Costello, Donald P. and Catherine Henley (1971) *Methods for Obtaining and Handling Marine Eggs and Embryos.* Woods Hole, MA: Marine Biological Laboratory, 247 pp.

PROTOCHORDATA

(ENTEROPNEUSTA)

Saccoglossus (Synonyms: *Balanoglossus=Dolichoglossus*) *kowalevskii*

LIVING MATERIAL:

These worm-like animals are 15 cm. or more in length; the proboscis is pinkish-yellow in color and the body orange-yellow. They are collected from sand flats in the Woods Hole, Mass., area. The sexes are separate.

BREEDING SEASON:

No specific information on this subject has been recorded, for animals in the Woods Hole region. Presumably they are ripe during the latter half of July, and in August.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals may be kept in fingerbowls supplied with a layer of clean sand. A gentle stream of running sea water should be provided.

B. Procuring Gametes: Colwin and Colwin (1953) report that females will shed spontaneously in the laboratory, the number of eggs spawned ranging from a few dozen to more than a thousand; the usual number (depending somewhat on the size of the female) is 100–300. Sperm may be obtained by cutting into the testis of a ripe male and collecting the sperm, which ooze out, in a pipette.

C. Preparation of Cultures: Colwin and Colwin (1953) placed naturally-shed eggs in small glass dishes containing sand-filtered sea water; spermatozoa (obtained as described above) were added directly from the pipette in which they were collected (Colwin and Colwin, 1950). Several changes of fresh, sand-filtered sea water should be made following insemination, and at least two or three times daily thereafter. The egg of Saccoglossus is sensitive to a number of environmental factors, including temperature, and the Colwins routinely took precautions to assure that the temperature of the sea water did not rise above 25° C. The eggs should not be crowded. It is advisable to keep the culture dishes in moist-chambers which are surrounded by running sea water.

Pre-hatching larvae should be transferred to dishes containing small amounts of fine, clean sand. If this is not done, free-swimming larvae tend to become caught in the surface film of the water, and the older, crawling larvae may become trapped by the adhesion to glass surfaces of the mucus which they secrete. Feeding is apparently not necessary; Colwin and Colwin (1953) supplied older cultures with unfiltered sea water, which contains an adequate amount of food to supply the larvae for at least as long as 36 days.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The eggs are opaque, and vary in color from whitish-grey to dark grey-blue (Colwin and Colwin, 1953); they are rather irregular in shape before fertilization, and measure, on the average, about 330 microns by 420 microns. The germinal vesicle usually breaks down before the eggs are shed, and is at the metaphase of the first maturation division when it passes from the body of the female. The egg may remain fertilizable for a considerable period of time after shedding.

B. Fertilization and Cleavage: Fertilization takes place at the metaphase of the first maturation division, the sperm apparently entering at any point on the egg surface (Colwin and Colwin, 1953). The first polar body is given off about ten minutes after insemination, and the second 30 to 40 minutes later. The position of the polar bodies bears no very constant relation to the exact position of the animal pole. The details of fertilization (including the formation of the fertilization cone and membrane elevation) are described by Colwin and Colwin (1954a, 1954b).

After fertilization, the eggs become spherical in shape and are about 350 microns in diameter; soon after the first polar body appears, a broad, shallow "girdle" constricts the equatorial zone, and 15–30 minutes after insemination, a pear-shaped stage is attained, the vegetal hemisphere constituting the large, blunt end and the animal hemisphere the narrower portion. Shortly before the second polar body appears, the egg again becomes spherical, and after the second polar body is given off, a "reversed pear-shape" is evident, in which the animal hemisphere is the large, blunt end and the vegetal hemisphere is the more pointed one. Once again, the egg rounds up, and shortly before the first cleavage, there is a shortening of the animal-vegetal axis.

The first cleavage is usually approximately equal, the furrow passing from the animal to the vegetal pole; the same holds true for the second division. The third cleavage is latitudinal, resulting in an animal and a vegetal tier of cells; the size ratio of the two tiers, with respect to one another, is variable. At the fourth cleavage, the animal tier of cells divides somewhat sooner than the vegetal tier, so that a transitory 12-cell stage is present, followed shortly by a 16-cell stage after the vegetal cells divide. The eight animal cells are divided into a single tier composed of two more or less parallel rows, each consisting of two large central cells with a smaller cell at each end. These two long rows of cells represent the dorsal and ventral sides, respectively, of the embryo, and their two ends are the future left and right sides of the embryo (Colwin and Colwin, 1953). The vegetal cells at the 16-cell stage are arranged in an upper tier of four large cells and a lower tier of four small cells.

The early blastula is somewhat flattened at the vegetal end, but later it becomes more nearly spherical. Gastrulation is by invagination, and the embryo rotates actively before closure of the blastopore begins; cilia are present (as a transverse ciliated band) in a ring around the blastopore.

C. Time Table of Development: The following schedule is based on data from the paper by Colwin and Colwin (1953); temperatures were from 20° to 25° C., and the times were recorded from insemination.

Stage	Time
First polar body	10 minutes
"Equatorial girdle"	12–17 minutes
Pear-shape	15–20 minutes
Second polar body	40–50 minutes
"Reversed pear-shape"	40–55 minutes
Sphere-shape	50–70 minutes
First cleavage	1¾ to 2½ hours
Second cleavage	2½ to 3¼ hours
Third cleavage	3½ to 3¾ hours
Fourth cleavage	3¾ to 4½ hours
Blastula	6–15 hours
Gastrula	13–24 hours
Elongation of gastrula	18 hours
Appearance of first pair of gill slits	3 days
Hatching	7 days

D. Later Stages of Development and Metamorphosis: About 18 hours after insemination, the late gastrula begins to elongate in an antero-posterior direction. The division of the body into proboscis, trunk and collar regions is next accomplished; this process usually is complete 48 hours after insemination. The larva acquires pigmentation except in the area of the posterior collar groove. The first pair of gill slits appears when the pharyngeal pouches become perforated at three to four days, and two or three additional pairs subsequently develop. Emergence of the larva from its membranes occurs by the seventh day, and an adhesive sucker is developed. Further details, and photographs of many stages in the development of this form, are to be found in the paper by Colwin and Colwin (1953).

REFERENCES:

BATESON, W., 1884. The early stages in the development of Balanoglossus (sp. incert.). *Quart. J. Micr. Sci.*, **24**: 208–236.

BATESON, W., 1885. The later stages in the development of *Balanoglossus kowalevskii*, with a suggestion as to the affinities of the Enteropneusta. *Quart. J. Micr. Sci.*, **25**: Suppl. 81–122.

COLWIN, A. L., AND L. H. COLWIN, 1950. The developmental capacities of separated early blastomeres of an enteropneust, *Saccoglossus kowalevskii*. *J. Exp. Zool.*, **115**: 263–295.

COLWIN, A. L., AND L. H. COLWIN, 1951. Relationships between the egg and larva of *Saccoglossus kowalevskii* (Enteropneusta): Axes and planes; general prospective significance of the early blastomeres. *J. Exp. Zool.*, **117**: 111–137.

COLWIN, A. L., AND L. H. COLWIN, 1953. The normal embryology of *Saccoglossus kowalevskii* (Enteropneusta). *J. Morph.*, **92**: 401–453.

COLWIN, L. H., AND A. L. COLWIN, 1954a. Fertilization changes in the membranes and cortical granular layer of the egg of *Saccoglossus kowalevskii* (Enteropneusta). *J. Morph.*, **95**: 1–45.

COLWIN, L. H., AND A. L. COLWIN, 1954b. Sperm penetration and the fertilization cone in the egg of *Saccoglossus kowalevskii* (Enteropneusta). *J. Morph.*, **95**: 351–371.

