ECHINODERMATA

(ASTEROIDEA)

Asterias forbesi and A. vulgaris

LIVING MATERIAL:

Both species are moderately abundant, and are obtained by dredging off Vineyard Haven, near East Chop, Martha's Vineyard, Mass. The most reliable feature for distinguishing between them is the shape of the arms or rays; in A. forbesi the rays are stout and cylindrical and tend to be blunt at the tip, and in A. vulgaris they are flattened and taper to a point. The sexes are separate but cannot be distinguished on the basis of external characteristics.

BREEDING SEASON:

May, June and early July in the Woods Hole region. A few ripe females can be obtained in late July; however, it is not practicable to do experiments on a large scale after July 15. Formerly, starfish from the Hole produced viable gametes as late as the middle of August (R. S. Lilie, personal communication).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Adults can be kept in good condition for a considerable period of time in aquaria supplied with running sea water, provided they are occasionally given mussels or small clams for food. If an animal sheds, it should be removed immediately from the aquarium, since it may induce shedding among the others. Dying or dead individuals should also be removed as promptly as possible.

B. Procuring Gametes: Shedding may sometimes be induced in mature animals by removing them from sea water and placing them on a board in the sun, or on the cement floor of the laboratory under the heat of a desk lamp. This method is reasonably effective in the case of the west coast starfish Patiria miniata, which normally sheds in the warm shallow water of tide pools at low tide. Usually, however, it is easier to obtain gametes as outlined below, than to depend on the occurrence of natural spawning.

Only animals with soft, bulging arms are fully ripe; it is a waste of time and material to open small, hard-skinned starfish in an attempt to obtain gametes. A small-bore pipette may be easily inserted into the arm of a ripe starfish, preferably near the central region, without causing injury; the few gametes thus obtained enable one to ascertain the sex of the animal rapidly.

After identifying a female, remove the punctured arm from the disc, and return the animal to an aquarium of running sea water to be used again. Injured animals will not, however, keep indefinitely; the gametes are rarely usable at the time the animal begins to undergo autotomy. Slit the detached arm along the mid-dorsal line to expose the bulging pair of ovaries, which are typically pale salmon in color. Then, with a pair of forceps, carefully detach each plume-like ovary by grasping it near its point of attachment at the proximal end, thus closing the gonoduct; rinse
it in a large fingerbowl of filtered sea water. Transfer the ovary to a second bowl and allow the eggs to exude from the blunt end. Do not cut up the ovary. At the end of five minutes, remove the gonads to another container or discard them; the best eggs are those which exude first. Gently stir the contents of the fingerbowl and allow the eggs to settle. When they have done so, pour off the supernatant fluid and carefully replace it with an equal volume of filtered sea water. Leave the eggs undisturbed for 20 to 30 minutes; during this time small samples may be removed with a pipette for examination. Eggs carefully obtained from a ripe female, kept under proper conditions of coolness and with an adequate oxygen supply from the time of collection, should show 85 to 90 per cent germinal vesicle breakdown at approximately the same time.

From the detached arm of a male remove a single testis, white or ivory in color, and rinse it in clean sea water. A small piece of the blunt end should be cut off and placed in 200 cc. of filtered sea water.

C. Preparation of Cultures: The optimum period for fertilization is after the breakdown of the germinal vesicle and before the extrusion of the first polar body. Eggs with intact germinal vesicles are non-fertilizable; they may elevate a fertilization membrane but will not develop further. It is convenient, therefore, to inseminate when the distal end of the first maturation spindle begins to protrude above the previously smooth surface of the oöcyte in a moderate percentage of the eggs which have undergone germinal vesicle breakdown. To do so, add about five cc. of the sperm suspension, prepared as directed above, to a large fingerbowl of eggs. Immediately rotate the dish gently to ensure complete mixing. When the eggs have settled, the supernatant fluid should be decanted and an equal amount of sea water added. This procedure should be repeated at half-hour intervals, to eliminate the excess sperm which would otherwise foul the culture and prevent development of the late embryonic stages. There should be only one layer of well-spaced eggs on the bottom of a dish.

When the first swimming stages appear (after about 20 hours), pour off the upper half of the culture, containing the more normal swimming blastulae, into a series of tall battery jars. Add enough filtered sea water to fill the jars. Care must be taken to eliminate dead embryos or unfertilized eggs. In these tall jars evaporation is considerably reduced; however, the original level should be maintained by the addition of distilled water. It is essential that relatively few embryos be placed in a jar, if one wishes to raise older larvae. Aerate the cultures gently, using glass tubing rather than rubber hose for this purpose. Early bipinnaria may be obtained without special feeding, but diatoms must be added to the cultures in order to obtain brachiolaria or later stages. The culture jars should be kept at temperatures between 17 and 20° C., away from the direct sunlight (Larsen, 1937).

The egg of Asterias is very delicate as compared with most eggs used for routine laboratory work. Satisfactory results are not obtained unless adequate precautions are observed, including the following: (1) Avoid contamination of either type of gamete with perivisceral fluid; (2) avoid over-insemination; (3) avoid crowding of eggs; and (4) use only fresh, motile sperm.

D. Methods of Observation: The presence of a jelly-hull around these eggs may be demonstrated by using dim illumination, or by adding a trace of Janus green. Vital staining with neutral red is helpful in studying the larval stages.
An aqueous (sea water) extract from squid egg-string jelly has proved useful in the Embryology Course at Woods Hole, for slowing down or immobilizing the larval forms of certain echinoderms. (It is apparently not effective for Callocardia veliger larvae, however.) The extract is prepared as follows: Peel the outer covering from about four egg-strings, and cut up the strings in approximately 10 cc. of sea water. Allow the strings to remain in the sea water for one or two hours, then filter through a moderately coarse grade of filter paper. The extract will retain the ability to slow down or immobilize echinoderm larvae for at least two days, if it is kept under refrigeration. Two or three drops of the extract will quiet the larvae contained in one drop of sea water, in a depression slide. It is not necessary that the squid jelly used contain squid embryos; empty jelly-strings are equally effective for preparing extracts.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg of Asterias is very delicate and is surrounded by a jelly-hull. It is shed in the germinal vesicle stage, and on contact with sea water proceeds spontaneously to the first and second maturation divisions. The mature ovum contains a lightly pigmented yolk, pale yellow in color, through which (in later stages) the spindles of mitotic figures may sometimes be seen. The egg of A. forbesi measures about 110 microns in diameter (Fry, 1937).

B. Fertilization and Cleavage: Immediately after insemination, sperm may be seen on the jelly-surface of the eggs. Some will be attached by a tenuous filament to a fertilization cone which has arisen on the egg surface. The fertilization membrane elevates in a wave which begins at the cone and spreads rapidly around the egg. The sperm is drawn passively through the jelly to the cone, and after a pause the head piece is pulled through the membrane and enters the cone. Six minutes after insemination, the delicate sperm aster is formed and moves through the egg to fuse with the egg nucleus. It was in the egg of the starfish that Fol (1879) first observed the actual penetration of an egg by a spermatozoon. See the papers of Chambers (1930) and Colwin and Colwin (1956) for comments and additional observations.

