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INITIATION OF METAMORPHOSIS IN LABORATORY CULTURED SEA URCHINS

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Many benthic marine animals release their gametes, embryos, or larvae into the water column. The offspring subsequently enter the adult population through settlement and metamorphosis.

Sea urchins are one such animal. They have highly differentiated larvae that undergo a complex metamorphosis. The scant information on their metamorphosis has been reviewed by Hyman (1955). MacBride (1903) was the first to prepare a comprehensive description of metamorphosis. He used *Echinus esculentus* eggs that were fertilized in the laboratory. The larvae were fed on organisms collected along with the seawater in which they were raised. Earlier workers used field-collected larvae. Neither of these methods could have yielded consistently healthy and uniformly developing animals. With the development of a culture technique for sea urchins (Hinegardner, 1969), large and uniform populations of healthy animals became available for study.

These laboratory raised larvae can reach a state of competence to undergo metamorphosis at about 21 days of age (Fig. 1a). Complete metamorphosis from feeding larvae to feeding adult takes 5 or 6 days. This includes the development of adult internal organs as well as the formation of the adult mouth and anus. The change in external shape from larval to adult, however, takes less than an hour. Parts of this sequence are illustrated in Hinegardner (1969). The terms we will use to designate larval and adult structures are from Hyman (1955). The series of events is: (1) the tube feet protrude from the opening in the vestibule. (2) The left postoral and left posterodorsal arms bend to the left of the anteroposterior axis. All the other arms bend to the right. This movement makes it possible for the urchin rudiment to reach the substratum (Fig. 1b). (3) The five primary tube feet attach to the surface and the spines protrude from the vestibule. (4) The thickened ciliary bands, called epaulettes, collapse and the tissue on the larval arms retracts from the spicules supporting them (Fig. 1c). The three pedicellariae come to lie evenly distributed on the aboral surface which has assumed a smooth rounded shape (Fig. 1d). These changes take an hour or less (Bury, 1895; Hinegardner, 1969).

After 24 hours the genital and ocular plates become more calcified. The spines, formed during the development of the urchin rudiment, elongate. The spines on the plates bearing the pedicellariae elongate also. At this point the exterior of the organism looks very much like a small adult urchin.

Very little is known about the factors that induce echinoderm larvae to settle. The larvae of the sea star, *Mediaster aequalis,* settle on the tubes of the polychaete, *Phyllochaetopterus prolifica.* If the tubes are absent, the larvae can delay metamorphosis up to 14 months (Birkeland, Chia and Strathmann, 1971). Sea

