4- Tunicates

becomes T-shaped, the stem of the T bordered by the yellow mesoderm-mesenchym cells.

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

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

Molgula.

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

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

Start some *Molgula* eggs developing, and follow the cleavage through gastrulation. If developing eggs are isolated in a watch glass, tadpoles and young stages of metamorphosis are obtainable. Tadpoles are fully formed in 8 hrs. (Conklin '05a) (Grave '2).

B. COLONIAL ASCIDIANS

1. AMAROUCIUM: TADPOLES, METAMORPHOSIS, EPICARDIAL BUDDING. (Usually not available until July)

a) Methods.

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

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

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

If colonies are kept in a shrouded aquarium the shedding can be postponed until a more seasonable hour. Swarms of active tadpoles usually appear within fifteen minutes of bringing ripe colonies out of the dark. About a third of the tadpoles will emerge within half an hour, if illuminated first at nine o'clock in the morning;

5- Tunicates

if the colony is kept dark until mid-afternoon about three quarters of them will emerge within half an hour. Nearly all of them will commence metamorphosis within an hour of being shed.

b. Early stages

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

c. Tadpole structure and behavior

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

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

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

d. Metamorphosis

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

e. Later Stages

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

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

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

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

f. Epicardial Budding

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

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

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

2. BOTRYLLUS: TADPOLES, METAMORPHOSIS, ATRIAL BUDDING

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

a) Structure of Tadpole

The tadpole is not as large as that of *Amaroucium*, but shows an interesting new feature. Just under the adhesive papillae is a ring of 8 ampullae which are diverticula of the body wall, destined to be parts of the as yet non-functional and incomplete circulatory system (Graves and Woodbridge '24). In metamorphosis they unfold

7- Tunicates

like petals of a flower, and spread around the edge of the developing tunicate. As the colony grows these increase in number.

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

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

b. Two-Day-Old Form Atrial Budding

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

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

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

c Week-old form.

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

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

8- Tunicates

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

3. PEROPHORA: SEPTAL BUDDING

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

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

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

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