The first two cleavages are meridional; they go through the animal and vegetative poles at right angles 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 occurs, and from this stage on, cleavage is irregular. Throughout the early cleavages the blastomeres exhibit a tendency to assume a spherical shape, resulting in a rather loose arrangement of cells. The perivitelline space is wider and the hyaline plasma membrane is thinner and weaker than in the Arbacia egg. These two conditions account, in part, for the loose connection between the blastomeres. Chambers has pointed out that in the absence of the fertilization membrane, the blastomeres tend to separate completely.

Eventually the cells become arranged in an epithelial wall enclosing the blastocoel. The surface cells acquire cilia and the blastula begins to rotate within the fertilization membrane. The two polar bodies are still visible, attached to the animal pole or lying loose within the perivitelline space. The embryo hatches in the late blastula stage.

C. Developmental Rate: No precise information is available. This is apparently due to the fact that few workers have obtained egg-batches showing uniform
spermatozoon and the nucleus of the egg, they form the zygote. According to Chambers and Chambers (1949), for example, the time of cleavage depends upon the exact time when the eggs are inseminated during the maturation stages. Their data indicate that if one waits until the egg is almost fully mature (i.e., shortly before the second polar body is to be extruded) before inseminating, the eggs cleave about 103.5 minutes later, at 36°C. Unfertilized Asterias eggs undergo maturation changes at 16-18°C, according to Chambers and Chambers (1949), as follows; the time is recorded from the moment of deposition of the eggs in seawater:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disappearance of nucleolus</td>
<td>8-9 minutes</td>
</tr>
<tr>
<td>Formation of first polar body</td>
<td>76-90 minutes</td>
</tr>
<tr>
<td>Formation of second polar body</td>
<td>105-119 minutes</td>
</tr>
</tbody>
</table>

D. Later Stages of Development and Metamorphosis:
Gastrulation: 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 and gradually becomes pear-shaped. The blind inner end of the archenteron becomes thin-walled and expands, and from it mesenchyme cells wander into the blastocoel. In a slightly later gastrula, two out-pocketings of the distal end of the archenteron can be seen; they are the primordia of the coelomic sacs. While they are forming, the first sign of the change from radial to bilateral symmetry may be seen, namely, the bending of the ciliated archenteron toward the future ventral side of the embryo.

Dipleurula larva: By the time this larva is fully formed, the blind end of the archenteron has made contact with an ectodermal depression, the stomodeum, on the ventral body wall and has broken through to form the mouth. Overhanging this is the oral lobe. The ventral side of the dipleurula is convex in shape. The entire surface is finely ciliated, and in addition there is a continuous ciliary band which is longitudinal with two cross-bars. The longitudinal bands above the upper cross-bar loop toward the mid-line where they eventually meet. Thus a frontal field, the pre-oral ciliary band, is separated on the upper ventral part of the larva, overhanging the oral field. This separate frontal field is characteristic of asteroid larvae. The alimentary tract consists of three parts, characteristic of echinoderm larvae: oesophagus (with a constriction near the entrance to the stomach), stomach, and intestine. In lateral views, the bend of the intestine can be seen. Ciliation occurs in the oral field and certain other parts of the tract. The two coelomic vesicles are clearly visible at the lower end of the oesophagus, near its entrance into the stomach. A subdivision of the vesicles is not yet clearly demarcated, but the narrow tube connecting the larger left coelomic vesicle with the dorsal body wall, the pore canal, and its opening, the madreporic pore, can be readily seen. Loose mesenchyme cells are scattered in the body cavity, which is the persisting blastocoel.

The dipleurula (early bipinnaria) larva represents an early larval type common to Asteroidea, Echinoidea, Ophiuroidea, and Holothuroidea (see the book of Korschelt and Heider, 1936, vol. 1, p. 499).

Bipinnaria larva: 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 along the
ciliary band. They are not supported by a skeleton, and young stages may not have all the arms developed. Pairs of arms follow each other in quick succession; unpaired median dorsal, and paired antero-dorsal, postero-dorsal, postero-lateral, post-oral and pre-oral arms can be identified. The coelomic vesicles grow out into long tubes and fuse in the anterior part of the larva. No further subdivisions have yet occurred.

Brachiolaria larva: Unlike the bipinnaria arms, which are long hollow tubes, those of the brachiolaria, the brachia, are short, and contain diverticula of the coelom. They are not ciliated but bear small papillae, differentiated from their end discs; they can adhere to the substrate. A sucker, the giant cells of which secrete a sticky substance, is formed between the brachia. The developing disc, the future young starfish, can be seen.

Metamorphosis: In late stages of metamorphosis, the anterior part of the brachiolaria, in front of the disc, shrinks to form a stalk. This attaches firmly to the substrate by means of the sucker and brachia, and bears the Asterias anlage at its distal end. About one week is required for the young starfish to complete development, rupture the stalk, and crawl away.

For further details of these stages consult papers by Agassiz (1877), Gemmill (1914) and the text-book of MacBride (1914), all of which contain figures.

REFERENCES:

ECHINODERMATA

(ASTEROIDEA)

Henricia (formerly Cribrella) sanguinolenta

LIVING MATERIAL:

This small red starfish is not abundant at Woods Hole, Mass., although some can be obtained by dredging at Lackey's Bay. The sexes are separate and similar in appearance.

BREEDING SEASON:

The species is said to breed in the early spring (Clark, 1902). Mead (1898) noted that eggs were frequently laid in aquaria at Woods Hole during the third week in April. Larvae were obtained in tows during the month of May (Bumpus, 1898), and motile sperm are found as late as June.

At St. Andrews, Scotland, the breeding period extends from the beginning of February to the end of April (Masterman, 1902).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Adults may be kept in aquaria or large fingerbowls, supplied with running sea water.

B. Procuring Gametes: It is not known whether artificial fertilization is successful in this species. Gametes can be obtained as follows: Cut off an arm along with a pie-shaped piece of the adjacent disc and, with forceps, remove the gonads (pale peach testis or orange-brown ovaries) from the base of the arm. Place them in a small dish of sea water, and allow the gametes to ooze out.

C. Preparation of Cultures: In normal development, the fertilized eggs are protected beneath the body of the female (Coe, 1912), where a brood-chamber is formed, roofed by the raised disc and enclosed by the bases of the arms. The embryos usually remain therein throughout their larval life, and during this period the female does not feed. However, if she is disturbed she will desert the brood permanently, and within a short time resume feeding. Meanwhile, the embryos develop quite normally.

Eggs and assorted larvae can be washed out of the brood-chamber with a pipette. They may be kept in fingerbowls, to which the more advanced larvae will attach. The sea water should be changed frequently, but no special feeding is necessary since there is an adequate supply of yolk.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature eggs are deep orange in color, and are opaque, due to the presence of a large amount of colored yolk. They are large and spherical in shape, and show some variation in size (800–1200 microns); the majority measure 1000 microns in diameter (Mead, 1898; Masterman, 1902).

B. Fertilization and Cleavage: Fertilization in this species has not been described. There is no definite pattern of segmentation, although the type most
frequently observed is holoblastic and unequal. In all cases the egg is transformed gradually into a solid morula, and then, by outward migration of cells, into a blastula with a large blastocoele. The forming blastula may be recognized by a number of transitory indented furrows which form on the surface. Gastrulation is by invagination. See the paper of Masterman (1902) for further details and illustrations.