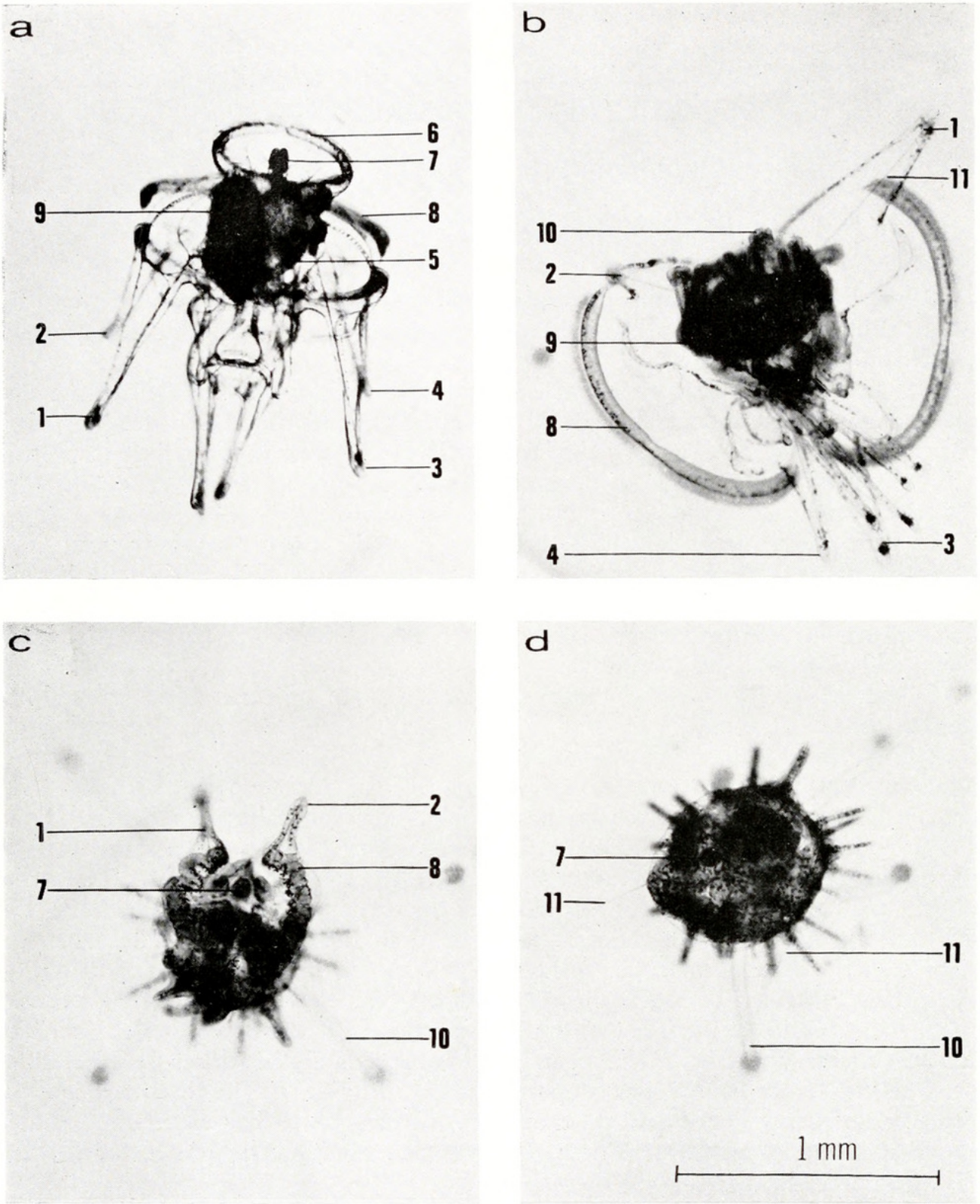

FIGURE 1. Metamorphosis of *Lytechinus pictus* (A.) A twenty-one day old larva competent to metamorphose is shown in frontal, or ventral, view with urchin rudiment on the left. (B.) A larva with arms bent, photographed 5 min after being placed in active seawater. This view is from below with the left side approaching the surface. (C.) This is an individual with much of the larval epithelium collapsed, 35 min after bending began, viewed from above, showing the folded ciliary band. (D.) An individual, viewed from above, in which the tissue collapse is completed, 60 min after bending began. One of the pedicellariae can be seen on the aboral surface. Legend is: 1, left postoral arm; 2, left posterodorsal arm; 3, right postoral arm; 4, right posterodorsal arm; 5, stomach; 6, upper epaulette; 7, apical pedicellaria; 8, lower epaulette; 9, urchin rudiment; 10 tube feet; 11, larval spicule.

urchins do not metamorphose spontaneously, either, but they will if presented with a film of bacteria and algae (Hinegardner, 1969). As we will show, only the bacteria are actually necessary.

Materials and Methods

The stimuli that initiate metamorphosis in sea urchins were analyzed using the following procedure.

Larvae of *Lytechinus pictus* and *Arbacia punctulata* were reared from the gametes of adults maintained in the laboratory at Santa Cruz. During the summer of 1971, larvae of *Arbacia* were reared at Woods Hole, Massachusetts using local material. The larvae were reared by the method of Hinegardner (1969) with slight modifications. At the onset of feeding (48 hours post-fertilization), 400 healthy plutei were transferred to 6 l of 0.45 μm Millipore filtered seawater. The cultures were maintained at 18° C and stirred with a paddle attached to a 30 RPM clock motor. The cultures were fed thrice weekly and the water changed weekly. *Lytechinus* larvae were fed an unidentified species of the cryptophyte, *Rhodomonas*, and *Arbacia* larvae were fed the chlorophyte, *Dunaliella tertiolecta*.