PROTOCHORDATA

(TUNICATA)*

Amaroucium constellatum

LIVING MATERIAL:

The adult colonies of this species form abundant dense clumps on rocks and piles, and may be collected from Lagoon Pond Bridge at Martha's Vineyard, Mass. They are conspicuous because of the orange color of the ascidiozooids, which shows through the tunics. The animals are hermaphroditic and viviparous.

BREEDING SEASON:

Late June to early September. Maximum reproduction has been observed during July and August (Scott, 1945).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: When material is brought to the laboratory, the healthy, uninjured colonies should be placed, uncrowded, in large glass dishes with running sea water. At convenient intervals, the colonies should be inspected; healthy ones can be identified by their expanded oral and atrial siphons, and all others should be discarded. Such cultures may be kept in running sea water for days and, by daily removal of dead members, these "seasoned" cultures will produce abundant tadpoles for at least two weeks.

B. Procuring Gametes: Fertilization of ovarian eggs has not been successful in this species.

C. Preparation of Cultures: The eggs are retained in brood-spaces along the length of the ascidiozooid. Segmenting eggs are found in the posterior and lower portions of the abdomen, and tadpoles are packed into the thoracic region. With the aid of a microscope it is possible to dissect out eggs and larvae, but they may be obtained with greater ease by squeezing a mass of freshly collected adults over a fingerbowl containing a small amount of sea water. Many highly colored fragments will be ejected along with the embryos. Fill the dish with water and decant the coarse particles which whirl to the top. Tadpoles and eggs of all stages of development will be found on the bottom of the dish (Grave, 1921). Pre-gastrula stages will not develop outside the brood-spaces, but later embryos can be cultured in fingerbowls, provided the sea water is changed several times daily.

The larvae are normally released at dawn. Freshly collected, adult colonies should be placed in flowing sea water overnight, and transferred at daybreak to a container near a window. Shedding can be postponed to a more suitable hour if ripe colonies are kept in shrouded containers or in a dark room. Within 15–30 minutes after the time when these colonies are brought into the light, swarms of active tadpoles usually appear. Following this procedure at 9 A.M. yields about one-third of the available tadpoles; if it is delayed until mid-afternoon, the yield

* We are grateful to Sister Florence Marie Scott, for a review of this and the subsequent sections on tunicate development, and for much helpful information.

is approximately doubled. The tadpoles gather at the top of the water, near the side of the container which is exposed to the light. They may be collected with a pipette and isolated in separate drops of sea water in Syracuse dishes. When they have attached firmly to the dishes, they can be stored in an inverted position in wooden racks, which are submerged in an aquarium supplied continuously with fresh sea water.

D. Methods of Observation: Since the inner follicle cells divide and become closely packed within the perivitelline space, they obscure most of the developmental processes following the first few cleavages. The early embryos, therefore, are best studied in the form of sectioned material. Temporary whole mounts may be made, however, and these are very useful. The material is fixed in Bouin's fluid and preserved and mounted in 70% alcohol in depression slides; coverslips should be sealed on with vaseline, to prevent evaporation. The eggs may be rotated, bringing all surfaces into view, by moving the coverslip. Since the yolk granules are now stained yellow, the relationship between the yolky cells and those containing only clear protoplasm is readily observed (Scott, 1945).

Metamorphosing and budding individuals may be examined in the watch glasses to which they are attached. Debris should be flushed out with care, and the specimens kept covered with sea water. They may be flattened, if necessary, by gently lowering a coverslip on them. Asexual reproduction begins in cultures about 17 days after the attachment of the tadpole.

Scott (1952) describes a method for making fixed and stained Feulgen preparations of metamorphosing individuals.

NORMAL DEVELOPMENT:

A. Egg Characteristics: The eggs, in metaphase of the first maturation division, are shed into brood-spaces. Because of the pressure there of surrounding eggs and larvae, they are often polyhedral in shape. When fixed, the egg measures 250 microns in diameter; it has a chorion, and an outer and inner layer of follicle cells. The outer follicle cells are tightly pressed against the chorion. The inner follicle cells multiply, as the egg cleaves, and completely fill the wide perivitelline space which formed after it was shed; the egg of Amaroucium contains more yolk than any of the ascidians whose embryology has been studied, and is therefore opaque (Scott, 1945).

B. Fertilization and Cleavage: The first polar body is extruded at fertilization. The basic pattern of mosaic development is essentially the same for all ascidians, but in Amaroucium, owing to the greater accumulation of yolk, the processes of cleavage and gastrulation are somewhat modified. The first cleavage is unequal. The four cells produced by the second cleavage are listed here in order of increasing size: right posterior, right anterior, left posterior, and left anterior. In the third cleavage, the dense yolky material becomes concentrated in the macromeres. Gastrulation occurs between the sixth and seventh cleavages. It is accomplished chiefly by epiboly, but this is accompanied by an involution of the mesoderm and a pseudo-invagination of the endoderm without the formation of an open archenteron. The closing blastopore is definitely skewed, due to a rapid growth of the right lip. (For further details, see the paper of Scott, 1945.)

C. Rate of Development: Development is relatively slow, because of the large, inert yolk mass. However, no specific data pertaining to the rate of development are available, since pre-gastrula stages will not develop when removed from the parent.

D. Later Stages of Development and Metamorphosis: A free-swimming urodele-like tadpole is formed, with a relatively large trunk, measuring 600 microns in length, and a tail which is approximately twice as long. The lateral tail fins are well formed. Three cup-shaped adhesive papillae are visible. The tunic is transparent and glassy; embedded within it are a few scattered test cells. The sensory vesicle is conspicuous and the sense organs within it are well developed. The "eye" is a complex structure, consisting of sensory and pigment cells and a series of three lenses. There is a hypophysis, with its associated ganglia, and a nerve cord which extends into the tail and lies to the left of the notochord. The atria are fused posteriorly, connecting at this point with the atrial siphon. Four horizontal rows of gill slits (7 to 9 to a row) pierce the large pharynx on either side, in the posterior region where it is in contact with the two atria. The pharynx bears a conspicuous endostyle along its antero-dorsal border, and contains a large, central yolk mass. The U-shaped digestive tract is well developed. In the body cavity, antero-ventral to the yolk mass, is a small pericardial sac containing the developing heart. Both these structures originate from the floor of the pharynx. Complete descriptions and diagrams of all larval stages can be found in papers by Scott (1934, 1946, 1952); descriptions of the free-swimming tadpole are available in papers by Grave (1920, 1921). Some of the factors affecting metamorphosis have been described by Lynch (1956).

The swimming tadpole moves in irregular spurts, rotating on the longitudinal axis in a manner similar to that of a paramecium (Grave, 1920). When first released, the tadpoles show an immediate positive phototropism which is then reversed, at times so rapidly that before they reach the lighted side of the container, they become negative to the light stimulus. They are negatively geotropic and are always found near the surface of the water until the time of metamorphosis; then this tropism also reverses and they seek the lower levels of the container.