C. Rate of Development: This is apparently rather slow. The two-cell stage is reached in 6 hours (Mead, 1898); the larvae hatch in 8 to 10 days, and 6 to 10 days later begin their somewhat slow transformation into the adult form (Masterman, 1902).

D. Later Stages of Development: The hatching larvae are demersal, uniformly ciliated, and nearly spherical in shape. They rapidly elongate to a somewhat barrel-shaped form, and develop a dorsal, tripod-like process. The larval stages have neither arms nor mouth. The orange-colored larvae swim about rather slowly for a few days, and then attach by means of the pre-oral process. The larval body, with the exception of this process (which is reduced), is gradually molded into the pentagonal form of the adult. Illustrations of the larval stages may be found in a paper by Masterman (1902).

REFERENCES:
BUMPUS, H. C., 1898. The breeding of animals at Woods Holl during the month of May, 1898. Science, 8: 58-61.
MEAD, A. D., 1898. The breeding of animals at Woods Holl during the month of April, 1898. Science, 7: 702-704.
ECHINODERMATA

(OPHIUROIDEA)

Amphipholis (formerly Amphiura) squamata

LIVING MATERIAL:

This small brittle star is obtained by dredging at the head of Lagoon Pond, Martha’s Vineyard, and at Provincetown, Mass., although it is not available in large numbers. It is found with Ophioderma brevispina. Amphipholis is hermaphroditic and viviparous, and is probably self-fertile (Fewkes, 1887).

BREEDING SEASON:

In New England, mature eggs and young in various stages of development have been obtained during the middle of the summer and on until the end of September (Fewkes, 1887; Coe, 1912). Animals obtained in the British Isles and New Zealand indicate that there may be a prolonged breeding period, which possibly extends throughout the year. At Plymouth, England, they are reported to breed from May to September (Garstang, 1931), but specimens collected in February, during an exceptionally cold winter, carried embryos of various stages (Fell, 1946).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals may be kept in aquaria or petri dishes, supplied with fresh sea water which should be inoculated at intervals with diatoms, such as Skeletonema (Fell, 1946).

B. Procuring Gametes: The ova are produced singly, and are liberated directly into the bursae where they are fertilized. Attempts at artificial fertilization have been unsuccessful.

C. Preparation of Cultures: Embryos in various stages of development can be removed from the bursae or brood-pouches of a mature adult, using the method described by Fell (1946): The animal is anaesthetized using a 2½-5% solution of menthol in sterile sea water. With fine tenotomy scalpel and forceps, the disc, with bursae attached, is separated from the arms and mouth skeleton and turned oral side upward. The free-swimming embryos are disentangled from the membranous walls by directing a stream of water from a hypodermic syringe into the bursae. The younger, attached embryos are excised with fine scissors.

The embryos should be pipetted through several washings of sterilized sea water, and then isolated in five-cm. watch glasses containing two to three ml. of “Erdschreiber” medium (Gross, 1937), which provides certain vital nutrient substances. The watch glass is set in a larger petri dish, together with a sterile swab of wet cotton wool. The whole set-up is placed on a water table in order to keep the temperature moderately constant. The medium should be renewed every fourth day. The embryos are very susceptible to bacterial toxins, but by using this method they may be kept for several weeks and will differentiate normally.

D. Methods of Observation: Polarized light is recommended for studying the developing skeleton in these rather opaque embryos.
AMPHIPHOLIS

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature ovum is orange-red in color, and measures approximately 100 microns in diameter (Fell, 1946). It is enclosed within a thin membrane, which is closely associated with the underlying protoplasm. The egg is opaque, due to the presence of yolk, and is shed at the germinal vesicle stage.

B. Fertilization and Cleavage: Little is known about fertilization and cleavage in this species. Two polar bodies are extruded and are retained within the fertilization membrane. The early cleavages are presumably total and regular. A modified form of invagination produces a gastrula with a reduced blastopore and no true archenteron. The pigmentation becomes restricted to the cells giving rise to endoderm (Fell, 1946).

C. Developmental Rate: No details are available.

D. Later Stages of Development and Metamorphosis: The young embryo becomes attached soon after hatching to the wall of the bursa. It is a vestigial, bilaterally symmetrical and oval-shaped pluteus, which corresponds approximately to the central portion of a normal pelagic ophiopluteus. A reduced calcareous skeleton, oesophagus, stomach and intestine are present, but anus and mouth openings are lacking. The embryos are very opaque and contain a cluster of orange pigment near the "anal" pole. The primitive gut degenerates as the growing embryos are converted to a more transparent, pentagonal form. At this stage, the attachments atrophy, and the embryos break off and lie free in the cavities of the bursae. Here they complete their metamorphosis. The young animals crawl out of the bursae when they have attained the adult form. Figures of these stages are to be found in papers by Fewkes (1887), MacBride (1893), Metschnikoff (1869), and Fell (1946).

REFERENCES:


ECHINODERMATA

(OPHIUROIDEA)

Ophioderma (formerly Ophiura) brevispina

LIVING MATERIAL:

These brittle stars are common in Lagoon Pond on Martha's Vineyard, Mass., where they are dredged from sandy bottoms among living and dead grasses and algae. The sexes are separate, and are similar in appearance.

BREEDING SEASON:

Grave (1916) states that the breeding season at Beaufort, N. C., is in June and July. At Woods Hole, Mass., observations indicate that spawning occurs during July and at least the first part of August.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals should be kept in aquaria provided with a constant supply of sea water. They do not need to be fed, and will remain in good breeding condition for a considerable period of time. Fertile eggs, which developed normally through metamorphosis, have been obtained from Ophioderma after a month in a laboratory aquarium. During this period the same animals had previously produced several batches of eggs.

B. Procuring Gametes: Artificial insemination has not proved successful in this species, but naturally-shed eggs may be readily obtained during the evening, provided that the animals are in breeding condition. Grave (1916) states that the males begin to spawn about 8 P.M., and it has since been observed that this process of spawning may continue until midnight.

The animals should be removed at sunset from the aquarium to a fingerbowl containing sea water, placed near a window. The males will begin to shed sperm; the presence of sperm in the water seems to induce the females to release their eggs. When the sperm concentration is very dense, a great number of immature eggs are shed along with the mature ones (Grave, 1916).

C. Preparation of Cultures: It is important to transfer the eggs to fingerbowls of fresh sea water as soon as possible after shedding, as the dense sperm suspension tends to induce abnormal development. Since the eggs are relatively large, the transfer is easily accomplished with the aid of a pipette. Both eggs and young larvae should be removed to fresh sea water at least twice a day. The cultures should be covered and kept on a water table.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is pelagic and floats to the surface as soon as it is released. It is very opaque, due to the presence of yolk. The color may vary from yellow to dark green, but within a single batch of eggs it is uniform. The average diameter of this egg is approximately 300 microns (Grave, 1916). The egg is probably shed after the formation of the polar bodies.
B. Fertilization and Cleavage: The eggs are fertilized as they are shed into the water. Cleavage is total and equal, closely resembling that of Asterias. A thick-walled hollow blastula is formed. Gastrulation is by invagination.