Tests for the factors involved in the initiation of metamorphosis were performed in polystyrene petri dishes of 2 sizes: 60 × 15 mm and 35 × 10 mm. Sometimes glass petri dishes of the larger size were also used. In each experiment at least two controls were used. One was to insure the competence of the larvae to undergo metamorphosis. This was prepared by placing a plastic petri dish in an established aquarium for several days, whereby it acquired a bacterial film which is a potent stimulus for metamorphosis. The other control was for handling and ambient conditions. It consisted of sea water known not to initiate metamorphosis, either sea water from the larval cultures or fresh, filtered sea water. Ten larvae were added to each of the control and test dishes. The results were recorded after 4–6 hours and the dishes set aside to be checked for normal development after 24 hours.

When necessary, controls for both the possible inhibiting and enhancing effects of the materials used in chemical separations were included in the experiments. The material in question was added to a dish of sea water capable of initiating metamorphosis (*active sea water*). If no decrease in the number of larvae which underwent metamorphosis was observed, then the material was regarded as not inhibitory. Similarly, if no increase in metamorphosis was observed when the material was added to the sea water incapable of initiating metamorphosis (*inactive sea water*), then the material was regarded as not enhancing metamorphosis. Between the test and control dishes, a difference of 20% in the behavior of animals was regarded as insignificant.

The source of the sea water throughout these experiments was the Hopkins Marine Station, Pacific Grove, California. This water was collected and stored in opaque 60 l polyethylene barrels.

Quantities of crude active sea water were prepared in various polyethylene and glass containers of 4 to 20 l by incubating the particulate material from our aquarium filters, or the sediment from the bottom of the storage barrel, in sea water at 15° C for at least 2 days. Both pH and osmolarity were measured for each batch.

Initiation of metamorphosis can be induced electrically. We used standard neurophysiological apparatus, including a suction electrode with a tip diameter of 90 μm. The electrode was used in other experiments for just holding the larvae.

The role of surfaces in initiation of metamorphosis was tested using both glass and plastic petri dishes. The glass dishes were roughened with grinding compound and the plastic dishes with a small rotary brush. Glass spheres of 140 μm diameter were also added to a set of dishes.

Results

The first three events of metamorphosis (extension of the tube feet, bending of the arms, and attachment of the tube feet) are reversible. The larva may bend its arms, then later return to the original larval shape. Sometimes a larva attaches its tube feet only to let go and swim away. The bending, but not the epaulette collapse and tissue retraction, can be induced by acid pH (6.5) or a slight decrease in osmolarity (from 1080 to 960 milliosmoles). The collapse and retraction are irreversible and, if they begin, metamorphosis follows.

Table I

Per cent metamorphosis of Lytechinus pictus *larvae on various combinations of inactive or active sea water, bacterial film and surface. See text for further explanation of experimental conditions.*

Sea water	Film	Surface	% Metamorphosis	No. tested
Inactive	Absent	Glass	0	10
	Absent	Glass spheres	0	10
	Absent	Rough glass	0	10
	Absent	Plastic	3	200
	Absent	Rough plastic	0	10
	Present	—	62	40
Active	Absent	Glass	97	30
	Absent	Glass spheres	70	10
	Absent	Rough glass	75	20
	Absent	Plastic	93	200
	Absent	Rough plastic	75	20
	Present	—	90	200

A crude system that induces complete metamorphosis in both *Lytechinus* and *Arbacia* larvae is a petri dish with a prepared bacterial film (see Materials and Methods). Tests of individual components of this system (bacterial film, surface of the dish and water) on *Lytechinus* larvae showed that while the film was important, the critical component was also in the water (Table I). Whether the surface was glass or plastic, rough, smooth or covered with glass beads made little difference. Neither adult *Lytechinus* from our aquaria nor the brown alga *Macrocystis* on which they fed provide the cue for metamorphosis when placed in freshly filtered sea water. The osmolarity of active sea water which provided the cue for metamorphosis of both *Lytechinus* and *Arbacia* larvae did not differ from that of filtered sea water. The pH was 7.4–7.6, while filtered sea water was

8.0–8.2. Raising the pH of active sea water to 8.2 did not alter its ability to induce metamorphosis.

