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Problems of the Mechanism Involved in the Metamorphosis of *Bugula* and *Amaroecium* Larvae

WILLIAM F. LYNCH[1]

Abstract. Various treatments which induce precocious fixation or inhibit attachment of *Bugula* and *Amaroecium* larvae are reviewed. Heilbrunn's general theory of stimulation and anesthesia is thought to provide a fairly suitable explanaton for the artificial induction or inhibition of metamorphosis of *Bugula* larvae, but the agents causing induced metamorphosis resemble those which affect cell division more than those which affect muscle contraction, a necessary correlate of the hypothesis. The hypothesis applied to *Bugula* larvae does not seem to offer a suitable explanation for induced metamorphosis in some ascidian tadpoles.

Larvae of the genus *Bugula* are pear-shaped organisms almost completely covered by cilia. At one end of the long axis, corresponding to the region of a pear where the stem is attached, is the apical plate surrounded by a crown of rigid cilia. Beneath the apical organ is a circular groove or collarette, the pallial furrow. At the opposite end of the long axis is an opening into the interior. This basal region has been called the oral pole by some of the older authors, although the term is misleading since *Bugula* larvae have no digestive tract. The most prominent external features are the apical plate, the pigmented spots, and the pyriform organ. The pyriform organ consists of an unpigmented groove surrounded by a glandular structure and extends about two-thirds of the distance from the basal pole to the apical plate. It widens out as it runs apically and contains vibratile flagella, active in feeling the surface of objects, in the region most distal to the basal pole. The number of pigmented spots varies in different species of *Bugula* and perhaps sometimes even in the same species, although the writer has never observed any variation in the number of pigmented spots in the species he has studied. In *B. neritina, B. simplex* (formerly called *B. flabellata,* but identical in appearance to those described by Ryland, 1958, as *B. simplex*), and *B. turrita* there are two small pigmented spots just apical to the pyriform organ and close to the vibratile flagella. In addition, there are two larger pigmented spots on the opposite side of the larva and closer to the basal pole. In *B. neritina* the pigmented spots are black, and the two larger ones are diamond-shaped. There are also two small pigment spots in the hemisphere opposite to the diamond-shaped ones, and the smaller spots are quite close to the crown of rigid cilia. In *B. turrita* the pigmented spots are all circular or oval

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and are brilliant red. In *B. simplex* the pigmented spots are of such a diluted pink color that they easily can remain undetected unless the light falls on them at the proper angle. Nitsche (1870) found that the pigmented spots of *B. flabellata* are equipped with lens-like structures, and the same seems to be true of those of *B. neritina,* if the observations made by the writer in 1944 are correct.

Internally the most prominent organ is the relatively voluminous internal sac, which bulges so much with adhesive fluid that it inflates the whole basal region. The internal sac has an opening at the basal pole for release of the adhesive fluid during attachment. In some ectoprocts there are nerve strands connecting the apical plate to the pyriform organ, vibratile flagella, and ciliated epithelium. These nerve strands, together with muscles, have been described by Prouho (1890) as part of the internal equipment of *Flustrella hispida.* Marcus (1926*a*) has described ganglion cells between the ectoderm and mesoderm and also in the ciliated epithelium in larvae of *Plumatella fungosa* (Phylactolaemata).

Bugula larvae are released when adult colonies are exposed to light, and within thirty minutes the photopositive organisms will gather in large numbers in the region of a finger bowl nearest the source of light. In *B. simplex* and *B. turrita* the photopositive reaction, lasting usually for three or four hours, is followed by a definite photonegative response to light. In *B. neritina,* however, there appears to be no definite photonegative response; at best, the reaction of these larvae towards light just before setting can be described most suitably as one of indifference (Lynch, 1947). This southern species, found at Beaufort, North Carolina, has a much shorter natatory period than that of either *B. simplex* or *B. turrita,* both of which are northern species found at Woods Hole, Massachusetts. The shorter natatory period of *B. neritina,* lasting from twenty to one hundred and twenty minutes as a rule, appears to be correlated with the higher temperatures prevailing in regions where adults of this species are found, for lowering the temperature of the sea water containing larvae of *B. neritina* can prolong the natatory period for hours; but probably some differences in the length of the natatory periods of northern and southern species are of genetic origin. The majority of larvae of *B. simplex* set by the end of ten or twelve hours, but some swim on for twenty-four hours or a little longer. Apparently larvae of *B. turrita* have a longer natant period than those of *B. simplex,* for there are always more swimming larvae of the former at twenty-four hours than there are of the latter. However, definite data on the percentage of larvae of *B. turrita* that set by the end of twelve and twenty-four hours are still lacking.

