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ANNELIDA

(POLYCHAETA)

Amphitrite ornata

LIVING MATERIAL:

These worms live in U-shaped, rather tough, mud tubes. The adults are fairly easy to obtain, but ripe individuals are never abundant. The best collecting grounds are at Barnstable, Mass., although some animals are found in Hadley Harbor. They should be collected during the day at low tide, washed free of mud, and placed immediately in a bucket of sea water. The sexes are separate, and may be distinguished by the darker abdominal segments of the females.

BREEDING SEASON:

June, July and August, the peak being in July. Lunar periodicity is marked, and ripe individuals are most plentiful within two days of the new or full moon (Scott, 1909).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: In the laboratory the worms should be washed and isolated in separate dishes. Injured worms tend to release their sexual products; for this reason, care should be taken to handle them gently, especially during transportation.

B. Procuring Gametes: Eggs obtained by cutting open the body wall can rarely be fertilized. However, if ripe, the worms will shed spontaneously during the afternoon or evening of the day of collection (Scott, 1909). The period of egg-laying lasts 30 to 60 minutes, with large, immature eggs appearing toward the end of that time. The eggs remain fertilizable for as long as one hour after entering sea water.

C. Preparation of Cultures: Half an hour after insemination, the excess sperm should be washed off the eggs, and the cultures placed on a water table. When the larvae develop, they should be decanted daily to fresh sea water; from the fifth day on, those larvae which are metamorphosing should be kept in dishes with fresh Ulva (Mead, 1897).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: Under normal conditions the oöcyte is retained in the body cavity until the metaphase of the first maturation division (Scott, 1906). When it is released from the ovary into the body cavity, the germinal vesicle breaks down and the egg, which until that time was spherical, flattens at the polar region. It measures 100 microns in diameter and is very opaque. A thin, wrinkled membrane is present (Mead, 1899), but it is slightly thicker than that of Lepidonotus (compare Figures 1 and 89 in the paper by Mead, 1897). There is a noticeable perivitelline space.

B. Cleavage: Cleavage is unequal and spiral. No polar lobes are formed. Gastrulation is by invagination (Mead, 1897).

C. Rate of Development: The rate of development is rapid. Swimming forms are present four to five hours after insemination, and well-formed trochophores in 20 hours. Larval segmentation starts at 36 hours, and metamorphosis begins at about the fifth day, when five trunk segments are present. By 11 days, metamorphosis is completed.

C. Later Stages of Development and Metamorphosis: The trochophore has a wide prototroch, a neurotroch, and a paratroch. The frontal bodies and gland cells are prominent. Larval segmentation occurs early, and when the larvae have developed about five trunk segments, they cease to swim about freely, and, sinking to the bottom, begin to metamorphose. (See text figures 6, and 9–18, in the paper by Mead, 1897.)

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ANNELIDA

(POLYCHAETA)

Arenicola cristata

LIVING MATERIAL:

The burrows of these animals are exposed at low tide on the mud flats at Lagoon Pond and Hadley Harbor, near Woods Hole, Mass. The sexes are separate, the mature males being creamy white, the females pinkish-brown.

BREEDING SEASON:

The latter part of June, and July (Bumpus, 1898). There is a definite periodicity, associated with the neap tides (Okada, 1941).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Several males and females should be kept together in an aquarium containing a layer of sand brought in from their natural habitat. A tank 60 cm. long, 55 cm. wide and 75 cm. high is recommended by Okada (1941); the sand layer should be about 20 cm. deep. A constant stream of sea water running through this aquarium keeps the water level at about 50 cm.

B. Procuring Gametes: The jellied egg-masses can be collected at the mouths of the burrows where they are deposited at nightfall. Okada (1941) has been successful in inducing these animals to shed eggs in laboratory aquaria.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is spheroidal in shape. In the Japanese form, the polar axis measures approximately 120 microns, while the diameter in polar view is 150 microns (Okada, 1941). The egg is faintly pink in color, granular and almost opaque. It is homolecithal. Germinal vesicle breakdown occurs immediately after discharge into sea water (Okada, 1941). The eggs are embedded in large, irregular jelly-masses.

B. Fertilization and Cleavage: Fertilization occurs at the metaphase of the first polar division, and is followed by the lifting of a thick fertilization membrane and the formation of the first and second polar bodies (Okada, 1941). Cleavage is unequal and spiral, and the micromeres are relatively large. No polar lobes are formed. Gastrulation is by a combination of invagination and epiboly.

C. Rate of Development: The following time table of development at 23 to 25° C. is given by Okada (1941). Time is calculated from shedding of the eggs.

Stage	Time
First cleavage	7 hours
Second cleavage	7½ hours
Third cleavage	8 hours
Early blastula	15 hours
Gastrula	24 hours

Stage	Time
Young trochophore	2 days
Trunk segmentation, loss of capsule	3 days
Three to five pairs of setae (embryos hatching from jelly-mass)	4 days

D. Later Stages of Development: A trochophore is developed during late gastrulation. The free-swimming larvae within the jelly-mass are oval organisms, with a narrow prototroch and telotroch; a small ventral neurotroch and a posterior tuft of cilia are also present. At this time, there is a single pair of eyespots in the pre-trochal region. The hatched larvae have three to five pairs of setigerous segments and an additional pair of eyespots. Diagrams of the larvae can be found in the papers by Wilson (1882), Child (1900) and Okada (1941).

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ANNELIDA

(POLYCHAETA)

Chaetopterus pergamentaceus

LIVING MATERIAL:

These worms live in parchment-like, U-shaped tubes in sand just below tide level; they can be dug only at low tide. The sexes are separate, and are distinguished by the parapodia on the posterior (sexual) segments. These parapodia are uniformly ivory white in the male, but in the female they contain yellow coils, which are the ovaries with their enclosed eggs.

BREEDING SEASON:

June, July, and sometimes the first two weeks in August.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: When brought into the laboratory, the animals are often still in their leathery tubes, which can be slit with scissors so that the worms can be removed gently. The sexes should be segregated, with no more than two or three animals per large fingerbowl. The dishes should be placed on a water table, and supplied with a constant, gentle stream of sea water. One or two females and one ripe male will give an adequate supply of eggs and sperm for ordinary embryological experiments.

B. Procuring Gametes: Animals may be kept in the laboratory for several days and parapodia removed as needed.

Female gametes: Unless the sexes have been kept separate for at least two days, rinse the female for a few seconds under a gentle stream of fresh water, to kill any sperm which may have adhered to the mucous film on the body. Cut off one or two parapodia and transfer them to a double layer of cheesecloth (which has been rinsed well in fresh water and then in sea water), allowing the eggs to filter into a fingerbowl of freshly filtered sea water. The straining will remove debris and most of the mucous matrix around the eggs. The parapodia may be teased apart, if necessary, to release the eggs.

Male gametes: Scissors are used to remove a posterior parapodium from a male, the tip of the segment being held with forceps. Allow the sperm to flow into a stender dish containing 10 cc. of filtered sea water. A drop of this suspension examined microscopically should contain highly motile sperm. If large numbers of motionless sperm are present, the suspension should be discarded and the procedure repeated with another male.

C. Preparation of Cultures: Procure eggs as directed above, and about 10 minutes later prepare the sperm suspension. Fifteen minutes after they are obtained, the eggs should be inseminated with one drop of the sperm suspension. This allows time for germinal vesicle breakdown. Thirty minutes after insemination, the eggs should be transferred to a fingerbowl of fresh sea water and placed

on a water table. The bowl should be covered and the water changed at least twice a day after trochophores develop.

D. Methods of Observation: No special technique is required for observing living Chaetopterus eggs, but it is often desirable to prepare permanent slides of various stages. For whole mounts, which are useful for determining stages of mitosis, fertilization, etc., see the paper by Henley and Costello (1957). For sectioning these eggs, consult the very complete directions given by Just (1939, p. 88).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: This egg is rather dark and granular, from the contained yolk-spheres. It is slightly more than 100 microns in diameter, and is often not quite spherical. When taken from the female the oöcyte, like that of Nereis, contains a large, central, immature nucleus, the germinal vesicle. However, in the egg of Chaetopterus maturation proceeds spontaneously to the metaphase of the first polar division after exposure to sea water. At this stage development is arrested until activation or death (Lillie, 1906; Pasteels, 1935). The spindle cannot be distinguished as such in the living egg without considerable flattening; the relatively clear region containing it is located quite excentrically. The spindle is attached to the egg surface in the region where the first polar body subsequently will be given off.