The volatility of the active factor in sea water was tested by placing two containers beneath a 500 ml bell jar. One was a test petri dish with larvae in 10 ml of inactive sea water and the other contained 200 ml of active sea water. After 24 hours, the ability to initiate metamorphosis was not transferred through the air. Furthermore, the active factor was not driven out of the active sea water by converting it to an aerosol which was then collected.

The ability of decolorizing charcoal (Norit A, Amend Drug and Chemical Co., New York, New York) to remove the potential to initiate metamorphosis from active sea water was tested. The charcoal was added to filtered active sea water and then removed by filtration through Whatman #1 filter paper. During this treatment active sea water changed from a pale yellow to colorless and lost the ability to initiate metamorphosis; however, it was no more inhibitory when mixed with active sea water than was filtered sea water. Other possible effects of the charcoal were excluded by the appropriate controls.

The organic nature of the active factor was tested by ashing. Eight grams of particulate matter were removed from one liter of active sea water by filtration through Whatman #1 filter paper and then ashed in a porecelain crucible to constant weight over a gas flame in air. This yielded a gray powder. No metamorphosis occurred when the ash was added to filtered sea water in one, five and ten times the original concentration. Appropriate controls excluded any inhibition by the ash.

The activity will pass through a 0.22 μm Millipore filter, indicating that it is not carried on small particles. Sometimes the activity becomes trapped on the particulate matter retained on the filter. This can be avoided by pre-centrifugation for half an hour at 13,000 g. The Millipore filter must be rinsed before use with at least 10 ml of sea water or glass distilled water per square cm of surface in order to remove toxic components.

Activity will also pass through a centrifuge ultrafilter (Centriflo filter, Amicon Corp., Lexington, Massachusetts), which excludes molecules of 50,000 MW and larger. The filter cones were thoroughly washed with glass distilled water and filtered sea water before use.

The ability of the active factor to pass through a dialysis membrane was tested. Dialysis tubing (Van Waters and Rogers, San Francisco, California, pore size = 24 Å) was prepared by boiling 20 minutes in sodium dodecyl sulfate, boiling 10 minutes in glass-distilled water and soaking 20 minutes in 10^{-4} M EDTA, followed by storage in glass-distilled water. In another method of preparation, the tubing was merely soaked in sea water before use. We found no difference between these two methods. A twenty milliliter aliquot of active sea water was placed inside the tubing and 5 ml of inactive water outside. The fractions were tested after 4 hours. The active factor passed through the dialysis membrane. Controls showed that the membrane had no effect itself. The rate the activity moved through the membrane suggests that the active element had a molecular weight less than 5,000.

We have found that electrical stimulation of competent *Arbacia punctulata* larvae induces the events of metamorphosis through tissue retraction from the

larval arms. A stimulus of 150 volts delivered for one millisecond at one second intervals induces bending of the arms and retraction of the tissue from them. In ten trials, the average time until the beginning of retraction from the posterodorsal arms was 223 seconds (s.d. = 148). Because the larval epithelium must be sucked into the bore of the pipette to hold the actively moving larva, the rest of the contraction would only occur if the larva was released. When it was, metamorphosis was normal and complete. *Lytechinus pictus* larvae did not undergo metamorphosis in response to electrical stimulation.

In about 80% of our tests, *Lytechinus* and *Arbacia* larvae would not undergo metamorphosis in active sea water if the rudiment tube feet were kept from touching a solid surface. This was done by holding the animals on a suction pipette or simply by turning them up side down and holding them that way. The remaining 20% did metamorphose without tube feet attachment. Repeated brief touching of the larval epithelium, vestibular pore, adult spines or even the tube feet would only cause bending of those that would not metamorphose. However, if the larvae were allowed to hold on to a solid surface for several minutes complete metamorphosis was induced.

Larvae will metamorphose in active sea water with various portions of the epithelium removed by surgery. Several individuals metamorphosed even though the entire upper epaulette was removed.

Discussion

We have identified two stimuli that are needed for the initiation of metamorphosis: (1) a chemical cue is of prime importance, (2) tube foot attachment to a surface is usually necessary. Experiments suggest that the chemical cue is a non-volatile compound of bacterial origin. It is probably organic since it is lost by ashing and removed by decolorizing charcoal. It is non-particulate since it passed through a 0.22 μm Millipore filter, a cellulose acetate ultrafilter, and a dialysis membrane. The characteristics of the ultrafilter put the molecular weight at less than 50,000. The speed of passage through the dialysis membrane suggests the molecular weight is probably less than 5,000.