C. Rate of Development: At a laboratory temperature of 24° C., the early cleavages occur at half-hour intervals. A rotating blastula is formed in 10 to 12 hours, and a gastrula 18 to 20 hours after fertilization. At 48 hours, the rudiments of the arms are visible. At 96 hours, the first tube feet appear as small protuberances, and at 120 hours, some of the larvae are able to crawl along the bottom.

D. Later Stages of Development: The young, yolky, larvae are cone-shaped with a flattened blastoporic pole at the time of gastrulation. They are completely covered with cilia which enable them to move slowly through the water. As they gradually elongate, the blastopore is displaced ventrally and the stomodeal depression becomes visible. Two lateral thickenings next appear, just below the equator of the embryo. The blastopore now closes, and the cilia become concentrated in the form of four bands. Five groups of elevations soon appear about the stomodeum, on the ventral surface of the flattened oral disc. Each group consists of three elevations, the rudiments of the end tentacle and the first two tube feet of each arm. In the course of further development the arms elongate, additional tube feet are added, the ciliated bands are raised on ridges, and the old "anterior" pole of the larva degenerates. For diagrams of the larvae and for a description of the internal development, see the paper by Brooks and Grave (1899).

REFERENCES:


ECHINODERMATA

(OPHIUROIDEA)

Ophiopholis aculeata

LIVING MATERIAL:

Ripe specimens of these brittle stars are usually not available at Woods Hole, Mass., but they may be dredged from the colder waters to the north. Bumpus (1898) received large numbers of them in good condition, shipped in artificially cooled containers from Nahant, Mass., to Woods Hole.

The sexes are separate. During the breeding season the gonads can be seen through the skin on the underside of the disc. The ovaries are yellowish-red and the testes white.

BREEDING SEASON:

Fewkes (1886) stated that animals at Eastport, Maine, breed in summer until mid-August. At Nahant, in 1898, ripe animals were obtained from mid-May to the end of June (Bumpus, 1898).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The adults may be stored in aquaria in a cool place. It is advisable to obtain fresh material quite often.

B. Procuring Gametes: Ripe animals will usually spawn about 15 to 30 minutes after they are placed in sea water at room temperature. The males usually, but not always, spawn first. Eggs may be obtained by removing the ovaries, cutting them in two, and allowing the eggs to roll out. Eggs which tend to cling together instead of rolling out are immature and should be discarded. The best way to obtain ripe eggs is to isolate a shedding female in a small container, first rinsing the animal in tap water. The eggs are shed within a short time, and it is probable that all which are ripe are released (Olsen, 1942).

C. Preparation of Cultures: The eggs should be transferred to a fingerbowl of fresh sea water, where they may be inseminated by adding a small amount of sperm suspension. All the eggs are fertilized, and should receive a change of sea water after a short time. They need little care until after hatching. Olsen (1942) gives methods for feeding the larvae with cultured phytoplankton.

D. Methods of Observation: The study of the developing skeleton of these embryos is greatly facilitated by the use of polarized light.

E. Removal of Membranes: The fertilization membrane can be removed with the aid of a pipette. This is most easily accomplished immediately after its formation. Development of the denuded egg proceeds normally.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature egg is spherical in shape and measures approximately 105 microns in diameter (Olsen, 1942). It is surrounded by a gelatinous capsule, which disappears about 10 minutes after fertilization. Fewkes
OPHIOPHOLIS

(1886) described the egg as faintly green in color; the Norwegian variety is reportedly reddish-yellow or yellowish-brown (Olsen, 1942). The ovum is shed after the breakdown of the germinal vesicle.

B. Fertilization and Cleavage: About one minute after fertilization, the fertilization membrane begins to elevate, leaving a small perivitelline space. The diameter across the fertilization membrane is approximately 130 microns. A transparent hyaline layer, consisting of two parts, can now be seen surrounding the yolky portion of the egg. The outer part, which is as clear as glass, remains on the egg surface throughout cleavage, while the inner, with dark radial stripes or shadows, sinks between the blastomeres during cleavage and surrounds them completely (Olsen, 1942). Soon after fertilization, the egg becomes irregular in shape, and remains thus until after the first cleavage. Meanwhile, two polar bodies are formed. They are retained for some time under the fertilization membrane, but not in any fixed position.

The cleavage pattern is usually irregular (Olsen, 1942). An ovoid, ciliated blastula is formed. It hatches and rotates slowly to the surface. Gastrulation is by invagination. The two portions of the hyaline layer can be followed through to late larval stages. For figures of cleavage and gastrulation, consult the papers by Fewkes (1886) and Olsen (1942).

C. Time Table of Development: Olsen (1942) reared the Norwegian form of this species in sea water at 8°C, and recorded the following times from fertilization for the early stages of development:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cleavage</td>
<td>3 hours</td>
</tr>
<tr>
<td>Second cleavage</td>
<td>5 hours</td>
</tr>
<tr>
<td>Third cleavage</td>
<td>5 hours, 45 minutes</td>
</tr>
<tr>
<td>Fourth cleavage</td>
<td>6 hours, 30 minutes</td>
</tr>
<tr>
<td>Fifth cleavage</td>
<td>7 hours, 30 minutes</td>
</tr>
<tr>
<td>Hatching (blastula)</td>
<td>24 hours</td>
</tr>
<tr>
<td>Gastrulation</td>
<td>27 to 48 hours</td>
</tr>
<tr>
<td>Young pluteus</td>
<td>3 to 4 days</td>
</tr>
<tr>
<td>Full-grown pluteus</td>
<td>about 30 days</td>
</tr>
</tbody>
</table>

These times agree with the few observations of the New England species recorded by Fewkes (1886); however, it is unlikely that his animals were reared at such a low temperature—none is indicated.

D. Later Stages of Development and Metamorphosis: The larval gastrula transforms into a young pluteus, with a calcareous skeleton and rudimentary arms. By the fourth day the alimentary tract and primitive coelom are formed, and the arms have increased in size. Older larvae (about 17 days) are characterized by the development of a fourth pair of ciliated arms, and the exceptional length of the postero-lateral pair. The developing hydrocoele may be seen after 20 days, and by 30 days the pluteus is full grown. Metamorphosis begins with the formation of the adult skeleton, and has been described in detail by Olsen (1942). Figures of these stages may be found in papers by Fewkes (1886, 1887) and Olsen (1942).
REFERENCES:
Bumpus, H. C., 1898. The breeding of animals at Woods Holl during the months of June, July and August. Science, 8: 856-858.
ECHINODERMATA

(ECHINOIDEA)

Arbacia punctulata

LIVING MATERIAL:

Although it is possible in some species of echinoids to distinguish the sexes by external characteristics (see the papers by Marx, 1932, and Harvey, 1956b), no differentiating characteristics have, as yet, been described for the American sea urchin, Arbacia punctulata. The sexes are readily identified after the animals are opened, by the deep red or purple ovaries and the white testes, or, if unopened animals shed spontaneously, by the red eggs and the white sperm. Hermaphroditic animals are occasionally found (Boolootian and Moore, 1956).

At Woods Hole, Mass., the animals are obtained by dredging; they are, at the present time, rather scarce.