The length of the free-swimming period varies from 10 minutes to as long as 100 minutes (Grave, 1920). In a large vessel, most of the larvae will attach on the side of the dish near the bottom, but in small dishes they often fail to make an attachment although they will continue to metamorphose normally. Temporary attachment is made by the suckers which come in contact with a solid object; final attachment is effected by the secretion of an adhesive substance by an adhesive organ. A secretion within the cup adheres to the surface and the larva can detach itself and attach in another place numerous times before metamorphosis commences. At the time of attachment, or even before this occurs, the tail tissue buckles and is drawn into the trunk region; more extensive test is formed, and metamorphosis has begun. At the end of two days, metamorphosis is completed; sensory pigment is scattered through the body or is being eliminated through the digestive tract (which has reached its adult status) and the animal is feeding. Within the first hour of metamorphosis the heart assumes its characteristic reversal of beat. By four or five days, the zooid is well formed. All regions of the body are in full evidence: the spacious thorax with oral and atrial siphons and expanded pharynx; abdomen

with the digestive loop, conspicuous in the bright orange tint of the stomach; and the post-abdomen, marked by a thin-walled, light orange epicardial tube throughout its length and a large heart at its distal tip. The zooid continues to grow through a period of about three weeks before asexual reproduction is initiated. During this time the post-abdomen increases in size and becomes filled with "blood" cells. The epicardium is the agent af asexual reproduction and colony formation. The process of metamorphosis is described by Scott (1952).

E. Asexual Reproduction: Asexual reproduction is accomplished in this species by strobilization, *i.e.,* segmentation of the post-abdomen which contains the epicardial strand. It is known as "pharyngeal" or "epicardial" budding. At the time of constriction, the buds consist of an inner vesicle of epicardial origin and an outer covering of parental epidermis. The cavity between these layers is congested with body ("blood") cells of various kinds, predominant among which are the large nutritive cells. Present, also, in each segment is a portion of the tube of neural tissue which develops in the post-abdomen as an extension of the abdominal nerve and which increases in size with growth of the post-abdomen. All internal organs of the new individual form from epicardial tissue, a pharyngeal derivative, and are, therefore, endodermal in origin. All other organs originate from neural and epidermal tissues.

During strobilization of the post-abdomen of the parent, the heart is isolated in the terminal bud where it persists as the heart of that member; all other members regenerate a new heart.

The buds, while developing into new zooids, move up and take their place around the parent, thus either forming a new colony around a metamorphosed individual or increasing the area of an old one. Swarms of buds in all stages of growth and migration can usually be found at the bases of the tiniest finger-like projections of a large healthy colony. (Details of this process are described by Kowalevsky, 1874; Berrill, 1935; and Korschelt, 1936.)

REFERENCES:

BERRILL, N. J., 1935. Studies in tunicate development. IV. Asexual reproduction. *Phil. Trans. Roy. Soc., London, ser. B,* **225**: 327–379.

BRIEN, P., 1930. Contribution à l'étude de la régénération naturelle et expérimentale chez les Clavelinidae. *Ann. Soc. Roy. Zool. Belg.,* **61**: 19–112.

GRAVE, C., 1920. *Amaroucium pellucidum* (Leidy) form constellatum (Verrill). I. The activities and reactions of the tadpole larva. *J. Exp. Zool.,* **30**: 239–257.

GRAVE, C., 1921. *Amaroucium constellatum* (Verrill). II. The structure and organization of the tadpole larva. *J. Morph.,* **36**: 71–91.

GRAVE, C., 1935. Metamorphosis of ascidian larvae. *Pap. Tortugas Lab.,* **29**: 209–291. (Carnegie Inst., Wash., Publ. no. 452.)

KOWALEVSKY, A., 1874. Über die Knospung der Ascidien. *Arch. f. mikr. Anat.,* **10**: 441–470.

KORSCHELT, E., 1936. Vergleichende Entwicklungsgeschichte der Tiere, vol. 2. Jena.

LYNCH, W. F., 1956. Factors inhibiting metamorphosis in tadpoles of the tunicate *Amaroecium constellatum. Biol. Bull.,* **111**: 308.

MAST, S. O., 1921. Reactions to light in the larvae of the ascidians, *Amaroucium constellatum* and *Amaroucium pellucidum* with special reference to photic orientation. *J. Exp. Zool.,* **34**: 149–187.

SCOTT, SISTER FLORENCE MARIE, 1934. Studies on the later embryonic development of Tunicata: *Botryllus schlosseri* and *Amaroecium constellatum.* Ph.D. Dissertation, Columbia Univ., pp. 1–53.

SCOTT, SISTER FLORENCE MARIE, 1945. The developmental history of *Amaroecium constellatum*. I. Early embryonic development. *Biol. Bull.*, **88**: 126–138.

SCOTT, SISTER FLORENCE MARIE, 1946. The developmental history of *Amaroecium constellatum*. II. Organogenesis of the larval action system. *Biol. Bull.*, **91**: 66–80.

SCOTT, SISTER FLORENCE MARIE, 1952. The developmental history of *Amaroecium constellatum*. III. Metamorphosis. *Biol. Bull.*, **103**: 226–241.

PROTOCHORDATA

(TUNICATA)

Botryllus schlosseri

LIVING MATERIAL:

Botryllus is a small, compound ascidian which is abundant around Woods Hole, Mass. It is found encrusting rocks, wharves, floats, and even the related genus Molgula. The daisy-like pattern, formed by the iridescent pigment bands extending between the siphons of the colony members, makes it easily recognized. Botryllus is viviparous.

BREEDING SEASON:

June to September, although the reproductive period for any one colony seems to be relatively short (Grave and Woodbridge, 1924).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: These animals are easily maintained in large fingerbowls, supplied with a continuous gentle stream of sea water. Dead material should be removed, as it will rapidly foul the water.

B. Procuring Gametes: As is the case for other viviparous species of ascidians, artificial insemination in Botryllus has not proved successful.

C. Preparation of Cultures: Early developmental stages must be dissected from the atrial cavity. This is done by slitting open the zooids and stripping the embryos from the atrial walls. They can be collected with a pipette and transferred to fingerbowls of sea water, where they will continue their development (Scott, (1934). In any one colony, all the embryos are at the same stage of development.

To obtain free-swimming larvae, a considerable number of adult colonies should be collected during the morning and placed in large fingerbowls of fresh sea water, near a window but out of direct sunlight. Sexually mature colonies contain zooids which are relatively thick and which tend to mat together. If they are fully ripe, some colonies will begin to release larvae within a few minutes. According to Grave (1937), the number of larvae released reaches a maximum at noon, with only an occasional tadpole being set free in the early morning or late evening.

For the study of metamorphosis and budding, tadpoles should be isolated in separate drops of sea water in watch glasses. When the larvae are firmly affixed, the dishes can be stored in an inverted position in wooden racks. These are in turn submerged in an aquarium supplied with a constant flow of sea water.

An easy way to collect tadpoles for the study of metamorphosis and budding is to stand slides around the inner wall of a fingerbowl containing Botryllus colonies. The tadpoles will attach to the slides, which may then be replaced in running sea water, in open slide-boxes. The advantage of collecting in this fashion lies in the fact that such slides of Botryllus may be killed, fixed, stained and mounted for further study.

D. Methods of Observation: Metamorphosing and budding individuals can conveniently be studied in the watch glasses to which they are attached; debris should be flushed out gently. The specimens may be slightly flattened if a coverslip is gently lowered to cover them.

NORMAL DEVELOPMENT:

A. Egg Characteristics: The living egg measures 420 microns in diameter, according to Berrill (1937); when fixed, it is 215 microns in diameter (Scott, 1934). The yolk is in the form of small, evenly distributed granules. The mature egg is shed into the atrial cavity, at metaphase of the first maturation division. It is surrounded by a chorion and an inner and outer layer of follicle cells. The inner follicle cells are sparsely scattered within the narrow perivitelline space; the outer follicle cells become fused with the outer wall of the peribranchial cavity, thus holding the developing egg in a fixed position. Two to six eggs are found in a single individual.

B. Fertilization and Cleavage: Fertilization probably occurs when the egg is shed into the atrial cavity, and cleavage is virtually the same as in other ascidians. Gastrulation occurs between the sixth and seventh cleavages, and is similar to that of Styela (Scott, 1934).