B. Fertilization and Cleavage: A few sperm may be seen adhering to the eggs almost immediately after insemination. Within five to six minutes the vitelline membrane separates slightly from the egg surface, and may now be called the fertilization membrane. Membrane elevation is inconspicuous in the egg of Chaetopterus, and there is little or no change in the membrane itself at this time; thickening and hardening do not occur. Later, however, the membrane undergoes a series of wrinklings which are quite pronounced (Pasteels, 1950). Ten to twelve minutes after insemination, the eggs, which become almost spherical after fertilization, elongate along an axis perpendicular to the polar axis. This is preparatory to the formation of the first polar body. In this division the egg thus assumes approximately the shape of a blastomere, although the polar body which results is a vestigial cell. The egg now rounds up, but elongates again in the same manner to produce a second polar body, which is usually formed under the first, pushing it away from the egg surface. The egg rounds up again, and the egg pronucleus may sometimes be seen migrating toward the center of the egg; occasionally, the sperm nucleus may be detected. The clear zone extends from the polar region toward the equator of the egg. A typical "pear-shaped" stage is reached, with the polar bodies in a position corresponding to that of the stem attachment in a pear. The bulge which forms the polar lobe appears quite suddenly at the antipolar end of the egg, reversing its shape.

The first cleavage furrow begins at the animal pole and passes to one side of the polar lobe, which thus becomes incorporated into one of the two smooth, unequal blastomeres. Abnormal three-celled eggs, resulting from polyspermy, may be seen. The two blastomeres become closely apposed, and about 10 minutes later the second cleavage occurs. The large blastomere again forms a polar lobe, and a four-cell stage results, in which one blastomere is larger than the other three.

The four clear nuclei become visible, and shortly after this the third division takes place, forming four relatively large micromeres. A profile view shows the rotated displacement of the micromeres resulting from spiral cleavage, although this displacement is neither great nor conspicuous in the egg of Chaetopterus.

The polar bodies are larger than those of Nereis. The inequality of the first two cleavage blastomeres is due to two factors: 1) an inequality of the poles and asters of the first cleavage spindle, and 2) the addition of the polar lobe material to the CD blastomere (Mead, 1897; Lillie, 1906).

C. Time Table of Development: Chaetopterus eggs develop rapidly. If eggs are fertilized after the partial maturation in sea water has been completed, they develop as rapidly as eggs inseminated when first placed in sea water 12 to 15 minutes earlier. A rise in temperature increases the rate of development, but temperatures above 26° C. are not desirable.

The following table includes a summary of the development of many batches of Chaetopterus eggs, at temperatures of 22–23° C. and 24–26° C. The times are calculated from insemination, and represent the averages of data obtained over a period of several years.

Stage	Time at 22–23° C.	Time at 24–26° C.
First polar body	14 minutes	11 minutes
Second polar body	28 minutes	18 minutes
"Pear" stage	42 minutes	36 minutes
Polar lobe	47 minutes	41 minutes
First cleavage	51 minutes	42 minutes
Second cleavage	71 minutes	59 minutes
Swimming trochophore	22–24 hours	8–20 hours

D. Later Stages of Development: Chaetopterus larvae differ from typical trochophores in having no pre-oral prototroch. A prominent apical flagellum (single except in rare cases) is present. In slightly older larvae, a second band of cilia, the mesotroch, is found below the prototroch (Wilson, 1882, 1929).

In the late trochophore, two to six days old, there is a gradual disappearance of yolk. The various regions of the digestive tract can be identified: the wide, slit-like mouth on the ventral surface, which leads to a short, ciliated oesophagus; the large, clear, sac-like stomach, which is separated from the short intestine by a double fold of endoderm; the anus which opens on the dorsal side, just anterior to the terminal papilla or holdfast. The mesotroch of the early larva is replaced in the older animal by a pair of lateral flagella, and a second ciliated band, the paratroch, appears in the region of the posterior boundary of the intestine. In the anterior region (the head vesicle) the apical flagellum is retained and a pair of lateral eyespots is now visible. (See the paper of Wilson, 1882, and Figures 49 and 55 in the paper of Wilson, 1929).

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ANNELIDA

(POLYCHAETA)

Cirratulis grandis

LIVING MATERIAL:

These worms live in muddy sand and are abundant in many locations at Woods Hole, Mass. The sexes are separate; mature males can be recognized by the bright orange body color which develops during the breeding season (Mead, 1898).

BREEDING SEASON:

The limits of the season have not been established for the Woods Hole region, but ripe individuals are available during July and at least the early part of August (Bumpus, 1898). Mead (1898) states that the height of the breeding season is early July, and that nearly ripe females were found as early as April 17.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The worms should be washed free of mud and placed in a fingerbowl of sea water. It is best if the water is changed daily.

B. Preparation of Cultures: Spawning occasionally occurs in the laboratory. After it is completed, the adults should be removed and the eggs allowed to remain undisturbed for an hour or so. Then the inseminated eggs should be transferred to a fingerbowl of fresh sea water. Change the sea water daily.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature ovum measures approximately 104 microns in diameter. It is opaque and pale yellow-green in color. A conspicuous refractive membrane is present. The egg is probably shed in the germinal vesicle stage, but the oöcyte nucleus quickly ruptures and the egg proceeds spontaneously to metaphase of the first maturation division.

B. Cleavage: Although no details of cleavage are available in the literature, it is presumably spiral.

C. Rate of Development: Development is relatively rapid. A trochophore-like form, which never becomes an active swimmer, appears within about 24 hours, but it is rapidly converted into a three-segmented larva. Metamorphosis is well on its way by the fourth day after insemination.

D. Later Stages of Development: The vestigial trochophore has an apical tuft and a poorly developed prototroch. A three-segmented larva is formed.

SPECIAL COMMENTS:

Although this animal was at one time routinely used in the Embryology Course at Woods Hole, practically no details of its culture or embryology are recorded. Since it is quite abundant in the Woods Hole region, it merits further investigation.

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ANNELIDA

(POLYCHAETA)

Cistenides (now *Pectinaria*) *gouldi*

LIVING MATERIAL:

The adults live in small tubes shaped like ice-cream cones, made of sand grains cemented together. They are found in muddy sand in shallow water, and can be collected readily by digging on sand flats around Woods Hole, Mass. The sexes are separate.

BREEDING SEASON:

The limits of the season have not been investigated, but it is possible to secure ripe animals at least during August.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: These worms are quite hardy and will survive in the laboratory if they are kept in dishes of running sea water, whether they are removed from their tubes or left in them.

B. Procuring Gametes: Eggs and sperm can be obtained by removing male and female animals from the tubes and pinching the bodies with a pair of fine forceps. When first released, the sperm are in packets, but these quickly break up into masses of free-swimming sperm when they come into contact with sea water.

C. Preparation of Cultures: Just (1922) reports that the larvae can be reared through metamorphosis, but without special feeding the trochophores die within a few days.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature ovum measures approximately 55 microns in diameter. It is pale yellow-green in color and very transparent, showing internal changes without staining. The egg contains a large germinal vesicle with a prominent nucleolus when shed, but the vesicle breaks down rapidly when the egg comes in contact with sea water.

B. Fertilization and Cleavage: A thin membrane is elevated at the time of fertilization. The formation of two polar bodies quickly follows. Cleavage is total, unequal and spiral; the D cell is markedly larger than the other macromeres, and the micromeres are usually large. Gastrulation is by invagination.

C. Time Table of Development: The following table shows the rate of development at 24° C. The time is recorded from insemination.