Spiral swimming movements are common in all three species of *Bugula,* but at times the larvae during their natatory phase emit a

fine strand of coagulated material which anchors them temporarily like a balloon at the end of a string and at a considerable distance from the bottoms of the Stender dishes containing them, while the larvae swim in circles of fairly large diameters. Under experimental conditions material may be shed from the internal sac, so that the whole bottom of the Stender dish contains clouds of milky-white material which entraps the larvae until they are able to free themselves by random back and forth movemnts. Towards the time of setting the larva swims in circles of constantly decreasing diameter, and if attachment is to take place to the bottom of the dish, the planes are more or less parallel to the top of the table on which the containers are placed. Eventually the larva stops swimming and revolves counterclockwise (like the larva of *Spirorbis*) while the stiff vibratile flagella inspect the surface. The pyriform organ is downward at this time. Sometimes a larva, apparently finding the surface unsuitable for attachment, swims off again and repeats the process several times. Eventually, however, the adhesive cement attaches the sac, now having two layers after its eversion, to the substrate. The larva simultaneously orients itself with the apical plate upwards, but often at an angle of 15° or 20° with the vertical. The larva now has a characteristic hour-glass appearance, for the amount of adhesive substance is almost as large as the larva itself. Very shortly the outer ciliated covering begins to migrate towards the everted internal sac and by a process of involution turns inside the larva. Simultaneously the old ciliated covering appears to pull out a new transparent larval covering from the circular, grooved collarette at the base of the apical plate. The new integument advances downward and slightly outward toward the adhesive cement and eventually all the ciliated covering of the larva becomes located inside the organism and the cilia no longer beat. Shortly afterwards the larva with a quick movement pushes itself into the adhesive cement and the larva now looks like an inverted cup on top of a flat plate, for the adhesive cement has a diameter somewhat greater than that of the larva. Within four or five hours *B. simplex* will have developed a club-shaped zoid attached either to the bottom or sides of the container, but most frequently to the surface film, by a stolon. The stolon of *B. simplex* is a tripod structure having two short dichotomous branches at the ends of each of the three segments. In *B. turrita* there are four rather than three segments which attach the zoid to the substrate. By 36-44 hours, but often sooner, the zoid of *B. simplex* will have developed a zooecial wall which contains chitin, according to Hyman (1958). Inside the zooecial wall the softer parts of the polypide will have differentiated into a gut opening into a circlet of tentacles which are active in feeding. By the end of another twenty-four hours a bud for the second individual of the colony is clearly recognizable.

Whereas, normally, ejection of the holdfast is the first event that occurs in metamorphosis, followed by migration of the various tissues, under experimental conditions this sequence of events may not take place. Thus, when larvae are suddenly immersed into sea water at 0° to −2° C., the holdfast is ejected but the cilia continue to beat, indicating the involution of the ciliated covering has not occurred. Furthermore, treatment of the larvae (*B. simplex,* for all experiments unless otherwise stated) with molar solutions of urea for about fifteen minutes causes tissue from the collarette to flare out widely before the holdfast is ejected, and the ciliated epithelium often migrates apically rather than basally. If larvae of *B. turrita* are employed in these experiments, the brilliant red pigmented spots can be seen lodged just beneath the apical plate (Lynch, 1958*a*). It is difficult to determine whether the holdfast is actually ejected or whether it merely seems to protrude because it has been denuded of the ciliated covering that surrounds it. Similarly, in solutions of sea water and isotonic glucose (80 cc. of the former / 20 cc. of the latter) containing neutral red in parts of 1:100,000 the body of the larva may become denuded of cilia without the holdfast being ejected at all.