C. Rate of Development: Development in this form is relatively rapid; a free-swimming larva is produced in about 12 hours.

D. Later Stages of Development and Metamorphosis: The neural plate of the young embryo is wide in the future brain region and narrows posteriorly. The neural folds, which encircle it, are visible shortly before the round blastopore closes; as they fuse to form the neural tube, the tail becomes marked off from the trunk and turns sharply to the left. Into the tail bud grow the dorsal neural tube, lateral muscle bands, notochord, and a ventral strand of endoderm. As the tail develops, it encircles the body meridionally, and by the time it has grown halfway around the body, the neuropore (seen in the region of the brain vesicle in early stages) has closed. A clear region in the anterior, ventral portion of the trunk marks the position of the primitive enteric cavity.

Shortly after the closure of the neuropore, a rapid series of changes occurs in the brain vesicle, resulting in the formation of a sensory vesicle, with a single sense receptor, and an adjacent hypophysis and associated ganglia. A conspicuous dorsal groove is present in the epidermis. In later development this groove stretches between the siphons. The atrium is formed by a single dorsal invagination in the posterior region of the trunk. A row of vertical gill slits is formed on each side where the two lobes of this invagination come into contact with the posterior wall of the pharynx. The tunic, siphons, papillae, and ampullae develop relatively late in embryonic life. When fully formed, the larvae drop off into the atrium and are released through the atrial siphon. (See the paper by Scott, 1934, for further details.)

The free-swimming tadpole is smaller than that of Amaroucium, having a body length of only 320–400 microns. The translucent tunic contains scattered cells, and extends out over the tail in the form of vertical fins. At the anterior end of the trunk can be seen eight conspicuous, sac-like outgrowths of the mantle, which are destined to be parts of the still non-functional and incomplete circulatory system

(Grave and Woodbridge, 1924). Also extending from the anterior region of the mantle are three projections arranged in the form of a triangle. Each of these contains a basal ganglion connected to the central nervous system, and they are believed to be sensory (rather than adhesive) in function (Scott, 1934). The siphons are inconspicuous and non-functional during the free-swimming period. The deep dorsal groove is visible between the siphons. The sensory vesicle appears as a large, clear sac located just behind the ampullae; suspended within it by a slender stalk is the statolith, a dense black cup associated with light-sensitive elements (Grave and Riley, 1935). The pharynx is large and contains a prominent endostyle along its anterior border. It extends posteriorly around the sensory vesicle in the form of two lateral lobes, each of which is perforated by a vertical row of four to six gill slits. The mass of yolk, which is so conspicuous in the pharyngeal floor of the Amaroucium tadpole, is completely lacking in the tadpole of Botryllus. A small, undeveloped heart lies below the pharynx. The short oesophagus leads to a sac-like stomach which narrows to a small intestine, coursing upward to the atrium. In the tail, the central notochord, dorsal neural tube, lateral muscle bands, and ventral cord of endoderm are clearly visible.

When first released, the tadpoles are strongly attracted to light; this attraction lasts throughout the greater portion of the free-swimming life. There is a period of indifference to light stimulus before metamorphosis, and some indication of a negative phototropism immediately before fixation (Grave and Woodbridge, 1924). The initial response to gravity is negative, but this decreases as metamorphosis approaches.

The length of the free-swimming period varies from 13 minutes to 27 hours, although on the average metamorphosis occurs in about two hours. Grave (1935) and Grave and Nichol (1939) have done some interesting work in an attempt to analyze the conditions which influence the onset of fixation. The anterior end of the tadpole attaches and metamorphosis is extremely rapid. One of the most striking features of the process is the unfolding of the ampullae, which spread out around the base of the developing tunicate like the petals of a flower.

Tadpoles which have attached and have been growing for two days are usually oriented so that the oral and atrial siphons are directed away from the substrate. The large pharynx, shaped like a truncate cone, bears three rows of stigmata (visceral clefts) which allow water to pass out into the atrial cavity on either side. A rod-like endostyle lies on the underside of the pharynx. The stomach ordinarily appears as a yellow body under the atrial opening. The intestine, near its junction with the stomach, turns to one side and loops to empty near the atrium.

E. Asexual Reproduction: Colony formation in Botryllus is often accomplished by the so-called "atrial" type of budding. The first bud, or blastozooid, is formed by an invagination of one side of the atrium, and its subsequently differentiated parts are thus derived solely from ectoderm. It is furnished with a blood supply. This first blastozooid is single, but all the later buds are formed in symmetrical pairs. By one week after attachment, four rows of stigmata have developed in the pharynx of the oözooid, and probably three or four rows in the blastozooid. Buds of the second and third order may have formed. The same organ structures are visible in all these individuals, notwithstanding their diverse embryology, with the minor exception that the oözooid does not develop gonads. By re-orientation

of the individuals, the completed colony develops a common atrial pit at its center, and separate pharyngeal openings at the periphery. (For further details consult the papers of Pizon, 1893; Berrill, 1941a, 1941b; Watterson, 1945.)

Recently, Oka and Watanabe (1957) have described a process of "vascular budding" in this form.

REFERENCES:

BERRILL, N. J., 1937. Culture methods for ascidians. *In:* Culture Methods for Invertebrate Animals, edit. by Galtsoff *et al.*, Comstock, Ithaca, pp. 564–571.

BERRILL, N. J., 1941a. The development of the bud in Botryllus. *Biol. Bull.*, **80**: 169–184.

BERRILL, N. J., 1941b. Size and morphogenesis in the bud of Botryllus. *Biol. Bull.*, **80**: 185–193.

GRAVE, C., 1935. Metamorphosis of ascidian larvae. *Pap. Tortugas Lab.*, **29**: 209–291. (Carnegie Inst., Wash., Publ. no. 452.)

GRAVE, C., 1937. Notes on the culture of eight species of ascidians. *In:* Culture Methods for Invertebrate Animals, edit. by Galtsoff *et al.*, Comstock, Ithaca, pp. 560–564.

GRAVE, C., AND P. A. NICHOL, 1939. Studies of larval life and metamorphosis in *Ascidia nigra* and species of Polyandrocarpa. *Pap. Tortugas Lab.*, **32**: 1–46. (Carnegie Inst., Wash., Publ. no. 517.)

GRAVE, C., AND G. RILEY, 1935. Development of the sense organs of the larva of *Botryllus schlosseri*. *J. Morph.*, **57**: 185–211.

GRAVE, C., AND H. WOODBRIDGE, 1924. *Botryllus schlosseri* (Pallas): The behavior and morphology of the free-swimming larva. *J. Morph.*, **39**: 207–247.

HERDMAN, E. C., 1924. Botryllus. *Mem. Liverpool Mar. Biol. Comm.*, **26**: 1–40.

OKA, H., AND H. WATANABE, 1957. Vascular budding, a new type of budding in Botryllus. *Biol. Bull.*, **112**: 225–240.

PIZON, A., 1893. Histoire de la blastogénèse chez les Botryllidés. *Ann. Sci. Nat., Zool., sér. 7*, **14**: 1–386.

SCOTT, SISTER FLORENCE MARIE, 1934. Studies on the later embryonic development of Tunicata: *Botryllus schlosseri* and *Amaroecium constellatum*. Ph.D. Dissertation, Columbia Univ., pp. 1–53.

WATTERSON, R. L., 1945. Asexual reproduction in the colonial tunicate, *Botryllus schlosseri* (Pallas) Savigny, with special reference to the developmental history of intersiphonal bands of pigment cells. *Biol. Bull.*, **88**: 71–103.