An exhaustive account of the life-history, embryology and metamorphosis of Arbacia is given by Harvey (1956a).

BREEDING SEASON:

From the middle of June until the middle of August (Harvey, 1956a), although the season may vary somewhat from year to year.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: In the laboratory, animals can be kept in aquaria provided with running sea water; they should not be crowded.

B. Procuring Gametes: The following methods may be used, (4) or (5) being preferable because of the present scarcity of Arbacia at Woods Hole:

(1) Cut around the peristome (on the oral surface) and remove the Aristotle's lantern, taking care not to injure the gonads. The perivisceral fluid should then be drained from the body and the animal, aboral surface down, placed to shed on a Syracuse watch glass which has been moistened with sea water. After each animal is opened, the hands of the investigator and all instruments used should be washed with running fresh water, to avoid contamination of the gametes of one sex with those of the other, or with body fluids. If the eggs are shed through the gonopores of the female into the Syracuse dish, they should be transferred within ten minutes to a fingerbowl containing 200 cc. of sea water. The sperm are best kept "dry," just as they exude from the testes.

(2) Cut around the test, about halfway between the mouth and the equator, and proceed as in (1) above. Shedding is more frequently obtained by this method, but there is also more likelihood of cutting the gonads, and of contamination with the perivisceral fluid.

(3) Cut as in (2) above, pour out the body fluid and remove the gonads (at the gonoduct end) with blunt forceps, spatula or spoon. The ovaries should be placed in 200 cc. of sea water in a fingerbowl, and allowed to shed. If undisturbed, the eggs are extruded in compact clumps or strings, without ovarian tissue,

184
and may be removed to a fresh dish by means of a wide-mouth pipette. If large quantities of eggs are desired, the ovaries should be allowed to shed for about 30 minutes, with occasional stirring; then pour them gently through cheesecloth (which has previously been washed in fresh water and soaked in filtered sea water) or bolting silk.

(4) Palmer (1937) induced spawning in Arbacia by injecting isotonic (0.53 M) KCl into the perivisceral cavity, and Tyler (1949) described a similar method, utilizing 0.5 cc. of 0.5 M KCl. Ripe animals begin to shed in a few minutes; the eggs can be collected by inverting the female in a dish of sea water, or by washing them gently from the surface of the test with a pipette. The sperm should be collected "dry." This method has the advantage that one is enabled to sex animals without sacrificing them, but there is some evidence (Harvey, 1956b) that eggs obtained in this manner do not develop normally. The animals may be returned to an aquarium, and will produce another batch of gametes in about three weeks, if fed periodically.

(5) Harvey (1953) has reported an electrical method (similar to a technique devised by Iwata, 1950, for Japanese sea urchins) for sexing Arbacia and inducing shedding. An alternating current of 10 volts (reduced by a transformer from ordinary 60-cycle, 110-volt current) is applied, using lead electrodes, to any two points on the test of the animal, which is placed, aboral side up, in a dish and covered with sea water. Almost immediately after the current is passed, the gametes will be extruded from each of the gonopores; when the current is stopped, the shedding ceases, to be resumed when the current is again applied. The gametes should be removed and used at once. Harvey points out that this method is of great value in laboratories where sea urchins have become scarce, since only the quantity of gametes desired is obtained and the animals need not be sacrificed.

C. Preparing Cultures: Two drops (0.2 cc.) of "dry" sperm may be diluted with 10 cc. of sea water (Just, 1939) in a watch glass, just before insemination; do not use sperm which have been diluted more than 20 minutes, and avoid a high sperm concentration, which leads to polyspermy and abnormal cleavage. Two drops of diluted sperm are sufficient for a fingerbowl of eggs. Stir the dish immediately.

NORMAL DEVELOPMENT:

A. The Sperm: After dilution with sea water, the sperm become intensely active, although they are immobile in concentrated suspension due, presumably, to the effects of high concentrations of carbon dioxide. Concomitantly, their ability to fertilize eggs is lost more rapidly in dilute than in concentrated suspensions. (See the papers by Lilie, 1915; Cohn, 1918; Hayashi, 1945.) "Dry" sperm kept in the cold (2° C.) may remain usable for several days; at room temperatures, dilute sperm suspensions often lose their fertilizing power in an hour or less.

The spermatozoon consists of three parts: head, middle piece and tail. These are 3.25 microns, 0.75 micron and 45 microns in length, respectively (Harvey and Anderson, 1943). The fibrilar axial filament of the tail protrudes a short distance beyond the end of the sheath.

B. The Unfertilized Ovum: A good batch of eggs from a ripe female should show uniformity of size, perfectly spherical form and complete absence of immature
eggs (which are in the germinal vesicle stage). Both meiotic divisions are completed while the eggs are still in the ovary, and the polar bodies very seldom remain attached when the eggs are shed (Hoadley, 1934). Occasionally (especially from relatively unripe animals, or after maceration of the ovaries), eggs may be found that are in the germinal vesicle stage; this is recognizable in the living state by the large clear nucleus (as opposed to the small nucleus of the ripe egg) and nucleolus. Such eggs may exhibit some surface response to sperm (including the formation of "papillae"), but they do not develop after insemination. The ripe egg, 72 to 75 microns in diameter, has a small, clear nucleus; it contains uniformly-dispersed, pale yolk granules, and slightly larger red granules containing echinochrome pigment, which is a substituted naphthoquinone related to the K vitamins (Ball, 1936; Hartmann et al., 1939; Tyler, 1939). The nucleus is usually eccentric in location. Since the polar bodies are not ordinarily 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 still attached, and in these, observations by 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 of the egg (about 30 microns wide), there is a funnel-shaped space which usually 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, because the "micropyle" (funnel) may disappear as the jelly swells after the eggs are shed into sea water. See the diagrams by Harvey and Dan (Harvey, 1956a, p. 84) of the membranes and layers of the fertilized, as compared with the unfertilized, egg.

C. Fertilization: Sperm penetration occurs very rapidly (apparently at any point on the egg), and it is usually difficult to study. Within a few seconds after insemination, the cortical responses of the egg begin; the vitelline membrane starts to elevate rapidly from the egg surface (in about five seconds), leaving a perivitelline space. This membrane hardens and thickens 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 formed about ten minutes after fertilization: the hyaline plasma membrane, which is apparently a calcium-proteinate, acting as a cement to hold the blastomeres together after cleavage. It disappears when the eggs are placed in calcium-free sea water. After insemination, the jelly-layer is often clearly delimited by the supernumerary sperm trapped near its surface. Moser (1939, 1940) 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 centrifugation (as are the granules of the underlying fluid endoplasm). Runnström et al. (1944) state that the cortical granules contribute to the formation of the fertilization membrane.