Experimentally, precocious attachment of *Bugula* larvae may be induced by various factors. These are exposure to heat (30°-31° C.) and cold (0° to -5° C.) (Lynch, 1949*a*; 1956 *a*); hypertonic sea water (Lynch, 1949b); a brief exposure to distilled water; removal of larvae from isotonic solutions of urea, KCl, $CaCl_2$ or NaCl (Lynch, 1956*b*, 1958*a*); sea water containing an excess of $CaCl_2$ (Lynch, 1952); x-raying of larvae (15,500 r) or their immersion in irradiated sea water; and exposure to sea water containing H_2O_2 (1:14,000 parts of 30 percent solution), 2,3,5-triphenoltetrazolium chloride ($1 \times 10^{-5}M$), sodium 2,6-dichlorobenzenoneindophenol (0.05 mg./liter) (Lynch, 1958*b*), methylene blue, neutral red (both 1:100.000 pts.), (Lynch, 1952), benzoquinone (Lynch, 1958*a*), 0.25 mg./liter, $CuCl^2$ (Lynch, 1949*b*), 2.5 mg./liter, or iodine ($5 \times 10^{-4}M$) (Lynch, 1956*b*); and finally Mg-free sea water (Lynch, 1952). On the other hand, attachment is inhibited but not prevented by darkness. Fixation is likewise inhibited by sea water containing cysteine ($1 \times 10^{-3}M$); sodium thioglycollate (1×10^{-2} *M*); certain antimitotic substances from the ovaries of the puffer and starfish; KCN ($1 \times 10^{-3}M$); chloral hydrate (0.15 percent); urethane (0.01 percent); alcohol (3.3 percent); thiourea (0.3 percent); sodium azide ($1 \times 10^{-3}M$); 2-4-dinitrophenol (0.01 percent); mixtures of sea water and isotonic solutions of KCl, $MgCl^2$, sucrose and glucose; temperatures of 5° to 10° C.; acidifying sea water below a pH of 6.0; and calcium-free media (Lynch, 1952, 1955, 1956*b*, 1958*a*, 1959). Of these agents calcium-free sea water, sodium azide, dinitrophenol, and a low pH are almost 100 percent effective; sodium thioglycollate, urethane,

and chloral hydrate cause about 95 percent inhibition of setting. Of the factors listed as inductors or inhibitors of setting in *Bugula* larvae, sixteen have a similar effect on ascidian tadpoles. Copper, dyes, iodine, hypertonic sea water, and a brief exposure to distilled water or to isotonic NaCl accelerate setting; on the other hand, potassium cyanide, azide, urethane, acidified sea water, $MgCl_2$, sugars, dinitrophenol, chloral hydrate, and temperatures at 5° to 9° C. cause inhibition (but sugars, dinitrophenol and chloral hydrate allow the first stage of metamorphosis to occur).

As an explanation of the reason that such diverse agents can induce precocious setting or inhibit fixation, a working hypothesis has been set up which postulates that setting, when artificially induced, is essentially a response to stimulation and that inhibition is a kind of anesthesia. According to Heilbrunn's theory (1952) of stimulation and anesthesia, the former is brought about by factors which release calcium from the cortex of cells and this free calcium enters the interior to activate an enzyme which can cause either clotting or liquefaction. If the factor is a stimulating agent, internal clotting results. Anesthetics likewise may release calcium from the cortex of cells, but the released calcium is prevented by the anesthetic from causing a clotting reaction; at least, this seems to be true of fat solvent anesthetics. It may be noted that many agents which cause artificially induced setting in *Bugula* larvae also activate marine ova, and according to the theory of calcium release these should be considered stimulating agents. Many factors which inhibit fixation of *Bugula* larvae also prevent cell division in marine ova. These agents which depress vital functions may be considered anesthetics in the broad sense. If one excludes from the list of inductors of fixation, benzoquinone, certain oxidizing agents, and both magnesium-free and irradiated sea water, all the others have been reported by various investigators as activators of marine ova. On the other hand, of the inhibitors of metamorphosis only darkness, cysteine, and mixtures of sea water and isotonic KCl have not been reported as agents which either inhibit mitotic division in marine ova or lower the viscosity of protoplasm (e.g., thioglycollate has been reported to keep the protoplasm of certain ova in a fluid condition by Wicklund *et al.*, (1953). Clearly some inhibitors of setting interfere with cellular enzymes (azide inhibits cytochrome oxidase) or block energy transport (dinitrophenol apparently does this). It should be noted, however, that the potassium ion can act either as a stimulant or as an anesthetic; and the action of potassium cyanide is quite anomalous, causing coagulation in some eggs (*Arbacia*) and preventing mitotic gelation in others (*Chaetopterus*). Indeed, one of the most baffling problems confronting the physiologist is that several agents may have quite opposite effects in different concentrations or when various experimental techniques are used. Thus, under factors which delay