Stage	Time
Polar bodies formed	29 minutes
Fusion of pronuclei	40 minutes
First cleavage	54 minutes
Second cleavage	72 minutes

Stage	Time
Third cleavage	92 minutes
Free-swimming blastula	5 hours
Gastrula	10 hours
Well-formed trochophore	22 hours

D. Later Stages of Development: The trochophore is small and transparent. It has a long tuft of apical cilia and a well-developed prototroch. Ciliated lateral and anterior lips overhang the mouth like a hood. From one of the lower corners of the mouth a tuft of long cilia protrudes; it is particularly noticeable in side view. There are indications of a telotroch. The tri-partite digestive tract is well ciliated and contractile. The diagrams by Wilson (1936), illustrating the larvae of a European species of *Pectinaria,* show that these larvae are almost identical with those of *Cistenides gouldi.*

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ANNELIDA

(POLYCHAETA)

Clymenella torquata

LIVING MATERIAL:

This worm lives in a tube fashioned of sand grains; the tubes are in a vertical position, in the sand of inter-tidal regions, and at Beaufort, N. C., are often found in association with the similar tubes of *Axiothella mucosa,* another tubicolous annelid (Bookhout and Horn, 1949).

At times, the animals are abundant, but the number seems to fluctuate widely from year to year. Since the breeding season apparently is very short, it is advisable to collect large numbers of the worms immediately before the onset of the breeding season.

Burbanck *et al.* (1956) report that Clymenella was regularly collected at Rand's Harbor, Mass., over a six-year period.

BREEDING SEASON:

According to Mead (1897), all mature individuals spawn during a restricted two- or three-day period which occurs between the latter part of April and the middle of May.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Mature females have eggs which show through the body wall during the breeding season. The sexes should be segregated and the females placed in an aquarium which is supplied with sand. New tubes are rapidly built by the worms.

B. Procuring Gametes: Eggs are deposited on the surface of the sand, at the mouth of the tube. They may be left in sea water for several hours before insemination.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg measures 150 microns in diameter. It is practically spherical, and is very opaque because of the large amount of yellow yolk. A closely fitting, thin, smooth membrane is present (Mead, 1897).

B. Fertilization and Cleavage: The sperm enters after the first maturation spindle has formed, and the polar bodies remain to mark the animal pole (Mead, 1897). Cleavage is equal and spiral, and no polar lobes are formed. Gastrulation is probably by epiboly. In general, the development is very similar to that of Amphitrite, according to Mead; see Figures 65 to 88 in his paper (1897).

C. Rate of Development: No precise information is available.

D. Later Stages of Development: The larva is a free-swimming trochophore, reported to be similar to the trochophore of Amphitrite.

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ANNELIDA

(POLYCHAETA)

Diopatra cuprea *

LIVING MATERIAL:

These are large worms (often 30 cm. long and 10 mm. wide), which live in dark grey parchment-like tubes embedded in hard-packed sand. The tubes are often encrusted with shells, algal particles and debris, and have a lateral vent (Hartman, 1945). Intact animals are not often obtained, since they tend to withdraw into their long tubes when disturbed. The animals are yellowish to dark brown in color, and the sexes are separate. Sexually mature males are white to yellowish in color; mature females are grey-green. It is very difficult to distinguish between the sexes (Allen, personal communication).

Sumner *et al.* (1911) state that Diopatra is "almost ubiquitous" in Woods Hole waters. North Falmouth and Hadley Harbor, Mass., are among the collecting grounds for this form.

BREEDING SEASON:

Bumpus (1898) reported that at Woods Hole the ova were "nearly ripe" in August; young larvae were obtained in tows at Beaufort, N. C. in July (Andrews, 1891a). Hartman (1945) found egg-strings exposed at low tide during June and July at Beaufort; she suggested that these egg-strings were probably deposited at night.

Allen (personal communication) has been successful in rearing embryos from the middle of June until the end of August, at Woods Hole. However, she has not found naturally-spawned egg-strings of Diopatra at Woods Hole any time during the period from April to the end of August.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals do well, if they are left in their tubes and supplied with adequate amounts of running sea water. They are carnivorous and thrive if fed pieces of Mytilus every day or two (Allen, personal communication).

B. Procuring Embryos and Gametes: The naturally-fertilized eggs are surrounded by a gelatinous substance, and are deposited on the sand in long, slender, cylindrical egg-masses (Hartman, 1945). Various stages of embryonic development are contained within the jelly; when a freshly-laid string is placed in a culture tank, the larvae leave the jelly and seek the upper, light side of the container (Hartman, 1945). Ordinarily, however, they remain within the jelly-mass for a period of several days.

In the female, the ripe eggs are packed into the body cavity (Andrews, 1891b), apparently unattached to the ovary. The sperm are likewise found in masses in

* Much of the information on which this section is based was obtained from Dr. M. Jean Allen, to whom we are most grateful.

the body cavity of the male. Allen (personal communication) states that when the adults are held with forceps, they readily pinch off posterior segments. Eggs may be obtained from such isolated posterior sections by slitting the body wall; sperm ooze out when the body wall at the base of a parapodium is pricked with a dissecting needle.

C. Preparation of Cultures: Andrews (1891b) was not successful in obtaining development of artificially-inseminated Diopatra eggs. However, Allen (1951, 1953) reported that eggs could be successfully inseminated *in vitro;* the percentage of fertilization under such conditions is often not very high (Allen, personal communication). Just (1922) stated briefly (p. 477) that he was successful in artificially inseminating Diopatra eggs cut from the females.

After they are obtained, the eggs should be washed in a fingerbowl containing sand-filtered sea water. Inseminate with several drops of milky sperm suspension (in sand-filtered sea water); polyspermy should be avoided. It is probably best to provide two or three changes of fresh, sand-filtered sea water within a few minutes after insemination, and at least once daily thereafter.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The eggs of this form are produced in the ovary by a remarkable process which was described by Andrews (1891b). The ovarian tissue is composed of cell-strands which are thrown into loops, projecting into the body cavity of the female. One of the cells at the apex of each loop becomes enlarged and specialized, and gives rise to the ovum. The remaining cells (often 15 in number) of the loop continue to be attached to the ovum (even after it is detached from the ovary and lies free in the body cavity) in the form of two long strands. These strands are retained until the ovum is almost ripe; however, Andrews suggests that the function of the "sister cells" is supportive rather than nutritive. Their subsequent fate, after detachment from the ovum, is not known.

The ripe egg, free of the two strands, is ovoid in shape; it is approximately 235 microns high and 205 microns wide (Allen, 1951). A considerable amount of yolk is present, rendering it heavy and opaque in the living condition. There is a striking aggregation of green pigment, in an area which is near the large nucleus (see Figure 4, Plate I, of the ripe egg of *D. magna,* in the paper by Andrews, 1891b).

B. Fertilization and Cleavage: Fertilization is external and takes place at the germinal vesicle stage (Allen, 1953). Two polar bodies are given off, and the first cleavage results in the formation of two unequal blastomeres. Subsequent divisions are of the spiral type (Allen, 1953), the four micromeres of the eight-cell stage being polar in position and somewhat smaller than the macromeres. Within three hours after insemination, functional cilia penetrate the egg membrane; anterior vacuolated cells form four plates, which surround a central mass (the source of the future apical tuft of the larva, according to Allen, 1953). Gastrulation is probably by epiboly.

C. Time Table of Development: The cleavage of Diopatra proceeds very rapidly (Allen, 1951). The following schedule is based on her data, at temperatures of 22–24° C.; the times are recorded from insemination and represent approximations, only, inasmuch as egg-batches vary considerably.

Stage	Time
First (and sometimes the second) polar body	30 minutes
Two- to four-cells	40–60 minutes
Eight-cells	50–90 minutes
Mid- to late cleavage	90–120 minutes
Functional cilia	3 hours
Apical tuft	12 hours
Rotating trochophores	24 hours
Elongated larvae	2½ days

D. Later Stages of Development: The young larvae are spherical in shape, and provided with equatorial and terminal cilia, according to Andrews (1891b), which enable them to rotate within the jelly-mass. The egg membrane is apparently retained as a "cuticle" in the larva (Andrews, 1891b), and irregular patches of green pigment (presumably derived from the pigment of the egg) are scattered over the body.