PROTOCHORDATA

(TUNICATA)

Ciona intestinalis

LIVING MATERIAL:

These large, solitary ascidians can sometimes be collected from the wharf piles at Oak Bluffs and Vineyard Haven, Mass., and from the salt water tanks on the roof of the Marine Biological Laboratory, at Woods Hole, when the tanks are emptied in September; they are not always obtainable. The animals are hermaphroditic and, under some conditions at least, self-fertile (Just, 1934a, 1934b). They are oviparous.

BREEDING SEASON:

Almost any time of the year, since Ciona is sexually mature above a certain size limit. Reproduction is seasonal only to the extent of the rhythm of the growth cycle (Berrill, 1937).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Mature adults will continue to produce normal eggs for several days in the laboratory, provided they are not crowded. Place only a few together in a large fingerbowl and insure a constant supply of fresh sea water.

B. Procuring Gametes: The eggs are normally shed at daybreak, but Rose (1939), using Styela, has developed a method for postponing this until a more convenient time. The adults are stored in a dark place, such as a desk drawer, until eggs are needed; shedding occurs almost immediately upon return of the animals to light.

Artificial insemination in this species is highly successful, since the genital ducts contain only ripe gametes. These may be obtained by slitting open the test and pipetting the eggs and sperm from the oviduct and sperm duct, respectively. If necessary, fine scissors can be used to puncture the ducts. The eggs remain viable for 18 hours after removal. They should be passed through several changes of sea water before insemination, to free them of perivisceral fluid.

C. Preparation of Cultures: Naturally-spawned eggs should be collected with a small-mouthed pipette and placed in fingerbowls of fresh sea water. The artificially-obtained eggs should be inseminated with a sperm suspension sufficiently concentrated to impart a faint milkiness to the sea water in which the eggs are contained. Berrill (1937) routinely uses eggs and sperm from different individuals, which is a wise precaution even in this self-fertile species. After fertilization, the essential requirements for normal development are the complete removal of excess sperm and oviducal fluid, and, above all, the use of glassware chemically and organically clean, as Morgan (1945) has demonstrated.

The development of Ciona tends to become abnormal during the period of tail elongation; placing the cultures in a larger volume of water prevents this tendency

and makes it possible to rear individuals through metamorphosis to maturity. The sea water should be replaced three or four times during the course of development. Feeding must be initiated once the small ascidiozooid has attached. Ciona, according to Berrill (1947), grows readily in an inverted bell jar or in a battery jar, equipped with an aerator. The diatom Nitzschia is used as a basic food, and its culture, within the bell jar, is regulated by controlling the amount of light with a dark paper shield. Nutrient salts are added from time to time.

D. Removal of the Chorion: Berrill (1932, 1937) gives several methods for the removal of the chorion for experimental purposes; this technique involves the use of crab stomach juice or proteolytic enzymes. Berg (1956) digested the chorion off unfertilized eggs with a 3% solution of protease in sea water. For observation, the chorion may be removed by simply rolling the eggs under a coverslip (Conklin, 1905).

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The diameter of this egg is between 150 and 170 microns, according to Conklin (1905) and Berrill (1935). The egg is surrounded by a chorion, and inner and outer layers of follicle cells, the latter being elongated and pyramidal in shape (Berrill, 1929). There is a perivitelline space (Conklin, 1905). The egg has a clear, transparent cortical layer, and either green or red pigment in the yolk granules; the former color indicates a physiologically young egg, the latter a physiologically old one (Berrill, 1929). The oöcyte proceeds to the metaphase of the first maturation division when it enters the oviduct, and is shed into the water at this stage.

B. Fertilization and Cleavage: Although possible, self-fertilization is not at all common in Ciona. Following insemination, two polar bodies are extruded. They are larger than the inner follicle cells and remain attached to, or embedded in, the egg, thus constituting an important landmark. The first two cleavages are equal and divide the egg into future right and left halves.

Berg (1956) isolated Ciona blastomeres at the four-cell stage, and by spectrophotometric methods demonstrated that the cytochrome oxidase activity of posterior blastomeres is about 2.7 times that of anterior blastomeres. He interprets his results to indicate a localization of mitochondria in the posterior blastomeres.

Gastrulation is by invagination and epiboly (Castle, 1896). (For further details of development, consult the papers by Conklin, 1905, and Duesberg, 1915.)

C. Rate of Development: Duesberg (1915) states that cleavage begins one hour after insemination, and that hatching occurs at 19 hours, whereas Conklin (1905) reported that the latter event took place 12 hours after insemination; the temperature was not recorded in these papers. At 16° C., hatching occurs at about 25 hours, according to Berrill (1935). He also noted gastrulation at 7 hours, closure of the blastopore at 11 hours, and the appearance of sensory pigment 19 hours after insemination at 16°C.

D. Later Stages of Development and Metamorphosis: The tadpole, which hatches by means of a proteolytic enzyme (Berrill, 1932), is urodele-like in appearance. It has vertical tail fins, three adhesive papillae for attachment, a sensory vesicle with both a statocyst and a light-sensitive organ, and a short intestine. The siphons are not prominent. The endostyle is easily seen. (See the diagrams by Willey, 1893; MacBride, 1914; Berrill, 1929.)

The free-swimming period may last from 6 to 36 hours, usually more than 12 hours (Berrill, 1935). At metamorphosis the tail is resorbed and the mouth and atrial siphons rotate to a dorsal position. A heart and two primary gill slits appear soon after attachment. The affixed anterior region of the tadpole grows out to form a stalk which lifts the trunk away from the substrate. Details and figures of metamorphosis are given by Willey (1893) and Berrill (1929).

REFERENCES:

BERG, W. E., 1956. Cytochrome oxidase in anterior and posterior blastomeres of *Ciona intestinalis*. *Biol. Bull.*, **110**: 1–7.

BERRILL, N. J., 1929. Studies in tunicate development. I. General physiology of development of simple ascidians. *Phil. Trans. Roy. Soc., London, ser. B*, **218**: 37–78.

BERRILL, N. J., 1932. The mosaic development of the ascidian egg. *Biol. Bull.*, **63**: 381–386.

BERRILL, N. J., 1935. Studies in tunicate development. III. Differential retardation and acceleration. *Phil. Trans. Roy. Soc.. London, ser. B*, **225**: 255–326.

BERRILL, N. J., 1937. Culture methods for ascidians. *In:* Culture Methods for Invertebrate Animals, edit. by Galtsoff *et al.*, Comstock, Ithaca, pp. 564–571.

BERRILL, N. J., 1947. The development and growth of Ciona. *J. Mar. Biol. Assoc.*, **26**: 616–625.

CARLISLE, D. B., 1951. On the hormonal and neural control of the release of gametes in ascidians. *J. Exp. Biol.*, **28**: 463–472.

CASTLE, W. E., 1896. The early development of *Ciona intestinalis*, Flemming (L.). *Bull. Mus. Comp. Zool., Harvard*, **27**: 201–280.

CONKLIN, E. G., 1905. The organization and cell-lineage of the ascidian egg. *J. Acad. Nat. Sci., Philadelphia, ser. 2*, **13**: 1–119.

DUESBERG, J., 1915. Recherches cytologiques sur la fécondation des Ascidiens et sur leur développement. *Contrib. to Embryol.*, **3**: 33–70. (Carnegie Inst., Wash., Publ. no. 223.)

HARVEY, L. A., 1927. The history of the cytoplasmic inclusions of the egg of *Ciona intestinalis* (L.) during oogenesis and fertilisation. *Proc. Roy. Soc., London, ser. B*, **101**: 136–162.

JUST, E. E., 1934a. On the rearing of *Ciona intestinalis* under laboratory conditions to sexual maturity. *Carnegie Inst., Wash., Year Book*, **33**: 270.

JUST, E. E., 1934b. Zoological researches. *Carnegie Inst., Wash., Year Book*, **34**: 280–284.