D. Cleavage: About 15 minutes after insemination (at 20° C.) a sperm aster is visible as a spherical region containing clear rays which extend from a clear center; this configuration attains its maximum development 20 to 30 minutes after insemination. Then a clear streak appears in the egg, slightly above the equator, and 45 to 50 minutes after insemination, it is replaced by two clear areas, the
asters of the first cleavage spindle. The first three cleavages divide the egg into eight blastomeres of equal size. The planes of the first two cleavages are meridional (in the polar axis), while that of the third is equatorial or horizontal (at right angles to the polar axis). The progress of the cleavage furrows in dividing eggs can be followed; the hyaline layer forms the surface of the furrow and later, when the cells flatten against one another, it forms the boundary between them. At the fourth cleavage, the four upper cells divide meridionally, forming eight equal mesomeres, while the lower four cells divide unequally and horizontally, to form four large macromeres; below them, at the vegetal pole, are four small, clear micromeres.

At the fifth division, the eight mesomeres divide equally and horizontally, forming two tiers of cells termed an1 (at the animal pole) and an2 (see the paper by Hörstadius, 1939), while the macromeres and micromeres divide meridionally. At the sixth cleavage, the an1 and an2 cells divide in a more or less radial direction, while the macromeres divide horizontally to form the veg, and veg, tiers of cells. Veg, cells are next to the micromeres, which have also divided at this time but which do not form distinct layers. Tiers of cells are not readily distinguished in later cleavage stages, and no special cell-layer designation is used after the 64-cell stage. It has been shown by Hörstadius (1939) that the an1, an2, and veg, cells form the ectoderm, the veg, cells form endoderm (gut) and part of the mesoderm (coelom), while the micromeres form the mesodermal skeleton components.

E. Time Table of Development: The following schedules of development have been observed at temperatures of $23^\circ$ C. (data from Harvey, 1956a) and $20^\circ$ C.; the time is recorded from insemination.

<table>
<thead>
<tr>
<th>Stage</th>
<th>$20^\circ$ C. Time</th>
<th>$23^\circ$ C. Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formation of fertilization membrane</td>
<td>5 minutes</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Formation of hyaline layer</td>
<td>10 minutes</td>
<td>2 minutes</td>
</tr>
<tr>
<td>Sperm aster appears</td>
<td>15 minutes</td>
<td>8 minutes</td>
</tr>
<tr>
<td>&quot;Streak&quot; stage</td>
<td>35 minutes</td>
<td>20–35 minutes</td>
</tr>
<tr>
<td>Cleavage asters</td>
<td>45–50 minutes</td>
<td>35 minutes</td>
</tr>
<tr>
<td>First cleavage</td>
<td>56–67 minutes</td>
<td>50 minutes</td>
</tr>
<tr>
<td>Second cleavage</td>
<td>107 minutes</td>
<td>78 minutes</td>
</tr>
<tr>
<td>Third cleavage</td>
<td>145 minutes</td>
<td>103 minutes</td>
</tr>
<tr>
<td>Blastula</td>
<td>6 hours</td>
<td>7–8 hours</td>
</tr>
<tr>
<td>Hatching of blastula</td>
<td>10 hours</td>
<td>12–15 hours</td>
</tr>
<tr>
<td>Gastrula</td>
<td>20 hours</td>
<td>24 hours</td>
</tr>
<tr>
<td>Pluteus</td>
<td>48 hours</td>
<td></td>
</tr>
<tr>
<td>Metamorphosis</td>
<td>2 weeks</td>
<td></td>
</tr>
</tbody>
</table>

Different batches of eggs vary slightly (1–2%) in average cleavage times; within a batch, most eggs will develop at the average rate, but some may vary by about 10%. Temperatures above $30–32^\circ$ C. are lethal for Arbacia eggs.

F. Later Stages of Development: At the eight-cell stage, there is a very small central cavity which enlarges as cleavage continues, to form the blastocoele. About six hours after fertilization, a smooth-surfaced, spherical young blastula is formed.
the wall of which is one cell in thickness. Cilia soon develop on the surface, and the blastula is rotated by their action within the fertilization membrane. The blastula hatches out of the fertilization membrane in about ten hours. It has been shown that the blastula releases a "hatching enzyme" at this time, which weakens and dissolves the membrane so that the blastula can break through. A tuft of long cilia develops at the animal pole of the blastula, which is the forward end when the embryo 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 blastocoele, forming the rudimentary mesenchyme which gives rise to the skeleton.

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 blastocoele in about five hours. The gastrula contains approximately one thousand 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, the coelom are budded off from the tip of the archenteron.

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 beginning of the skeleton. Where the tip of the archenteron touches the ectoderm, there is formed a depression which later acquires an opening into the archenteron to become the stomodeum. The archenteron becomes divided by two constrictions into oesophagus, stomach and intestine. The apical tuft disappears, a ciliated band surrounds the oral field, the embryo begins to elongate in the dorso-ventral axis, and the direction of swimming changes, so that the ventral side is forward.

After about 48 hours, the embryo enters the pluteus stage, which is fully developed by the end of the third day. The original apical plate grows out in a ventral direction, to form the oral lobe which includes the stomodeum and the 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 post-oral) 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). Each of the rods is made up of three or four parallel parts joined by cross-bars. Different species of sea urchins differ in this regard, so that 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 through oesophagus, stomach and intestine becomes J-shaped. The stomach expands, to become a spherical structure which fills a large part of the body of the pluteus; sphincter muscles connect it with the oesophagus and intestine. The two coelomic sacs extend postero-laterally from the oesophagus; the one on the left side becomes larger and later acquires a dorsal opening called the pore canal. The
right coelomic sac buds off cells to form the madreporic vesicle; but otherwise remains rudimentary. The left coelom undergoes extensive later development in the formation of structures of the adult sea urchin. These changes do not occur until the second week, when metamorphosis begins in properly fed larvae. The adult organs are built up in and around a structure called the "echinus rudiment," which is formed by the fusion of an invagination (the amniotic invagination) of the ectoderm on the left side with the mid-portion (hydrocoele) of the left coelom. The left side of the pluteus becomes, then, the future oral surface of the adult.

G. Special Methods of Observation: Dark-field illumination shows a bright, reddish "luminous" layer on the surface of the unfertilized egg. The luminosity diminishes and becomes paler after fertilization (Runnström, 1928; Ohman, 1945). The skeletal spicules and rods are best demonstrated by the use of a polarization microscope.

REFERENCES:

ARBACIA


ECHINODERMATA

(ECHINOIDEA)

Echinarachnius parma

LIVING MATERIAL:

The common sand dollar is found in isolated areas on sandy bottoms below low tide level, and is obtained in abundance by dredging at West Barnstable on Cape Cod Bay, Mass.

The sexes are separate, but it is impossible to distinguish the male from the female by superficial examination.

BREEDING SEASON:

The animals breed abundantly at Woods Hole, Mass, from at least the last week of March to the end of July; even in August, a limited amount of ripe gametes may be obtained (Bunouus, 1898a, 1898b; Mead, 1898).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The adults may be kept indefinitely in an aquarium provided with a layer of clean sand and a continuous supply of fresh sea water. They can be fed on kelp, mussels, and other marine plants and animals.

B. Procuring Gametes: Tyler's method (1949) for procuring gametes is to inject isosmotic KCl (0.5 M) with a hypodermic syringe into the body cavity of the sand dollar, inserting the needle (25-27 gauge, ½- to ¾-inch) through the mouth in a direction as nearly parallel as possible to the oral surface. A single injection of 0.5 cc. of 0.5 M KCl into an animal about 30 cc. in volume will induce shedding of virtually all the ripe eggs or sperm. For smaller animals, the dose should be proportionately smaller (0.5-0.2 cc.). Shedding starts within a few seconds and is completed in 5 to 15 minutes. Unripe animals usually fail to respond. The eggs can be collected readily by immersing the animal in a large fingerbowl containing about 250 cc. of sea water. The sperm can be removed "dry" or in concentrated suspension.