Allen (1951, 1953, and personal communication) described the later stages of development in artificially-inseminated eggs as follows: An apical tuft forms within about eleven or twelve hours after insemination, and during the next 24 hours, elongated trochophores develop, which have a broad prototroch, a narrow telotroch, and red eyespots. These larvae continue to elongate, and by 60 hours have developed two or three sets of setae and a Y-shaped gut. Swimming is by a rotating movement. Allen (1951) reported that she was able to grow larvae in culture for as long as 21 days, by which time six sets of setae, pharyngeal muscle fibers, cerebral ganglion, tentacles, jaws and anal cirri were among the structures present.

SPECIAL COMMENTS:

Andrews (1891a) and Hartman (1951) comment on the fact that regeneration of the anterior end of Diopatra is a common phenomenon, and one which would be worthy of further study.

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ANNELIDA

(POLYCHAETA)

Harmothoë imbricata

LIVING MATERIAL:

These animals are found between tide levels, in pile scrapings and under stones. They may be distinguished from Lepidonotus, which is collected in the same localities, by having 15 rather than 12 pairs of scales. The sexes are separate.

BREEDING SEASON:

Mid-April through May. The season is about one week earlier than that of Lepidonotus (Bumpus, 1898).

PROCURING AND HANDLING MATERIAL:

Ripe females can be recognized by the bright pink color of the ventral surface. Eggs teased from the body cavity are easily fertilized, according to Mead (1898). Shedding of eggs may be induced by the methods suggested for Lepidonotus (p. 81).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is pink and rather clear, with minutely granular yolk. It is small; Sars (1845) records 50 microns for the egg diameter of a European form of this species, and diameters ranging between 56 and 78 microns are reported by McIntosh (1900) at St. Andrews, N. B.

B. Cleavage: Cleavage is equal and spiral, and no polar lobes are present. Gastrulation is probably by invagination.

C. Rate of Development: No precise data are available, although Mead (1898) states briefly that the eight-cell stage was attained in less than two hours.

D. Later Stages of Development: Mead (1897) reports that the development of Harmothoë is very similar to that of Lepidonotus. The larval stages are well described and illustrated by McIntosh (1900) and Thorson (1946). Young trochophores are positively phototropic (McIntosh, 1900).

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ANNELIDA

(POLYCHAETA)

Hydroides hexagonus

LIVING MATERIAL:

Adults of *Hydroides hexagonus* live in white, twisting, calcareous tubes which they secrete on old mollusc shells, stones, or timbers which are dredged in abundance from the harbor. For a description of features which distinguish this worm from Sabellaria, see p. 93 of this manual. The sexes are separate, but similar in appearance.

BREEDING SEASON:

According to Grave (1933), this opens between June 10 and 15, and closes between October 1 and November 1. During the early part of the season, more than 50% of the shed eggs are immature and undersize; after the middle of July, nearly all the eggs are mature and fertilizable.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: If the worms are left in their tubes and placed in aquaria with a good supply of running sea water, they may be kept almost indefinitely in the laboratory.

B. Procuring Gametes: The gametes, which are carried in the coelomic cavity, will be released through the nephridiopores almost immediately after the worms are removed from their tubes. This is done by chipping away the tubes with forceps. Place each animal in a separate stender dish containing about 25 cc. of sea water, and observe shedding. Clouds of white sperm will flow from the male, while the female will release a large number of peach-colored eggs. This is a convenient way of distinguishing the sexes. Remove the male when the sea water is cloudy with sperm, and use this sperm suspension without dilution, as indicated below.

C. Preparation of Cultures: Eggs may be successfully fertilized as long as four hours after shedding. Sperm, once activated, remain viable for as long as eight hours. The sperm are inactive when first shed, but become activated slowly after dilution with sea water. For this reason it is best to delay insemination until at least half an hour after shedding. At this time examine a sperm sample under a compound microscope. If the sperm appear active, add five or six drops of the suspension to the eggs, which have been transferred to a stender dish of fresh sea water. Allow the inseminated eggs to stand undisturbed for about 30 minutes and then decant the upper layers of sea water, replacing it with fresh sea water. Cover the dish and place it on the water table. In ten hours or less, actively swimming gastrulae will be present; they should be decanted to a fingerbowl of fresh sea water. Discard the debris and the undeveloped eggs. The larvae are very hardy, and will develop for days without extra care, although if they are to be kept over long periods, they should be decanted daily to fresh sea water. They have been

successfully reared through metamorphosis (about two weeks after hatching), but to do this, feeding with diatoms is necessary.

D. Methods of Observation: Since these eggs are small, they are best studied under high magnification, mounted on glass slides covered with unsupported coverslips. The older, moving larvae can be temporarily quieted by adding a drop of very dilute Janus green solution (1:1000 in sea water) to the mount. This will also serve to indicate clearly details of the digestive tract.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The eggs are small (67–72 microns in diameter), peach-colored, and have a thick, refractive, vitelline membrane. They are spherical and very opaque, due to the presence of a considerable amount of yolk, but the outline of the large germinal vesicle can be seen.

B. Fertilization and Cleavage: Insemination is not immediately followed by any noticeable changes, no entrance cone nor fertilization membrane being formed. Colwin, Colwin and Philpott (1956b) report that a narrow perivitelline space is present, however, after fertilization. The outline of the germinal vesicle becomes irregular and lobulated, and 15 minutes after insemination, it ruptures completely. Although the maturation spindle is not visible (because of the opacity of the egg), it is forming and moving to the periphery of the egg.

Prior to the appearance of the first polar body, two changes may be seen to occur. The egg flattens at the animal pole, and the vitelline membrane rises from the surface in this vicinity to form a cap-like space into which the first polar body is elevated. This polar body usually divides once, soon after its formation. Following this division, the second polar body is produced. A second cap-like space is formed at the vegetal pole, by the separation of the egg surface from the membrane. This precedes the first cleavage, which is equal; the AB cell cannot be distinguished from the CD cell. The second cleavage divides the egg further, into four approximately equal blastomeres, and very shortly after this, a dexiotropic, horizontal cleavage cuts off the first quartet of micromeres. Further cleavages follow the typical course of spiral cleavage, and produce a ciliated, moving blastula in about five hours. (See the paper by Shearer, 1911.)

C. Time Table of Development: The exact relationship between temperature and developmental rate has not been established in this form, but the following table will give an approximate chronology of stages observed at 24° to 25° C. The time is recorded from insemination.

Stage	Time
Germinal vesicle breakdown completed	14 minutes
First polar body	40 minutes
Second polar body	60–70 minutes
First cleavage	1 hour, 20 minutes
Second cleavage	1 hour, 36 minutes
Third cleavage	1 hour, 46–50 minutes
Swimming larva (blastula)	5–6 hours
Gastrula	9–12 hours
Well-formed trochophore	20 hours
Metamorphosis	12 days–2 weeks

D. Later Stages of Development and Metamorphosis: The process of gastrulation may be followed by observing embryos 6–10 hours after insemination. The vitelline membrane is not cast off when the cilia develop and the larvae start to swim; the cilia grow through it, and the membrane is not lost, eventually forming part of the cuticle of the worm body. In the young blastula, it is still possible to see the membrane raised from the surface in the polar area. The polar bodies, however, are no longer visible at this time. As gastrulation progresses, the elongated endodermal cells invaginate into the blastocoele to form the archenteron, which opens to the surface by way of the blastopore. In older gastrulae, the apical tuft and prototroch are well developed; they are therefore trochophores. (See the paper of Hatschek, 1886.)

Larval stage: The larva is a typical annelid trochophore. For details of structure, the excellent figures of Hatschek (1886) and Shearer (1911) may be consulted. The larvae show positive phototaxis, and gather on the illuminated side of the dish. Trochophores, three to five days old, can be mounted on a slide with a few shreds of lens paper to entangle them, or they can be quieted with a drop of dilute Janus green (1:1000 in sea water). The larvae are transparent, and proper illumination (obtained by adjusting the microscope mirror and condenser) will help to bring out the details of structure. The apical tuft and the anal vesicle are landmarks for the animal and vegetal poles, respectively; the mouth is on the ventral side, the eye on the right.