Bacterial films and the sea water associated with bacterial and organic particulate matter kept in the dark, can both induce metamorphosis. This indicates that the factor is of bacterial origin. Bacterial films have been implicated in the settlement of other larvae (Wilson, 1955; Meadows and Williams, 1963). Large beds of the sea urchin *Strongylocentrotus purpuratus* associated with leptopel near sewage outfalls have been reported (North and Pearse, 1970). Perhaps these populations arose from larvae settling in regions rich in organic matter and bacteria. Formation of the factor appears to require special conditions. So far we have not been able to isolate a bacterium from the active sea water preparations that can initiate metamorphosis or that can impart that capacity to the sea water in which it was grown. Several enriched and defined media have been tried.

Efforts have been made to understand the stimuli for settlement of other marine animals (see review by Meadows and Campbell, 1972). For example, the serpulid polychaetes, *Spirorbis* spp., exhibit striking substrate preferences. Four different species of this genus each prefer a different species of alga for settlement (de Silva, 1962; Gee and Knight-Jones, 1962). The specific stimulus that

Spirorbis larvae receive from algae is chemical (Williams, 1964; Gee, 1965). The cyprid larvae of barnacles settle in response to an adsorbed chemical (Crisp and Meadows, 1963). Sediments are important in inducing the settlement of the larvae of the snail, *Nassarius obsoletus*. The inducing properties of these sediments can be transferred to the adjacent water (Scheltema, 1961). On the other hand, Wilson (1952) has shown that *Ophelia bicornis* larvae settle on sands taken from the area in which the adults live even if these sands are treated to remove organic matter. Also his experiments suggest that some sands carry a substance repellant to settlement. The work reported here suggests the presence of a specific attractant to metamorphosis in sea urchins.

We have not been able to extract and recover the chemicals necessary for the initiation of sea urchin metamorphosis using published methods (Jeffrey and Hood, 1958; Siegel and Degens, 1966). The active element is probably present in very low concentrations, as suggested by the fact that it is sometimes removed by particles caught on membrane filters. Another problem has been our inability to remove all toxic contaminants from organic chemicals used in extraction procedures. The acid pH of active sea water suggested the use of anion exchange resins as a method of separation. But even the eluents from extensively washed resins were toxic to the larvae.

The form assumed by a dying larva looks enough like a metamorphosed individual to be confused with it. Only by observing test animals after 24 hours can healthy young urchins be distinguished from dying larvae. None of the early workers did that and as a consequence erroneous conclusions have been drawn. For example, experimental work by Huxley (1928) using mild mercuric chloride poisoning, led him to suggest that metamorphosis was due to metabolic differences between larval and adult tissues. He was probably seeing dying animals.

How the larvae bend during metamorphosis is not well understood. Von Ubisch (1913) reports muscle bundles in the wall of the larval somatocoel. These bundles could provide the force to bend the larva. This is compatible with the reversible nature of the bending and its initiation by changes in pH and osmolarity.

The manner in which the larvae interact with the chemical cue is unknown. Since all portions of the larval epithelium have been surgically removed in one or another of the ablation experiments, it is safe to say that a discrete sensory structure does not exist there. Since the tube feet respond to a surface, possibly they or other parts of the urchin rudiment begin the response to the chemical cue. Another possibility is that the chemical interacts with the cells in the epithelium. Changes in surface properties could be allied with cell movement. Electrical stimulation could bring about such changes. Studies are being undertaken to address these questions. Further characterization of the chemical cue must await more sensitive and efficient organic chemical methods for sea water.

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Summary

A method is described for studying the initiation of metamorphosis in laboratory cultured sea urchins. Two stimuli are usually needed to initiate metamorphosis: a non-particulate organic chemical cue and a surface. The chemical probably has a molecular weight of less than 5,000 and is of bacterial origin. Electrical stimulation can be used to initiate metamorphosis of *Arbacia punctulata* larvae.

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