This method does not injure the animals. More gametes are matured, provided the animals are well fed, and successive sheds may be obtained at two-week intervals during the season. If only a small number of gametes is desired, a correspondingly small amount of KCl should be injected, and the animals may then be kept for further use.

The eggs of Dendraster excentricus, the common sand dollar of the Pacific Coast, may be obtained by this same method (Tyler, 1949). Their development is similar to that of Echinarachnius parma.

C. Preparation of Cultures: The eggs should be washed several times in sea water. To them add one drop of sperm suspension (one drop "dry" sperm in 10 cc. of sea water) and mix thoroughly. After about 10 minutes, when the eggs have settled, decant the sea water to remove the excess sperm, and add fresh sea water. Store the fingerbowls on the water table. The sea water should be
changed once a day. Normal development will not take place at temperatures above 24°C.

The larvae can be reared to metamorphosis if they are transferred to large battery jars of sea water and provided with some surface sand from a stock aquarium containing a diatom culture. No further care is necessary. Details of the method for diatom culture may be found in a short paper by Grave (1902).

D. Removal of Membrane: The fertilization membrane may be easily removed by shaking the eggs in a test-tube, half-full of sea water, a few seconds after the membrane has elevated. Echinarachnius eggs are fragile and will not stand as much shaking as those of Arbacia.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature egg of this species measures approximately 135 microns in diameter, and is surrounded by a thick jelly-layer in which are suspended fine red pigment granules. The diameter across the jelly varies from 220 to 250 microns (Fewkes, 1886). The egg itself, free of the jelly, is yellow due to its yolk content.

B. Fertilization and Cleavage: In a matter of seconds (7-22) after sperm penetration, the vitelline membrane begins to elevate in a wave from the entrance point of the sperm around the egg cortex (Just, 1919a). The resulting perivitelline space is quite large. Cleavage is usually equal and regular until the 8-cell stage (Fewkes, 1886). There is a thinner hyaline plasma layer than in the Arbacia egg, and less regularity of cleavage. However, the cleavage pattern is not markedly different from that of Arbacia. A hollow, ciliated blastula, which rotates slowly within the fertilization membrane, is produced. Gastrulation is by invagination.

C. Time Table of Development: No temperature was recorded for the following schedule of development observed by Fewkes (1886). The times are calculated from fertilization.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>First cleavage</td>
<td>1½ hours</td>
</tr>
<tr>
<td>Second cleavage</td>
<td>2 hours</td>
</tr>
<tr>
<td>Third cleavage</td>
<td>3 hours</td>
</tr>
<tr>
<td>Blastula</td>
<td>10 hours</td>
</tr>
</tbody>
</table>

D. Later Stages of Development and Metamorphosis: The early larva is nearly spherical in shape and rests on a tripod formed by the two posterior arms and the single anterior lobe. Pigment can be seen at the anal pole. A rudimentary skeleton, primitive alimentary tract, and mouth and anal openings are present. As the larva matures, it becomes more and more helmet-shaped. At four days the oral, or anterior, arms begin to form, and by the end of a week the antero-lateral and antero-internal arms have been added.

The body of the older pluteus is elongated, and rounded at the anal region. The four pairs of ciliated arms are of equal length; all except the antero-internal pair contain clusters of dark red pigment granules at the distal ends of the calcareous supporting rods. The pigmentation extends along the arms and over the body wall. The pluteus is propelled freely, but not rapidly, through the water.
The young sand dollar, which is very different in shape from the adult, is formed about the left hydrocoele of the pluteus, which is gradually absorbed. It is almost completely opaque, due to the formation of pigment, spines, calcareous rods and plates. The body is rounded and plump, and the spines are proportionately large when compared with those of the adult. Further details and illustrations of the larval development can be found in a paper by Fewkes (1886).

REFERENCES:


ECHINODERMATA
(HOLOTHUROIDEA)

Leptosynapta inhaerens and L. roseola

LIVING MATERIAL:
Both these species may be obtained readily by digging near low water mark at the Northwest Gutter or Sheep Pen Cove, near Woods Hole, Mass., at Lagoon Pond, on Martha's Vineyard, or at Rand's Harbor. *L. roseola*, which is small and rose-colored, is usually found under stones, and *L. inhaerens*, which is 10 to 20 cm. in length and white in color, is found burrowing in sand and occasionally in black mud. Both species are hermaphroditic, but neither is self-fertile. The American form of *Leptosynapta inhaerens* has been considered by some workers to be distinct from the European form with this name, and perhaps should be called *L. tenuis* (Ayres). *Leptosynapta roseola* is sometimes referred to as *Leptosynapta (Eptimapta) roseola* (Verrill).

BREEDING SEASON:
The height of the breeding season in the Woods Hole region is at the end of June and early in July (Clark, 1899), but mature gametes are probably available throughout both months (Bumpus, 1898; Coe, 1912).

PROCURING AND HANDLING MATERIAL:
A. Care of Adults: The animals are very sensitive to environmental conditions and will fragment if they are not handled with extreme care. They can be kept in aquaria provided with running sea water and a deep layer of clean sand.

B. Procuring Gametes: The gonads, in the form of long, contractile white strands, are visible through the transparent body wall during the breeding season. If the body wall overlying them is punctured, the gonads will ooze out. Dissection of the strings with fine needles releases the gametes. In some animals, the gonad strings will contain mature sperm and immature ova; in others, they are full of eggs and the sperm-bearing parts are inconspicuous.

Free-swimming larvae have been raised from artificially-inseminated eggs of *L. roseola* (Coe, 1912), but for *L. inhaerens* it is necessary to obtain naturally-shed ova. Spawning in the latter species begins between 3 and 4 o'clock in the afternoon and continues until 5 or 6 P.M. In the laboratory, it is most frequently observed on the first two days following collection, although it does occur occasionally after that (Runnstrom, 1927). The males shed first and continue for a longer period than do the females. Details of the natural spawning of *L. roseola* have not been recorded.

C. Preparation of Cultures: Artificially-obtained eggs of *L. roseola* may be inseminated by adding a few drops of sperm suspension to a dish of eggs. Mix well, allow the eggs to settle, and then decant the supernatant fluid. Add filtered sea water. Naturally-inseminated eggs of either species can be collected with a
pipette and transferred to a fingerbowl of filtered sea water. The cultures should be stored on a sea water table.

When free-swimming forms appear, pour the upper layers of the culture, containing them, into another dish. Fill the dish with fresh sea water. No feeding is necessary since the larvae develop through metamorphosis exclusively on their own yolk content.

**NORMAL DEVELOPMENT:**

_A. The Unfertilized Ovum:_ The egg of _L. inhaerens_ is very transparent and fragile. It is surrounded by a narrow coat of jelly. The ovum, on removal from the egg-string, is not quite spherical and measures between 190 and 209 microns in its longest diameter. The germinal vesicle is very large and contains a conspicuous nucleolus.