The following may be observed:

1. The shape of the trochophore, with pre-trochal and post-trochal regions.
2. Apical tuft; several long cilia probably functioning as a sense organ.
3. Apical organ, a thickening of the ectoderm at the animal pole; a nerve center and the primordium of the cerebral ganglion.
4. The prototroch, an equatorial band of large cilia. In older trochophores, two rows will be found, with a row of short cilia anterior to the large cilia.
5. The metatroch (paratroch), a circular band of cilia in the middle of the post-trochal hemisphere.
6. A ciliated groove on the mid-ventral line connecting the mouth and the anus. It marks the line of closure of the blastopore, the mouth being the remnant of the blastopore, the anus a secondary opening at the lower end of the blastoporal slit.
7. One eyespot (with red pigment) on the right side in the pre-trochal hemisphere.
8. Two statocysts on the ventral side.
9. The digestive tract, consisting of mouth opening, stomodeum or oesophagus (ectodermal), enlarged stomach (endodermal), narrow intestine (endodermal, with the exception of terminal, ectodermal proctodeum), and the anus, an opening dorsal to the vegetal pole. The whole tract is lined with cilia. The mechanism of food intake may be studied if the larvae are fed Chinese ink.
10. The anal vesicle, a large, vacuolated cell at the posterior end, not found in most other species of trochophores.
11. The cavity between the outer body wall and the intestine; not a true coelom but a primary body cavity, it is the persisting blastocoele.
12. The larval kidneys (paired), typical protonephridia with flame cells; they open near the anus, and appear as slender cords near the statocysts, extending between

oesophagus and anus. (Consult the figures in the papers by Hatschek and Shearer.)

13. Muscles. Two fine strands may 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 at the oesophagus. These are longitudinal muscles. Other longitudinal muscles extend from the stomach to points in the upper hemisphere. A strong circular muscle is located near the metatroch; the constriction of the larva caused by its contraction will be frequently observed. There are circular, sphincter muscles in the digestive tract.

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

15. The important entomesodermal cells (derivatives of the 4d teloblasts), which 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 larval kidney.

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ANNELIDA

(POLYCHAETA)

Lepidonotus squamatus

LIVING MATERIAL:

These animals are relatively abundant, and can be found in pile scrapings and under stones at the tide level. The sexes are separate; the males are whitish and the females dark on the ventral surface (Mead, 1897; Bumpus, 1898).

BREEDING SEASON:

From the last of April to nearly the beginning of June (Mead, 1897; Bumpus, 1898).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Males and females should be segregated in individual finger-bowls when brought into the laboratory, and supplied with running sea water.

B. Procuring Gametes: The animals shed during the evening of the day of collection, usually between 8 and 10 o'clock, although sometimes earlier. The shedding of eggs may be induced during this time by plunging the female into a dish of colder water and then placing the dish close to a lamp. The eggs can stand for several hours in sea water before insemination without impairing normal development (Mead, 1897).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is irregular in shape when first shed, but soon becomes spherical. It measures 65 microns in diameter, and is rather opaque, although it contains a relatively small amount of yolk. A smooth, thin, closely fitting membrane is present.

B. Fertilization and Cleavage: There is apparently no information as to the time of sperm entrance. Cleavage is equal and spiral; no polar lobes are formed. Gastrulation is by invagination (Mead, 1897).

C. Rate of Development: In general, development proceeds rather slowly. Swimming forms are present 8 to 10 hours after insemination; gastrulation occurs in about 20 hours, well-formed trochophores by 48 hours. After this, there is little change for several days. The rate of development, however, is greatly increased with a rise in temperature (Mead, 1897).

D. Later Stages in Development: During the gastrula stage the trochophore assumes a remarkable shape: the membrane stands out from the body except in the regions of the apical tuft and the wide prototroch. The older trochophores are thin-walled and have a narrow, well-developed prototroch with longer cilia, and a neurotroch. An eyespot is present. (See text figures 19 and 20, and plate figure 104 in the paper by Mead, 1897.) The larvae show several interesting reversals of phototropic response during development according to Mead (1897).

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ANNELIDA

(POLYCHAETA)

Nereis limbata

LIVING MATERIAL:

The heteronereis form of *Nereis limbata* lives in the mud of Eel Pond at Woods Hole, Mass., and also, in smaller numbers, in the Fisheries Basin and in Great Harbor. The sexes are separate. During certain phases of the lunar cycle (from full to new moon), these worms swarm at the surface, beginning about an hour after sunset. The males can be recognized by their smaller size, more active movements, and more vivid coloration— they are bright red, with white posterior segments. The larger, more sluggish females are a pale yellow-green in color.

It is convenient to collect the animals from the Eel Pond floating dock of the Supply Department, at Woods Hole. The light of a 100-watt lamp is used to attract the worms (the dock being wired with electricity for the collecting lamps). A long-handled net (having a flat, oval-shaped head, about 10 inches in length in the long axis, and with gauze stretched tightly over the framework) is used to scoop the worms from the water. On nights when there is a "run," a few males will appear first, swimming in wide circles. The females appear later; fewer in number and swimming more slowly than the males, they are first seen at the outer boundary of the circle of light. As a female spirals slowly towards the illuminated surface of the water, males which approach within a certain orbit will deviate from their original spiral paths to swim actively around her in rapidly narrowing circles, shedding sperm as they do so. This action is stimulated by substances which originate in the eggs and which are given off by the body of the ripe female. In turn, the presence of sperm in the water is a stimulus which induces the female to circle and shed. The females sink slowly to the bottom when they are spent. If females are to be collected before any shedding occurs, it is necessary to obtain them before they begin to circle.

Exceptions to the "dark of the moon" swarming are as follows: (1) In the first run of June, the animals may swarm every night until the full moon of July. (2) On stormy or windy nights, or nights following very cloudy days, Nereis may fail to appear. (3) In late September on cold nights they do not swarm even in the dark of the moon. (4) The curve of swarming is bimodal, with a depression several days before new moon.

Other striking examples of lunar periodicity have been described by Clark and Hess (1940a, 1940b), Hempelmann (1911), Izuka (1903), Just (1914), Mayer (1908) and Woodworth (1907).

BREEDING SEASON:

June through September, as discussed above (see, also, the paper by Lillie and Just, 1913).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals should be collected one by one, and each female placed in a separate fingerbowl of sea water; several males may be kept together. It is best to prepare the fingerbowls in advance of the collection, placing a piece of Ulva (or a piece of paper towel, previously thoroughly soaked in clean sea water) in each dish, which is then half-filled with clean sea water. These dishes can be carried in a wooden tray to the collecting dock, and the animals placed directly in them. When they are brought back to the laboratory, the sea water should be changed and fingerbowls containing the animals should be covered and placed on a water table, surrounded by running sea water. *Do not keep the worms in a refrigerator.*

B. Procuring Gametes: An excess of sperm, which should be avoided, is usually obtained if the males and females are placed together in a dish, unless the male is removed as soon as it has shed its first cloud of sperm. Instead, gametes should be procured by pinching the animals with fine forceps, near the middle of the body; a single strong pinch should result in extrusion of the sex cells. The eggs from each female should be placed in a 250-ml. (or larger) fingerbowl, half-filled with clean sea water; no more than a single cell-layer of eggs should be present on the bottom of the dish, after the eggs have settled. "Dry" sperm are obtained by placing a male worm in a dry Syracuse dish and pinching the middle of the body. Adults should be removed from the dishes after shedding is completed.

Clipping the body with scissors is not a good practice, since it increases contamination of the gametes with coelomic fluid (which apparently adversely affects the normal fertilization reaction).

C. Preparation of Cultures: Add a few drops of dilute sperm suspension (one drop of "dry" sperm in 50 cc. of sea water) to the fingerbowl of eggs, and stir at once with a rapid circular movement of the dish. Care must be taken to avoid polyspermy, which results in interference with cleavage or in abnormal cleavage and development. In some forms, polyspermic eggs develop more rapidly than normally fertilized eggs, but those of Nereis usually fail to cleave.

To obtain later stages of development, allow the fingerbowl to remain undisturbed for about 30 minutes; then transfer the contents to a large dish and change the sea water. Keep the dish covered and on a sea water table, changing the water at least twice a day. After they leave the jelly, pour off the swimming trochophores to a clean dish of sea water; discard the jelly and dead eggs.