cleavage in *Arbacia* eggs Harvey (1956) lists at least ten agents, including vital dyes, urethane and chloral hydrate, which can either activate marine ova or accelerate mitosis in fertilized eggs. The action of calcium-free sea water is especially hard to understand, for it inhibits fertilization but has also been reported to accelerate cleavage (Harvey, 1956, p. 155).

Perhaps the most convincing evidence in favor of the working hypothesis that artificially induced fixation involves calcium release can be obtained from data on the behavior of larvae of *B. simplex* at moderately low temperatures. It has long been known that temperatures of 0° C. and below have a stimulating effect on organisms and tissues. Thus, a frog's heart which has stopped beating in a calcium-free Ringer's solution can be made to beat again by a sudden exposure to –2° C. (Heilbrunn, 1952, p. 539). And temperatures of zero and below have been found somewhat effective in causing parthenogenesis in *Chaetopterus* ova (Heilbrunn and Wilson, 1955). Yet work done on *Arbacia* eggs has shown that temperatures of 7° to 10° C. greatly increase the time between fertilization and the first cleavage (Harvey, 1956, p. 98). Now it had been noted in 1944 that larvae of *B. neritina* placed in sea water at about 5° C. had a prolonged natatory period, as reported by Marcus (1926*b*) for other bryozoans; later it was confirmed that larvae of *B. simplex* behaved in much the same way in sea water at 5° to 12° C. (and incidentally *Amaroecium* tadpoles are inhibited at 9° C.). If, on the other hand, larvae of *B. simplex* are suddenly immersed in sea water at 0° to –3° C., they will eject their holdfasts within thirty minutes. If the larvae become rigidly attached and the sea water is then allowed to reach room temperature, the larvae will complete metamorphosis and form zoids; but if the Stender dishes containing the larvae are left for too short a time in finger bowls containing mixtures of salt and ice, the adhesive substance is often withdrawn within the bodies of the larvae and they eventually swim away. But temperatures of 0°C. and below will not induce fixation in larvae kept in calcium-free media. Nor will the larvae attach at room temperature in either oxalated or calcium-free sea water. Apparently, then, calcium *seems* to be involved in artificially induced fixation. It is proposed, therefore, that in *Bugula* larvae, ejection of the holdfast and the subsequent gelation of the adhesive substance triggers the mechanism of metamorphosis.

The hypothesis that the artificial induction and inhibition of fixation are essentially similar to stimulation and anesthesia, while serving as an excellent guide for a program of organized research, introduces problems that have not yet been solved. It is not too difficult to consider that inhibition of fixation would be brought about by narcotics or by agents which inhibit enzymes or interfere with

energy transport. Presumably the mechanism of metamorphosis fails to respond in the presence of these agents just as a dividing cell ceases to function in a similar environment. And metamorphosis, being explosive in nature, probably requires the expenditure of considerable energy. But if the working hypothesis is accepted that prematurely induced fixation is a kind of stimulation involving calcium release, one may well ask what mechanism or anatomical part of the organism is affected by the released calcium or by an enzyme activated by this ion. There are many lacunas in our knowledge of the mechanism involved, but there are some possibilities to consider.