MACBRIDE, E. W., 1914. Text-Book of Embryology. Vol. I. Invertebrata. Macmillan and Co., Ltd., London.

MORGAN, T. H., 1945. The conditions that lead to normal or abnormal development of Ciona. *Biol. Bull.*, **88**: 50–62.

ROSE, S. M., 1939. Embryonic induction in the Ascidia. *Biol. Bull.*, **77**: 216–232.

WILLEY, A., 1893. Studies on the Protochordata. I. On the origin of the branchial stigmata, praeoral lobe, endostyle, atrial cavities etc., in *Ciona intestinalis*, Linn., with remarks on *Clavelina lepadiformis*. *Quart. J. Micr. Sci.*, **34**: 317–360.

ZEUTHEN, E., 1955. Mitotic respiratory rhythms in single eggs of *Psammechinus miliaris* and of *Ciona intestinalis*. *Biol. Bull.*, **108**: 366–385.

PROTOCHORDATA

(TUNICATA)

Molgula citrina

LIVING MATERIAL:

Members of this species are sometimes gathered, together with individuals of *M. manhattensis.* from Eel Pond at Woods Hole, Mass. While their anatomy is almost identical, the two species are radically different in their mode of reproduction. *M. citrina* is viviparous, *M. manhattensis* oviparous.

BREEDING SEASON:

Mid-June to mid-September (Grave, 1926). The period of reproduction seems to depend on the size of the individual rather than on the time of year.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: Sexually mature individuals (those over five mm. in length) will continue to reproduce and release larvae when brought into the laboratory, if they are kept in vessels supplied with a gentle stream of sea water.

B. Procuring Gametes: It is apparently not possible to remove and then fertilize ovarian eggs of this species.

C. Preparation of Cultures: Rearing embryos outside the parent is difficult. Berrill (1935) suggests that they be raised in a thistle-tube, the large end of which is covered with bolting silk on which the eggs are placed, the other end being attached to a T-tube through which air is bubbled. The whole apparatus is submerged in sea water; further details can be found in the original article. Even with this set-up, the mortality rate is high, and it is better to take the stages desired for study directly from the atrial brood-chamber of the parent.

Larvae are released when the adults are exposed to light; no particular time of day seems to be optimal. Culture directions for the tadpole are similar to those given for *M. manhattensis* (see p. 216). This form is favorable for use in a study of metamorphosis.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The egg measures approximately 210 microns in diameter, and is very opaque due to the presence of a yellow-orange pigment in the densely packed yolk. The outer follicle cells form a markedly flattened layer over the surface of the chorion, while the inner follicle cells are closely packed into a narrow perivitelline space (see the paper by Berrill, 1931, Figure 2). The egg is shed from the oviduct at the metaphase of the first maturation division.

B. Fertilization and Cleavage: Fertilization and development take place within the atrial chamber of the parent. The first few cleavages are equal, dividing the egg into a right and a left half. Gastrulation is by invagination, between the sixth and seventh cleavages (Berrill, 1935).

C. Rate of Development: Details are not available, but development in this form is relatively slow. At 16° C., the tadpoles hatch by rupturing the chorion 150 hours after fertilization.

D. Later Stages of Development and Metamorphosis: The free-swimming larvae, although they bear the same superficial resemblance to urodele larvae as do those of Amaroucium or Botryllus, seem to be less highly specialized. They lack organs for attachment, and no gill slits are visible. The most conspicuous larval organ is a huge sensory vesicle containing a statolith; there is no "eye." The alimentary tract is very yolky and poorly differentiated. The atrium consists of two sacs joined dorsally and posteriorly; the siphons are inconspicuous. The tail-fins are vertical. A small bilobed pericardial sac lies anterior and ventral to the intestine. Eight thickenings in the mantle precede the formation of the ampullae (see the paper of Grave, 1926, for a diagram).

The free-swimming period is short, averaging less than three hours (Grave, 1926). Fixation can be accomplished at any region of the adhesive test, and is accompanied by tail shrinkage and the extension of 8 or 10 mantle projections, the ampullae. A very small percentage of larvae metamorphose within the egg membrane inside the atrial cavity. Details and diagrams of metamorphosis are available in papers by Grave (1926) and Berrill (1931).

REFERENCES:

BERRILL, N. J., 1931. Studies in tunicate development. II. Abbreviation of development in the Molgulidae. *Phil. Trans. Roy. Soc., London, ser. B,* **219**: 281–346.

BERRILL, N. J., 1935. Studies in tunicate development. III. Differential retardation and acceleration. *Phil. Trans. Roy. Soc., London, ser. B,* **225**: 255–326.

GRAVE, C., 1926. *Molgula citrina* (Alder and Hancock). Activities and structure of the free-swimming larva. *J. Morph.,* **42**: 453–471.

PROTOCHORDATA

(TUNICATA)

Molgula manhattensis

LIVING MATERIAL:

Adults are abundant on the floats and wharf piles around Woods Hole, Mass. They are far more common than *M. citrina,* but in the past workers have often confused the two species. Since *M. citrina* is viviparous and releases larvae instead of eggs, there is little reason for this mistake in identification. The animals are hermaphroditic.

BREEDING SEASON:

Probably there is no fixed season. Individuals over 12 mm. in length seem to breed continuously (Berrill, 1931).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: These animals keep very well when placed in large fingerbowls on the water table. A continuous gentle stream of sea water should be supplied and dead material removed promptly, since the water will foul very rapidly.

B. Procuring Gametes: For experimental work it is usually advisable to obtain naturally-shed eggs. Molgula sheds soon after dawn; however, this process may be delayed by placing several animals in large fingerbowls, and keeping them in the dark until they are needed. They will usually shed about 15 minutes after they are brought into the light.

Eggs and sperm may also be obtained by cutting open the tests of individuals over 12 mm. in length, and pipetting gametes from the genital ducts. This procedure may yield immature as well as mature eggs, since the oviducts in Molgula are short. The eggs should be passed through several changes of sea water, to free them of perivisceral fluid.

C. Preparation of Cultures: Naturally-shed eggs should be pipetted to fingerbowls of fresh sea water. To artificially-obtained eggs, enough sperm should be added to cause a faint milkiness in the water. The water should be replaced after one or two hours. It is probably advisable to use gametes from different individuals.

The bowls of fertilized eggs should be kept on a water table, and the water changed three or four times during subsequent development. As soon as the larvae begin to swim, they should be decanted or pipetted to fingerbowls of fresh sea water or, if a study of metamorphosis is desired, isolated in separate drops of sea water in Syracuse dishes. When they have firmly attached to these dishes, sea water should be added. The dishes with attached larvae can be stored in an inverted position in wooden racks which are submerged in aquaria of running sea water.

D. Removal of the Chorion: The chorion can be digested off with the stomach juice of crabs or with proteolytic enzymes, before or after fertilization (Berrill, 1932, 1937). This technique is useful for experimental purposes.

NORMAL DEVELOPMENT:

A. The Unfertilized Egg: The egg is opaque, with colored yolk; measurements of its diameter vary from 100 microns (Conklin, 1905) to 115 microns (Grave, 1926). Outer follicle cells, which are rounded, form a compact layer around the chorion, and a few inner follicle cells are present. A perivitelline space is visible. In the oviduct the germinal vesicle breaks down, and the egg proceeds to the metaphase of the first maturation division. Eggs are shed at this stage and remain in it until fertilization or death. (See the paper by Berrill, 1931, for further details.)

B. Fertilization and Cleavage: The eggs are fertilized as they are shed into the water. Cleavage is equal up to the fourth division, and separates the egg into future right and left halves, as in Styela. Gastrulation occurs between the sixth and seventh cleavages, and is a rather specialized form of true invaginative gastrulation.