D. Methods of Observation: One or two minutes after insemination, place the eggs in a drop of thick Chinese ink suspension (made by rubbing a piece of the solid ink, wet with sea water, on a finely-ground glass surface). As the jelly is secreted by the eggs, it flows past the attached sperm, leaving a funnel-shaped cavity in which the ink particles remain, serving as an indicator of the point of sperm entrance.

Another method of studying sperm entrance is by the production of exaggerated entrance cones. Place a drop of eggs, inseminated 5 to 8 minutes earlier, in a stender dish containing 50 cc. of alkaline NaCl (pH 10.3–10.5) and mix rapidly and thoroughly. 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 (Costello and Young, 1939). The vitelline mem-

brane 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 a marked indentation of the membrane. It is sometimes necessary to use two or three changes of alkaline NaCl to obtain maximum exaggeration of the entrance cones. If the eggs (or the females from which they were obtained) have been kept in a refrigerator, they may become polyspermic when inseminated, and show numerous exaggerated entrance cones following this treatment. About ten minutes after treatment, the sperm head and middle piece may be seen moving across the perivitelline space to fuse with the egg surface. The membrane indentation is relaxed as soon as the sperm head has passed through. If the eggs are now carefully removed to sea water and washed several times, some will develop normally within the raised membranes. If they are left in the solution an 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. For further details of obtaining these denuded eggs, see the papers of Costello (1945a, 1949). For details of useful procedures for fixing, sectioning and staining Nereis eggs, consult the book by Just (1939b).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg of Nereis is approximately 140 microns in diameter and 100 microns high. Because of its shape, it tends to orient on a flat surface with the animal pole either above or below, only rarely to the side. It has a large germinal vesicle, with many small oil droplets and yolk spheres in the cytoplasm surrounding it. The egg has a cortex about seven microns thick, of jelly-precursor granules.

B. Fertilization and Cleavage: Very soon after insemination, a transparent jelly-layer is secreted by the egg, external to its vitelline membrane. This jelly arises from the cortical granules. In 20 minutes the zone of jelly will be as wide as the diameter of the egg it surrounds; its margin can often be observed with the aid of supernumerary spermatozoa or other particles (such as Chinese ink) at its edge or in the medium.

There is little visible change in the vitelline membrane at fertilization, although it is called the fertilization membrane after this event. However, a narrow perivitelline space is present shortly after insemination, resulting from the breakdown of the jelly-precursor granules and release of the jelly. The sperm entrance cone now becomes clearly visible, and is best seen in the profile view of an egg with a sperm at its periphery. 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, tending to obscure the sperm from view. About 20 minutes after insemination, the egg wrinkles, becoming distorted and almost amoeboid in appearance. The entrance cone has flattened considerably but is still present, and although the sperm is partially concealed from view, the entrance of its head into the egg is not completed until some time later (Just, 1912; Lillie, 1911, 1912). Its final penetration through the membrane (about 48 minutes after insemination) leaves the middle piece and tail outside.

The egg then rounds up, and elongates in a direction perpendicular to the polar axis, as the time approaches for the formation of the first polar body. The prepara-

tion should be shaken if no eggs lie so that the forming polar body is on the periphery. The polar body is given off into the space between the egg and the vitelline membrane, which is wider in the region of the animal pole than elsewhere. The second polar body forms under the first, thus lifting it away from the egg surface. The first polar body of Nereis rarely, if ever, divides.

The egg cleaves into two unequal blastomeres, and the second cleavage is also unequal. The third cleavage, from four to eight cells, produces four micromeres by spiral cleavage (Wilson, 1892; Costello, 1945a).

C. Time Table of Development: The following schedule is based on the development of 16 batches of eggs, at temperatures of 22–24° C.; times are calculated from insemination.

Stage	Time
Disappearance of membrane of germinal vesicle	10–15 minutes
First polar body	42 minutes
Sperm penetration	48 minutes
Second polar body	58 minutes
First cleavage	81 minutes
Second cleavage	108 minutes
Third cleavage	132 minutes
Fourth cleavage	162 minutes
Ciliated trochophore	8–10 hours
Pigmentation in trochophore	24–38 hours

There is a high temperature coefficient for the cleavage process; Lovelace (1949) gives data from which the following mean times have been calculated for first cleavage in 50% of the eggs in a given batch.

Mean temperature, ° C. (within a 1° range)	Time after insemination
20.0	97 minutes
21.3	86.9 minutes
22.1	84.2 minutes
23.1	77.3 minutes
24.1	70.8 minutes

D. Later Stages of Development and Metamorphosis: Gastrulation is by epiboly. The products of the first three quartets of micromeres overgrow the four large oil-bearing cells, 3A, 3B, 3C and 4D. These four endodermal cells, after giving off a few small cells, persist unchanged for a relatively long period. The four large oil droplets (which result from coalescence of the smaller oil droplets of the egg) may be used as a criterion of normal development, since those in the C and D quadrants are larger than those in the A and B quadrants. For further details, consult the papers of Wilson (1892, 1898) and Costello (1945a).

The trochophore larva metamorphoses into a segmented worm in about seven days. The trochophore is somewhat atypical, and there is an abbreviated, "telescoped" larval development. The first signs of the segmented adult organization appear very early. To study, mount the larvae on a slide, and either entangle

them in a few shreds of lens paper or quiet them by adding a drop of very dilute (1:1000) Janus green solution.

Distinctive features of the trochophore larva at 40 hours (Wilson, 1892; Figure 84) include:

1. An equatorial prototroch consisting of 12 very large, ciliated cells, instead of the 16 typical of most annelidan and molluscan trochophores. There is the characteristic interruption in ciliation, in the mid-dorsal line. A narrow paratroch is present, near the vegetal pole.
2. Pigmentation, consisting of (a) a pair of red-pigmented eyespots in the pretrochal hemisphere; (b) orange-brown "prototrochal" pigment, in cells adjacent to the prototroch; (c) greenish-black anal pigment in the region of the proctodeum.
3. The four large macromeres (each still containing a single large oil drop) have not yet differentiated into the parts of the intestine. Short, blind ectodermal invaginations constitute stomodeum and proctodeum.

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ANNELIDA

(POLYCHAETA)

Platynereis megalops

LIVING MATERIAL:

Platynereis megalops may be found in Great Harbor and in Eel Pond at Woods Hole, Mass. The swarming heteronereis form is attracted to light and may conveniently be collected with the same type of flat net as is used to obtain *Nereis limbata.* The sexes are separate. The reddish males swim with rapid, jerky movements, rotating in spirals tangential to the surface of the water. The large females, pale yellow in color, swim slowly at a greater depth. They travel either in a straight line, or, with head bent at right angles to the body, describing a circle about the head (Just, 1914). The males are smaller than those of *Nereis limbata* (which swarm at the same times) and swim more rapidly. Although it may be somewhat difficult to distinguish these two species when swarming, they are easily differentiated when examined in the laboratory. The eyes of Platynereis are much larger and form conspicuous dark spots on the prostomium, which, unlike that of Nereis, lacks palps and protrudes anterior to the eyes as a transparent, oval lobe.

The animals rarely appear in large swarms, and as a rule, the number appearing during an evening can be easily counted.

BREEDING SEASON:

July, and the first three weeks in August. Platynereis shows a lunar periodicity, appearing in varying numbers from full to new moon. The frequency curve does not correspond precisely to that of *Nereis limbata.* See the paper of Just (1914) for details of swarming.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals should be isolated in separate fingerbowls of sea water as soon as they are collected. As with Nereis, trays of bowls containing clean sea water and a piece of Ulva should be used. The water should be changed in the laboratory, and the dishes placed on the sea water table.

B. Procuring Gametes: It is imperative to obtain gametes by allowing males and females to mate. Just (1914) succeeded in artificially inseminating eggs only when he mixed "dry" eggs with "dry" sperm. Artificial insemination was not successful, when the eggs were diluted with more than an equal volume of sea water. Mating, fertilization and subsequent egg-extrusion will occur when a male and female are placed together. The mating habits, as described by Just (1914), are of special interest; polyspermy does not ordinarily occur.