_B. Cleavage:_ Segmentation is total and equal in both species. It is reported to be very regular in _L. inhaerens_ (Coe, 1912); gastrulation is by invagination, and a free-swimming gastrula is produced (Runnström, 1927).

_C. Rate of Development:_ Little has been recorded concerning the rate of development for these species. However, the free-swimming gastrula of _L. inhaerens_ can be seen about 24 hours after fertilization.

_D. Later Stages of Development and Metamorphosis:_ Both species produce free-swimming larvae. The three-day larva of _L. inhaerens_ is barrel-shaped, with four distinct transverse bands of cilia. No auricularia larva is developed. The free-swimming life is short and the larva remains near the bottom. Illustrations of the larval development of _L. inhaerens_ may be found in a paper by Runnström (1927).

_E. Pre-Adults:_ Metamorphosed animals up to 5 cm in length may be collected at night, swimming near the surface in Eel Pond (Costello, 1946).

**REFERENCES:**

Bumpus, H. C., 1898. The breeding of animals at Woods Holl during the months of June, July and August. _Science_, 8: 850-858.


ECHINODERMATA

(HOLOTHUROIDEA)

Thyone briareus

LIVING MATERIAL:

This sea cucumber is abundant at Woods Hole, Mass. It lies almost completely buried in mud or sand in shallow water, and may be collected from many of the inlets in the vicinity. One recommended location is Bowen’s Pond, near Falmouth Heights; another is in the Gutters at Hadley Harbor.

The sexes are separate and similar in appearance. The genital papilla, found between the two dorsal tentacles, is reportedly larger in males, but this has proved too unreliable a characteristic for sex determination. The sex ratio is approximately five females to six males (Colwin, 1948).

BREEDING SEASON:

June and July, according to Bumpus (1898), but June and possibly earlier is suggested by Colwin (1948), who found that the capacity to spawn decreases markedly during the latter half of June. Mead (1898) observed that every female examined at the end of April was full of nearly ripe eggs, a fact which would certainly indicate a season beginning in May, but Just (1929) claimed that eggs obtained in April and May showed atypical responses to insemination. He believed they were unripe oocytes, capable of responding to insemination but unable to develop. Until this has been further investigated, Colwin (1948) recommends the first half of June for embryological experiments with this material.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Any medium- or large-sized animal should be a suitable source of gametes. It is advisable to store the animals overnight in containers supplied continuously with fresh sea water, at a temperature between 15° and 17° C.; the latter precaution discourages shedding, and the former ensures that much of the debris, which Thyone pumps through its gut, will be removed, thus facilitating the recovery of gametes. The animals should then be isolated in containers with enough sea water to permit them to expand fully—6 × 7½-inch battery jars are excellent. The sea water should be changed once or twice a day (Colwin, 1948).

B. Procuring Gametes: Although active sperm and apparently mature eggs can be teased from the gonadal lobes, artificial insemination of such eggs has not proved successful (Ohshima, 1925a; Just, 1939). This failure is perhaps explained by the fact that ovarian eggs contain a large germinal vesicle, while those shed normally are in metaphase of the first maturation division.

Spawning is preceded by full expansion and the so-called “feeding movements” of the tentacles. Ohshima (1925a, 1925b) noted that although shedding normally occurs late in the afternoon of the day of collection, it could be induced at any time by placing the animals in a dim light; it did not occur in darkness. However,
Colwin (1948) observed some normal shedding throughout the day and even in total darkness, although evening seemed to be the optimum time to obtain gametes. She discounts light intensity as a factor primarily involved in the shedding reaction. Any stimulus strong enough to cause a contraction of the animal will interrupt the process.

Colwin's method (1948) for obtaining naturally-shed gametes is to isolate the animals in sea water warmed to room temperature (20–22°C) for about five hours. Shedding may begin immediately or after an interval; it may not occur until after the sea water has been re-cooled (16–18°C). A period of warming is, however, apparently necessary for the occurrence of shedding in the laboratory. Other unknown factors may increase the frequency, but injections of KCl have no such effect. A concentrated sperm suspension is obtained by placing a shedding male into a fingerbowl containing only a very small amount of sea water. The adults are removed from their respective containers as soon as shedding ceases.

Colwin and Colwin (1956) have recently described another method for obtaining Thyone gametes, involving electrical stimulation of the adults. However, they state (p. 252) that they have not observed cleavage in inseminated cultures prepared from such gametes.

C. Preparation of Cultures: A small amount of sperm suspension should be added to the dish of eggs. After a short interval, the sea water should be changed, and the dish covered and placed on a water table. The sea water must be changed several times a day.

The larvae emerge in about three days. They may be successfully reared if placed in large battery jars of sea water, provided with some surface sand from a stock aquarium containing a diatom culture; then they may be left without further care. Details of the method for diatom culture may be found in a short paper by Grave (1902).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg is hemispherical in shape, measuring 260 to 300 microns in the equatorial plane, and 200 to 300 along the egg axis, and is enclosed in a thick, striated, jelly hull, approximately 55 microns wide (Colwin and Colwin, 1956). It is opaque and yellow-brown in color. The ovum is shed at metaphase of the first maturation division, although Colwin and Colwin (1956) point out that in living eggs, the germinal vesicle often appears to be intact, perhaps due to persistence of its "residual substance."

B. Fertilization and Cleavage: The eggs are fertilized as soon as they are shed into the water, the sperm most frequently entering near the equator. The polar bodies are soon formed and extruded, the egg rounding up during their formation (Ohshima, 1921a). An equal and somewhat modified form of radial cleavage was briefly noted by Ohshima (1925a). Gastrulation is by invagination, and appears to be similar to that of Cucumaria (Ohshima, 1921). Illustrations of the fertilization cone and polar body formation may be found in papers by Ohshima (1923b), and by Colwin and Colwin (1956).

C. Time Table of Development: The following schedule is taken from the paper of Ohshima (1925a). The time is calculated from egg-laying, the temperature not being indicated.
Stage | Time
---|---
First polar body | 20-30 minutes
Second polar body | 50-60 minutes
First cleavage | 120 minutes
Second cleavage | 150 minutes
Third cleavage | 180 minutes
Gastrula | 18-20 hours
Emergence | 3½ days

D. Later Stages of Development: The ciliated gastrula, with polar bodies still attached, enclosed, within its membrane, transforms into a diplopula, and then, just before emergence, into a metadoliolar larva. This type of development is similar to that of Holothuria floridana, described by Edwards (1909). The metadoliolar larva has a large, overhanging pre-oral hood, five unbranched tentacles and a pair of ventral pedicels. This form creeps out of the egg membrane. During the course of later development, the pre-oral hood is resorbed (pentactula stage), the tentacles branch and increase in number to ten, the ventral pedicels increase in number, and calcareous plates appear in the skin. There is no freeswimming stage (Ohshima, 1925a).

REFERENCES:
Bumpus, H. C., 1898. The breeding of animals at Woods Holl during the months of June, July and August. Science, 8: 850-858.
Mear, A. D., 1898. The breeding of animals at Woods Holl during the month of April, 1898. Science, 7: 702-704.