C. Rate of Development: Berrill (1931) states that at 19° C. the blastopore is closed and the tail bud visible in four hours; hatching occurs in 8 to 11 hours. Secretion of the test, caudal degeneration, and outgrowth of the ampullae (*i.e.*, metamorphosis) occur 18 to 24 hours after insemination.

D. Later Stages of Development and Metamorphosis: The larvae normally hatch by means of enzymatic digestion of the chorion. The free-swimming urodele-like tadpoles have vertical tail-fins and a large sensory vesicle containing an otolith which is not destroyed during metamorphosis. There are no gill slits or adhesive papillae visible, and the siphons are undeveloped. The alimentary tract is poorly developed and very yolky. For diagrams of larvae, see the papers by Berrill (1931) and Grave (1926).

A few of the early stages of metamorphosis are figured by Berrill and by Grave. As has already been indicated, fixation is followed by tail degeneration and the outgrowth of a long, primary ectodermal ampulla. When this is fully formed, additional ampullae appear, and in the final state there are two present on one side of the body and three on the other. Pulsations appear early in the primary ampulla, which probably has a respiratory function.

REFERENCES:

BERRILL, N. J., 1931. Studies in tunicate development. II. Abbreviation of development in the Molgulidae. *Phil. Trans. Roy. Soc., London, ser. B,* **219**: 281–346.

BERRILL, N. J., 1932. The mosaic development of the ascidian egg. *Biol. Bull.,* **63**: 381–386.

BERRILL, N. J., 1937. Culture methods for ascidians. *In:* Culture Methods for Invertebrate Animals, edit. by Galtsoff *et al.,* Comstock, Ithaca, pp. 564–571.

CONKLIN, E. G., 1905. The organization and cell-lineage of the ascidian egg. *J. Acad. Nat. Sci., Philadelphia, ser. 2,* **13**: 1–119.

GRAVE, C., 1926. *Molgula citrina* (Alder and Hancock). Activities and structure of the free-swimming larva. *J. Morph.,* **42**: 453–471.

PROTOCHORDATA

(TUNICATA)

Perophora viridis

LIVING MATERIAL:

These small green ascidians are found in abundance on sea-weed, wharf piles, etc., and may be collected from Lagoon Pond bridge at Martha's Vineyard, Mass. They are hermaphroditic and viviparous.

BREEDING SEASON:

August and September, according to Berrill (1937). However, Grave and McCosh (1923) indicate a shorter breeding period for Woods Hole, namely, the first half of August. Bumpus (1898) reports that ripe specimens of this species were taken throughout July.

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals are relatively hardy and will continue to breed and produce larvae in the laboratory, provided they are kept in large dishes with a constant supply of fresh sea water.

B. Procuring Gametes: Artificial insemination, using gametes pipetted from the genital ducts, has not been successful for this species.

C. Preparation of Cultures: Fertilized eggs, in various stages of development, can be obtained from the atrial brood-chamber by slitting open the test of an adult. They are difficult to rear outside the parent, although some success has been attained using the thistle-tube apparatus described by Berrill (1935a). The mortality rate decreases with advanced stages.

Older larvae can be obtained by placing dishes of adults before a window. The number of larvae released starts to increase at 8 A.M., reaches a maximum about 10 A.M., and declines by 11 A.M.; only a few are released throughout the rest of the day. This is a form well suited to the study of metamorphosis in ascidians; for such a study, the tadpoles should be isolated in separate drops of sea water in watch glasses. When the larvae have attached, the dishes may be stored in an inverted position in wooden racks which are submerged in aquaria constantly supplied with running sea water.

Bud formation is perhaps best observed in young cultures. If a small piece of the colony is affixed with vaseline to a watch glass which is stored in running sea water, stolons will be extended over the surface of the glass and new blastozooids formed at intervals along them. Within two weeks, a series of well-formed buds will be present.

D. Methods of Observation: To examine metamorphosing or budding individuals, remove a watch glass from the rack and gently flush out any debris; avoid

exposing the surfaces of the animals to air during the examination. If specimens are growing upright, they may be flattened by gently lowering a coverslip on them.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: This egg measures 240 microns in diameter. It has a very thin membrane and practically no perivitelline space. The ovum is yellowish in color. The germinal vesicle ruptures and the first maturation spindle is formed when the egg enters the oviduct.

B. Fertilization and Cleavage: Perophora eggs leave the oviduct one at a time, already fertilized, and pass into the atrial brood-chamber, where they are retained throughout development. Cleavage and gastrulation are similar to those of Styela (see p. 222). Gastrulation is by a rather specialized form of invagination, and occurs between the sixth and seventh cleavages (Berrill, 1935a).

C. Rate of Development: Development is relatively slow, and at 16° C. the interval between successive cleavages is approximately four hours. Gastrulation begins about 45 hours after insemination, the blastopore closes after 60 hours, sensory pigment appears after 120 hours, and the rupture of the chorion occurs about 185 hours after insemination (Berrill, 1935a). Grave and McCosh (1923) reported that the average free-swimming period lasts five hours.

D. Later Stages of Development and Metamorphosis: The translucent green larvae are rather similar to those of Amaroucium, having three cup-like adhesive papillae and horizontal tail-fins. The attachment papillae have cones of secretion projecting from the center of the cups. The sensory vesicle is enormous and contains an eye with lens, as well as a statocyst. Hypophysis, definitive ganglion, and sub-neural gland are present. There are four rows of horizontal gill slits on the right side and six on the left. Both the siphons and endostyle are well formed. The heart is functional and shows a characteristic reversal of beat. Further details and diagrams are available in the paper by Grave and McCosh (1923); Berrill (1935a) gives some details of metamorphosis.

E. Asexual Reproduction: Perophora exhibits the type of budding designated as "septal." The stolons, which branch irregularly over the substrate, have central mesenchymal septa separating the outgoing and ingoing blood streams. In bud formation, there first appears a hypertrophy of the epidermis between the tip of the stolon and the last formed zooid. Beneath this evagination, the cells of the vascular septum proliferate and grow out to form a hollow vesicle within the epidermal bulge. The epidermal covering of the bud forms the epidermis of the new zooid, and the indifferent mesenchyme of the inner sac forms all the remainder of the blastozooid. In Perophora, the bud never loses its connection with the stolon and is, therefore, vascularized by the common blood stream of the colony. For further details see the papers by Huxley (1921) and Berrill (1935b).

REFERENCES:

BERRILL, N. J., 1935a. Studies in tunicate development. III. Differential retardation and acceleration. *Phil. Trans. Roy. Soc., London, ser. B,* 225: 255–326.

BERRILL, N. J., 1935b. Studies in tunicate development. IV. Asexual reproduction. *Phil. Trans. Roy. Soc., London, ser. B,* 225: 327–379.

BERRILL, N. J., 1937. Culture methods for ascidians. *In:* Culture Methods for Invertebrate Animals, edit. by Galtsoff *et al.,* Comstock, Ithaca, pp. 564–571.

BUMPUS, H. C., 1898. The breeding of animals at Woods Holl during the months of June, July and August. *Science,* **8**: 850–858.

GRAVE, C., AND G. K. MCCOSH, 1923. *Perophora viridis* (Verrill). The activities and structure of the free-swimming larva. *Washington Univ. Stud., Sci. ser.,* **11**: 89–116.

HUXLEY, J. S., 1921. Studies in dedifferentiation. II. Dedifferentiation and resorption in Perophora. *Quart. J. Micr. Sci.,* **65**: 643–697.

RITTER, W. E., 1897. Budding in compound ascidians, based on studies on Goodsiria and Perophora. *J. Morph.,* **12**: 149–238.