C. Preparation of Cultures: The animals should be allowed to mate as soon as possible after collection and the adults removed immediately following shedding. After 20 minutes, decant the water from the dish of eggs and replace with fresh sea water. At the time of the first cleavage, gently break up the jelly-mass and distribute it equally among 7 to 10 fingerbowls of fresh sea water. After about

8 hours, the water should be changed again, and when the trochophores become free-swimming, they should be transferred daily to fresh sea water. The trochophores of this form are markedly sensitive to light, and if too many are kept in one dish, clumping and consequent smothering will occur. One way to prevent this is to keep the larvae in subdued light.

No feeding is necessary up to the three-somite stage, but as soon as all the endodermal oil drops are absorbed, diatom feeding should be initiated (Just, 1922).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The oöcyte from the body cavity is compressed and irregular. After they are shed, the few uninseminated eggs round up and become nearly spherical, although the polar axis remains slightly shorter than the diameter of the equator. The egg diameter varies somewhat, the largest measuring between 180 and 200 microns. The egg is almost perfectly transparent (much more so than the egg of *Nereis limbata*), with an equatorial ring of oil drops and a well-marked, clear cortex containing very fine granules and striations.

B. Fertilization and Cleavage: Insemination follows the curious copulation phenomenon (see the paper by Just, 1914), and is internal; therefore, although the egg is shed in the germinal vesicle stage, it usually has a sperm attached, and a jelly layer and perivitelline space are forming at this time. No sharply defined fertilization cone is present. Sperm penetration is completed about 30 minutes after egg-extrusion; the middle and tail piece are left outside the egg (Just, 1915b). Development is very similar to that of *Nereis limbata.*

C. Rate of Development: No detailed information is available. Well-formed, swimming trochophores are present in 24 hours; by the seventh day the larvae have three setigerous segments bearing parapodia. After this time, at least one new segment is added daily.

D. Later Stages of Development: The larvae resemble those of *Nereis limbata.*

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ANNELIDA

(POLYCHAETA)

Podarke obscura

LIVING MATERIAL:

These animals are relatively abundant at Woods Hole, Mass. They may be obtained during the day from the bottom or vegetation of Eel Pond, or during the evening, between 7:30 and 9:30, when the mature worms are swarming at the surface. They are attracted by the light of the Nereis-collecting lamps. The sexes are separate, and when the animals are ripe, they may be distinguished by the color of the gametes seen through the semi-tran parent body wall; the females are seal-brown, the males cream color.

BREEDING SEASON: July and August.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Transferred to clean dishes, with an occasional change of sea water, these worms will live indefinitely in the laboratory. It is best to segregate the sexes.

B. Procuring Gametes: Swarming animals usually will shed when taken. Females procured during the day shed eggs from 7:30 to 9 P.M. on the second or third, but rarely on the first, night after collection (Treadwell, 1901). The eggs sink to the bottom of the dish where they may be collected with a pipette. A simpler method of collecting them is to strain the water through a fine cloth which allows the eggs to pass through, but retains the spent adults.

C. Preparation of Cultures: Fertilized eggs may be obtained either by placing several males and females in a fingerbowl and allowing them to shed, or by inseminating naturally-shed eggs from isolated females with sperm from the body cavity of the males (Treadwell, 1901).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The eggs are slightly irregular when first shed, but soon become spherical. The average diameter is about 63 microns, and a thin, smooth membrane is present. Upon shedding, the egg proceeds spontaneously to the metaphase of the first maturation division and remains in this condition until fertilized (Treadwell, 1901).

B. Fertilization and Cleavage: There is no visible alteration of the egg membrane at fertilization. Cleavage is equal and spiral with especially large entomeres; no polar lobes are formed. Gastrulation is by invagination (Treadwell, 1898, 1901).

C. Rate of Development: Swimming forms appear five hours after insemination; well-formed trochophores are present in cultures 24 hours old.

D. Later Stages of Development: The trochophores are small, thin-walled and active. They have a large enteron; the intestinal portion of it seems to be almost severed from the remainder by a circular "shelf" of tissue. There is no paratroch,

but the neurotroch, prototroch, apical tuft, and an additional anterior tuft are well developed. Two ventral eyespots and five frontal bodies are present (Treadwell, 1899). (See Figure 12 in the paper of Treadwell, 1899, and Figure 60 of Treadwell, 1901.)

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ANNELIDA

(POLYCHAETA)

Sabellaria vulgaris

LIVING MATERIAL:

These tube-dwelling, polychaete worms are common at Woods Hole, Mass.; they live on old shells, stones, etc., which are dredged from the harbor bottom. The worms may be distinguished from Hydroides, which occurs in the same localities at Woods Hole, by their tubes and certain other adult features. The tubes of Sabellaria, often brown or pinkish in color, are formed by sand grains and are moderately soft and crumbly; those of Hydroides, greenish-gray or white in color, are calcareous and hard. The gill filaments of Sabellaria are filiform in general appearance, the "barbules" being very inconspicuous. Those of Hydroides, however, are much more brilliant, and may vary from purplish (sometimes striped) to brilliant scarlet in both males and females; each one has small "barbules" coming off the central shaft. Sabellaria has a tail-like abdomen which has no parapodia and which folds back on the thorax. The setae are more prominent than in Hydroides.

The sexes are separate. They are externally recognizable after the individuals have been removed from their tubes, but only if the worms are fully mature, containing large numbers of gametes. In these animals, the abdominal segments are swollen and appear opaque and white in the male, pink in the female. Individuals showing neither color distinctly may, however, shed abundantly. The sex of such animals can be ascertained by placing them in a few drops of sea water until shedding starts. Sperm will pour forth from the male in dense clouds, but egg masses will break up into small clumps on contact with water.

BREEDING SEASON:

Sabellaria is said to spawn naturally in May and June, but ripe individuals may be obtained throughout the summer months (Waterman, 1934).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: If worms are left in their tubes and placed in aquaria with a good supply of running sea water, they will produce normally developing eggs for as long as nine weeks.

Uninjured animals are most easily obtained by removing the sand tubes from the substrate, chipping away enough of the tube to expose the head and tail of the worm, and then gently forcing out the animal by inserting a blunt probe into the anterior end of the tube— the animal slowly withdraws, hind-end foremost, from the tube (Novikoff, 1939).

B. Procuring Gametes:

Female gametes: Place a female in a fingerbowl containing 200 cc. of sea water, in which it will shed if ripe. After it has shed for a few seconds, move it to a new spot and allow it to continue shedding. The eggs first shed should be dis-

carded, as they may have been exposed to air when the worm was out of water. Since the eggs are expelled by active contractions of the body, it is easy to tell when shedding has ceased; the female should be removed and discarded at this time.

Male gametes: Sperm may be obtained by placing a male in a dish containing four drops of sea water. When shedding is completed, remove and discard the worm.

C. Preparation of Cultures: Allow the eggs to stand in sea water for about 15 minutes after shedding. Toward the end of this period, prepare a dilute sperm suspension as follows: Add one drop of concentrated sperm suspension to four drops of sea water, and add one drop of this diluted sperm suspension to a fingerbowl of sea water. To this fingerbowl add the eggs, using a narrow-mouth pipette to transfer them. This method of insemination prevents polyspermy and its accompanying abnormalities. If only small amounts of eggs and sperm are available, however, culturing can be done in stender dishes instead of fingerbowls. Allow the culture to stand undisturbed for an hour; then change the water, cover, and place on a water table. After 24 hours, decant the upper layers of water, which contain the more normal, top-swimming trochophores, to a clean fingerbowl. Repeat this procedure at least once a day, adding water each time. After a day or so, larvae should be fed on a pure culture of Nitzschia.

D. Methods of Observation: Because of the small size of these eggs, they are best examined using a high magnification (440 ×). The eggs are too opaque to reveal internal changes other than the breakdown of the germinal vesicle. The ciliation of the early larvae can best be seen with dark-field illumination. A very dilute solution of Janus green (1:1000 in distilled water) will partially inactivate older swimming forms.