First, if one conceives that the mesodermal tissue comprising the internal sac contains contractile elements or that muscles actually cause ejection of the holdfast, as seems likely in *Flustrella hispida,* calcium may indeed cause contraction of the sac and expulsion of the adhesive substance. Heilbrunn (1956) has devoted a chapter to muscle contraction in which he has summarized evidence that calcium is involved in the contraction of a muscle. Secondly, if the adhesive substance is a mucoprotein, as it appears to be in several sessile organisms (Pyefinch and Downing, 1949), it might well be that calcium is involved in increasing its viscosity to such a degree that it rigidly attaches the larva to a substrate. It is well known that part of a mucoprotein consists of highly polymerized chondroitin sulfuric acid. And chondroitin sulfate has a great affinity for calcium to which much of it is bound in tissues (White *et al.,* 1954, p. 811). On the other hand, inhibitors of metamorphosis might prevent both ejection of the holdfast and coagulation of the adhesive substance. Even so active an organism as an *Amaroecium* tadpole ceases movement almost immediately on being placed in sea water containing potassium cyanide or sodium azide. It may remain in a state of "suspended animation" for hours. Since its motion depends on muscular contractions, it would appear that these substances as well as cold sea water, $MgCl_2$ and urethane, do prevent contraction of its muscles. And since several inhibiting agents of *Bugula* larvae, notably thiourea, phosphate buffers, and antimitotic substances from ovaries, cause leakage of the cementing material while the larvae are still swimming slowly, it would appear that some inhibiting factors cause the contents of the internal sac to be more fluid than it normally is. Nitsche (1870) has reported that this occurs in larvae under normal conditions, but the writer has never observed it.

The hypothesis just discussed has the obvious weakness of being based on analogies. If we accept the possibility that contraction of a muscle is activated by calcium, we are embarrassed by the fact that agents which inhibit or induce larval fixation resemble those which affect marine ova more than those involved in muscular con-

traction. Although Heilbrunn (1952, p. 410) considers a muscle cell to be essentially like a sea urchin egg or an ameba in having a stiff cortex and fluid interior, its responses to certain agents such as acids do not appear to be the same. Furthermore, we are not certain that the cementing substance would behave like the colloids of living cells.

We have discussed the possibility that artificially induced fixation may involve calcium release, but so far nothing definite can be said concerning the normal mechanism of attachment and metamorphosis. We may consider, perhaps, that the mechanism of fixation is in a state of inhibition and that some substance gradually accumulates while the larva is swimming (or before its release into the sea water). On reaching a certain critical concentration this substance may cause a sudden contraction of the internal sac. The substance need not be calcium, although it could be, for any factor causing calcium release would suffice. It would be of interest to determine with a spectrophotometer whether or not larvae do accumulate calcium as some marine ova do after fertilization (Oström and Oström, 1942). If this could be done, it would shed light on the inhibiting effect of calcium-free sea water. The possibility of thigmotropic stimulation arising from movements of the vibratile flagella should also be investigated.

At the present time no suitable explanation can be offered for the similarity of effect of at least sixteen different agents on bryozoan and ascidian metamorphosis. One student at Woods Hole who has observed attachment in *Amaroecium larvae* extensively has gained the general impression that metamorphosis in this organism is triggered by ejection of the holdfast material. The above hypothesis might explain why copper, hypertonic solutions, a brief exposure to distilled water, and other agents initiate metamorphosis by releasing calcium. But the stubborn fact remains that *Amaroecium* larvae can undergo metamorphosis to the advanced stage at which the branchial basket is clearly recognizable without being attached at all, as they do after being removed from sea water containing potassium cyanide (Lynch, 1959). In this respect they differ from *Bugula* larvae, for the writer has never seen metamorphosis occur in these organisms without fixation. Perhaps some inhibitors of metamorphosis in *Amaroecium* larvae block energy transport; others through a narcotic action may interfere with the movement of cells necessary for changes that occur during the transformation of a larva into an adult. It may also be possible that agents which induce premature fixation hasten the onset of metamorphosis, even though metamorphic changes can occur without attachment. Experiments involving removal of the tadpoles from sea water containing urethane, azide or potassium cyanide

seem to indicate that quiescence plus a favorable environment may be necessary for metamorphosis. When *Amaroecium* larvae are seeded in KCN-sea water and left in this medium for eight hours, swimming ceases immediately on placing the tadpoles in a new environment and only very rarely do any metamorphic changes ever occur. If the larvae are then removed to sea water they neither swim nor attach to the substrate, but metamorphic changes begin and the organism can reach at least the stage in which the branchial basket is formed. Observations have not been carried out further than this stage, for further development usually requires frequent additions of fresh sea water, and this is almost impossible with larvae that are not attached.

It will be evident from the foregoing remarks that considerably more work must be done on either *Bugula* or *Amaroecium* larvae before a clearly acceptable hypothesis of metamorphosis can be set up. The work done so far merely indicates the possibilities of investigation in an interesting but complicated field.

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