PROTOCHORDATA

(TUNICATA)

Styela (formerly *Cynthia*) *partita*

LIVING MATERIAL:

Styela is a simple ascidian with a hard tunic and small granular tubercles on and about the papillae bearing the apertures. It is brownish or yellow in color, and up to 25 mm. in length. Occasionally, the animals are found in groups although, as noted above, the form is not a colonial one. They are quite common around Woods Hole, Mass.

BREEDING SEASON:

June to September, according to Berrill (1937).

PROCURING AND HANDLING MATERIAL:

A. Care of Adults: The animals live well in the laboratory, if they are adequately supplied with sea water. High temperatures should be avoided.

B. Procuring Gametes: Although it is hermaphroditic, Styela is ordinarily self-sterile. Eggs and sperm are shed between 4 and 7 P.M., and fertilization takes place when the ripe gametes from two different individuals are mixed. The usual method of obtaining Styela eggs and embryos has been to mince the gonads from a large number of individuals, in a dish of sea water. This liberates all stages in the maturation of eggs and sperm, and usually at least a few eggs will be fertilized (whatever the time of day or night) and will begin normal development.

Rose (1939) has described a method of controlling natural spawning in the laboratory; it works well except for a few weeks in mid-summer, when the animals are spent. The adults are kept in the dark until eleven or twelve hours before fertilization is desired; then an artificial day is started, by turning on a 40-watt electric light, placed about 18 inches from the animals. Eggs and sperm are discharged in clouds at the desired time. The same batch of animals can be induced to shed a number of times on successive days.

NORMAL DEVELOPMENT:

A. The Unfertilized Ovum: The mature unfertilized egg is approximately 150 microns in diameter, and has a tough membrane, the chorion, to which a few follicle cells adhere at the outer surface. Between the chorion and the egg surface, there are small, spherical inner follicle cells ("nurse cells"), which contain yellow granules. The peripheral layer of the egg is clear and contains minute yellow granules, and the central part of the egg consists of grey yolk platelets. The germinal vesicle is large and clear, and is excentrically placed, near the animal pole; it ruptures and maturation begins at about the time when the eggs are discharged. The maturation spindle remains at the metaphase of the first division until the sperm enters.

B. Post-Fertilization Changes: The sperm enters at or near the vegetal pole (Conklin, 1905a); maturation continues, and two polar bodies are given off. An extensive re-arrangement of the cytoplasm now occurs: within two to eight minutes after fertilization, the clear, yellowish peripheral material streams to the lower pole, over the yolk, followed by the clear protoplasm from the animal pole. This process is best studied using daylight for illumination; the microscope diaphragm should be open as far as possible.

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

The different pigmented regions of the egg correspond closely to the various embryonic areas with specific presumptive developmental fates. The yellow pigment area, at the posterior vegetal region, forms the "yellow crescent," which is presumptive mesoderm. The ventral and anterior portion of the vegetal hemisphere, which has the slate grey color of the yolk, forms endoderm and small amounts of mesoderm; it also contributes to a portion of the neural plate. The animal hemisphere material, which is light grey in color because of the presence of clear protoplasm beneath the peripheral yolk, forms the body epidermis and a portion of the neural plate. The animal pole becomes the ventral-anterior side of the larva, while the vegetal pole is the future dorsal side.

C. Cleavage and Gastrulation: The first cleavage is equal, separating the two "horns" of the yellow crescent from one another and bisecting the clear protoplasm anterior to the yellow region. The second cleavage is nearly equal, vertical, and at right angles to the first. The two posterior cells contain only a small amount of yolk and practically all the yellow crescent substance. The two anterior cells, on the other hand, contain much yolk and almost no yellow crescent material. There is an equal division of the clear protoplasm to the four cells. At the third cleavage, which is horizontal, the yellow crescent substance is almost entirely confined to the two posterior dorsal cells. The planes of cleavage at the fourth division vary in different quadrants, but the cells do not overlap the sagittal plane of the embryo. Two of the antero-dorsal cells and two of the postero-ventral cells of the 16-cell embryo are crowded away from this sagittal plane, but all the other cells touch it. The dorsal and ventral hemispheres at this stage are mirror images of one another. The yellow pigment lies in four posterior cells. Division in the dorsal (vegetal) hemisphere precedes that in the ventral (animal) hemisphere at the fifth cleavage, and cleavage in the anterior part of each hemisphere precedes that in the posterior part. When the 32-cell stage is reached, the yellow substance is almost entirely confined to six dorso-posterior cells, three on each side of the midline. They give rise to mesoderm and mesenchyme. Six yolk-filled cells at the vegetal pole, anterior to the yellow mesoderm cells, give rise to endoderm. Four cells at the anterior border of the embryo (just below the equator) and two just above the equator produce the notochord and neural plate. All the other cells are ectodermal.

Gastrulation is by epiboly. The gastrula passes through disc-shaped, saucer-shaped and cup-shaped stages, starting at the seventh cleavage. As it finally becomes egg-shaped, the blastopore assumes the form of a "T," the stem of the "T"

being bordered by the yellow mesoderm-mesenchyme cells. The cells overhanging the cross-bar of the T-shaped blastopore constitute its dorsal lip. They overgrow it, finally engulfing the yellow cells which are then seen only dimly through the translucent ectoderm.

D. Time Table of Development: The following approximate schedule for the development of normally-shed Styela eggs is from the classic monograph of Conklin (1905a). If eggs are obtained from "minced" cultures, cleavage is delayed, the eggs apparently maturing at variable intervals after coming into sea water. Time is recorded from insemination; the temperature is not specified, although Conklin states that these observations were made during the evening hours.

Stage	Time
First cleavage	40 minutes
Second cleavage	70 minutes
Third cleavage	100 minutes
Fourth cleavage	120 minutes
Fifth cleavage	140 minutes
Sixth cleavage	160 minutes
Seventh cleavage (beginning of gastrulation)	180 minutes
Eighth cleavage	200 minutes
Neural plate	5 hours
Fully-formed tadpole	12 hours

REFERENCES:

VAN BENEDEN, É., AND CH. JULIN, 1884. La segmentation chez les Ascidiens et ses rapports avec l'organization de la larve. *Arch. de Biol.*, **5**: 111–126.

BERRILL, N. J., 1929. Studies in tunicate development. I. General physiology of development of simple ascidians. *Phil. Trans. Roy. Soc., London, ser. B,* **218**: 37–78.

BERRILL, N. J., 1937. Culture methods for ascidians. *In:* Culture Methods for Invertebrate Animals, edit. by Galtsoff *et al.*, Comstock, Ithaca, pp. 564–571.

CASTLE, W. E., 1896. The early embryology of *Ciona intestinalis,* Flemming (L.). *Bull. Mus. Comp. Zool., Harvard,* **27**: no. 7, pp. 203–280.

COHEN, A., AND N. J. BERRILL, 1936. The early development of ascidian eggs. *Biol. Bull.*, **70**: 78–88.

CONKLIN, E. G., 1905a. The organization and cell-lineage of the ascidian egg. *J. Acad. Nat. Sci., Philadelphia, ser. 2, part 1,* **13**: 1–119.

CONKLIN, E. G., 1905b. Mosaic development in ascidian eggs. *J. Exp. Zool.*, **2**: 145–223.

CONKLIN, E. G., 1905c. Organ-forming substances in the eggs of ascidians. *Biol. Bull.*, **8**: 205–230.

CONKLIN, E. G., 1931. The development of centrifuged eggs of ascidians. *J. Exp. Zool.*, **60**: 1–120.

ROSE, S. M., 1939. Embryonic induction in the Ascidia. *Biol. Bull.*, **77**: 216–232.