E. Removal of Membranes: It is sometimes desirable for study or experimental work to obtain Sabellaria eggs without their tough vitelline membranes. The method devised by Hatt (1931), and modified by Novikoff (1939), consists of treating the eggs with alkaline NaCl. The details of this method, as described by Costello (1945a) for the egg of Nereis, are given on p. 84 of this manual. Sabellaria eggs from which the membranes have been removed may still retain some of the perivitelline jelly; this can be demonstrated readily by placing the eggs in a suspension of Chinese ink in sea water.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: When first shed, the egg is very irregular in shape; usually it has a particularly deep indentation directly opposite the animal pole. This crater coincides with the point of former ovarian attachment. The small egg has a large, rather excentrically placed germinal vesicle, and a considerable amount of yolk distributed through the cytoplasm, making it appear opaque. A conspicuous vitelline membrane is very closely applied to the egg surface (Waterman, 1934).

A few minutes after shedding, a series of pre-maturation changes occurs. The germinal vesicle breaks down and its contents flow to one side of the egg, to form a clear, hyaline cap at the future animal pole. The first maturation spindle extends across not quite half the diameter of the egg. The egg rounds up, becoming spherical, and the vitelline membrane rises from the egg surface, leaving a perivitelline space about 12 microns wide. The surface changes accompanying this can

best be observed in the thin cytoplasm at the edge of the large, crater-like indentation. As the vitelline membrane elevates, a thin, transparent, hyaline plasma layer appears on the egg surface, presumably outside the egg's plasma membrane. The vitelline membrane and hyaline plasma membrane remain connected with one another by means of numerous fine, granule-free protoplasmic strands, which stretch across the perivitelline space. Elevation is due to the swelling of a transparent, dense jelly which is located between the membrane and the surface of the egg (Hatt, 1931). At first the membrane is smooth, but as it elevates and stretches further, it becomes wrinkled. An interesting series of cortical changes accompanies membrane elevation. Upon contact with sea water, the small, refringent spherules lying just beneath the vitelline membrane disappear, leaving a granule-free surface. It is this layer of hyaline material which is pulled out to form the radiating filaments. As elevation continues, many of the deeper cortical granules move toward the egg periphery and disappear, increasing the width of the cortical hyaline layer, the outer boundary of which becomes conspicuous (Waterman, 1936; Novikoff, 1939).

At the end of the pre-maturation stage, the egg is approximately 60 microns in diameter (Waterman, 1934).

The pre-maturation changes are very rapid, and are usually completed within ten minutes after shedding. To observe them, eggs should be transferred to a slide immediately after they are shed.

B. Fertilization and Cleavage: The phenomena of insemination uncomplicated by pre-maturation changes may be seen in eggs which have been allowed to stand in sea water for 15 minutes or longer. Except for the formation of an entrance cone and the withdrawal of the radiating filaments, the ovum is not visibly changed by insemination. No additional fertilization membrane is formed, and the cortical zone and existing membranes remain the same. When a spermatozoon attaches to the vitelline membrane, a large, rounded, hyaline entrance cone rises from the egg surface and pushes out toward the membrane. Novikoff (1939) and Waterman (1934, 1936) fail to agree on all details of sperm penetration, and those interested in the minor discrepancies are referred to the papers cited. According to Novikoff's account, when the entrance cone contacts the sperm, the head and middle piece separate from the tail and pass through the membrane. The discarded tail twitches a few times, and, after freeing itself from the membrane, may swim about with a rapid, whip-like motion for some time. The cone recedes, carrying the sperm head down into the egg cytoplasm. The filaments are withdrawn at this time, so that within 11 minutes of insemination, the egg surface is again smooth.

The formation of the first polar body occurs about twenty minutes after insemination, preceded by a distinct flattening of the egg in the polar region. The egg rounds up, but again flattens before the second polar body is produced. The egg then becomes spherical and remains in this condition until shortly before the first cleavage. At this time a large, first polar lobe is formed. A little more than an hour after insemination the first cleavage is completed, and the egg assumes a trefoil shape. Since the first cleavage plane passes just to one side of the polar bodies, the cleavage is slightly unequal. The polar lobe is soon resorbed into the larger CD blastomere. Soon a smaller second polar lobe is given off at the anti-polar region of this cell, and the second cleavage follows. At the completion of this division, the lobe flows into the D cell. The first quartet of micromeres (which

are almost as large as the macromeres) is given off by the usual dexiotropic division. During this division, a third polar lobe forms in the D cell, and afterwards it is incorporated into the larger basal 1D macromere. The later cleavages presumably follow the normal pattern of spiral cleavage, as exemplified by Nereis.

C. Time Table of Development: The exact relationship of temperature and developmental rate has not been worked out in detail. The following schedule is approximate for laboratory temperatures varying between 19° and 25° C. (Novikoff, 1937). Times are recorded from insemination.

Stage	Time
First polar body	19–23 minutes
Second polar body	28–34 minutes
First polar lobe	50–55 minutes
First cleavage	65–70 minutes
Second polar lobe and cleavage	80–85 minutes
Swimming larva	5½ hours
Apical tuft and prototroch	8 hours
Metamorphosis	7 weeks

D. Later Stages of Development and Metamorphosis: Consult the papers by Novikoff (1938a) and Wilson (1929) for details of development, and for illustrations of larvae 5 to 29 hours old. The larvae of Sabellaria are interesting because they show very long bristles which have probably both a suspensory and a protective function.

In Sabellaria trochophores two days old and older, some of the distinctive features include:

1. Stiff cilia in the apical region, which develop before the disappearance of the apical tuft (Novikoff, 1938a).
2. The prototroch, consisting of three rows of cilia with a gap on the dorsal side.
3. The neurotroch in the mid-ventral line.
4. One eye on the left side; more eyespots develop later.
5. Very long bristles with a fine structure, which develop in seta sacs. Ten pairs are formed, one after another. At metamorphosis they are replaced by ordinary setae.
6. The digestive tract, which is internally ciliated.

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ANNELIDA

(POLYCHAETA)

Sthenelais leidyi

LIVING MATERIAL:

The adults are rather flattened, elongate worms, covered with flat scales which alternate anteriorly with dorsal cirri. The body color is grey, with a mid-dorsal stripe; the head is brown with a central red spot, two pairs of eyes, and a single tentacle. The worms have been collected at Hadley Harbor, near Woods Hole, Mass., but are not abundant. They can be obtained by sand-sieving.

BREEDING SEASON:

The limits of the season have not been determined; however, mature animals have been obtained during the middle and latter parts of August (Bumpus, 1898).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The worms should be isolated in fingerbowls of sea water when brought into the laboratory.

B. Procuring Gametes: Mature animals usually shed a few hours after collection. Body-cavity eggs, obtained by cutting up a female, do not develop as well as those which are spawned normally. The males and females should be removed from their respective dishes as soon as shedding is completed.

C. Preparation of Cultures: Only one or two drops of dilute sperm suspension should be added to a fingerbowl of eggs; over-insemination results in abnormal cleavage. The embryos may be raised through the trochophore stage. Swimming larvae should be decanted to bowls of fresh sea water daily.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is approximately 110 microns in diameter, very opaque, and flattened and triangular in shape when shed. It has not been ascertained whether the germinal vesicle breaks down before shedding, or whether this occurs soon after contact with sea water.

B. Fertilization and Cleavage: The eggs in which germinal vesicle breakdown has occurred can be fertilized. As soon as fertilization occurs, a wrinkled fertilization membrane rises from the egg surface. Cleavage is spiral and almost equal; gastrulation is probably by invagination.

C. Time Table of Development: The following schedule indicates the development of an egg batch at a temperature of 23° to 24° C. Times are given from insemination.

Stage	Time
Polar bodies formed	30 minutes
First cleavage	40–45 minutes
Second cleavage	60 minutes
Third cleavage	85 minutes
Free-swimming embryos	3½ hours
Trochophores	17 hours

D. Later Stages of Development: The larva is a large, plump trochophore which swims with a peculiar end-over-end motion. When fully formed, it has a long, stiff, apical tuft, and a telotroch. In addition, a long tuft of cilia projects from the lower left corner of the mouth. A pair of eyespots and prototrochal pigment appear during development. The stomach is very large and vesicular; the intestine and oesophagus are small.

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