

GENERAL METHODS

Collection of animals

At the Marine Biological Laboratory, the collecting is done, for the most part, by the Supply Department, and the investigators usually do not obtain their own material. This system is a very convenient one when the collecting staff is experienced, so that they realize the advantages of freshly collected material over that aged in a floating stage; in addition, the collectors must know the best means of transporting the animals from the collecting grounds to the Supply Department, the handling and maintenance of material after it reaches the Department, and the importance of delivery to the investigator as soon after collection as possible. It goes without saying that they must also be equipped with the best possible boats, dredges and other collecting apparatus, and that they must have an accurate knowledge of the availability of a given form. The Supply Department has met all these requirements with a high degree of success.

In other laboratories, where experienced collectors are not a part of the staff, the investigator has an opportunity to learn at first hand the breeding-habits and general ecology of the animals with which he is working; he can ascertain the best methods of collecting, transporting and maintaining them in order to provide himself with the best possible experimental material. Some of the general factors to be kept in mind include the following:

- (1) Avoid trauma to the animals, both during and after collection.
- (2) Avoid marked temperature changes, maintaining the specimens at all times as nearly as possible at the temperature of their natural environment.
- (3) Do not crowd the animals; adequate supplies of sea water and oxygen should be provided during collection, transportation, and maintenance in the laboratory.
- (4) Conserve the supply of living material, taking no more of a given species than necessary and leaving an adequate breeding population. Whenever possible, unused animals, or spent animals which have been induced to shed, should be returned to their natural habitat.

Sexing adults

If the differences between the two sexes of a dioecious species are such that the investigator cannot distinguish them on the basis of external characteristics, there are several methods for ascertaining sex without wasting material. (1) A few drops of gonad material can be withdrawn, by means of a hypodermic syringe and fine needle. The needle is inserted at the hinge-line in the case of *Macra*, through the peristome of *Arbacia*, or through an arm for *Asterias*. This material, when examined, should contain eggs or sperm if the individual is mature. (2) Certain of the methods used for inducing shedding (electrical stimulation, KCl-treatment, or reactions to gamete-water) enable one to ascertain sex merely by looking at the gametes.

Obtaining gametes

After the adult animals are collected and brought into the laboratory, they are usually kept in aquaria or other containers supplied with running sea water. Only

a few species shed their gametes spontaneously, and it is therefore necessary to have methods for inducing them to shed at the time when the gametes are needed by the worker. The crude older technique of cutting open the animals and removing the gonads is wasteful of material, and often yields gametes so contaminated with body fluids that they are non-fertilizable. Recent methods that have been developed to induce shedding include (1) injection of isosmotic KCl (first described by L. P. Wilson for *Arbacia* and subsequently modified in various ways for use with a number of other forms); (2) temperature shock (an imitation of one of the tidal effects on littoral animals); (3) electrical stimulation (used very successfully by Iwata in Japan for several echinoderms and subsequently adopted, with modifications, by E. B. Harvey in this country); (4) mechanical stimulation, such as shaking the adults in a bucket of sea water; (5) altering the normal periods of light and darkness (see the section on *Styela*, for example); and (6) adding gametes (or gamete-sea water) of the opposite sex. For species which fertilize their eggs internally, of course, such methods do not apply, and there are certain other forms for which removal of the excised gonads to dishes of sea water is apparently the only practicable method for obtaining eggs and sperm. "Stripping" the gametes is the method of choice for most teleosts.

Insemination

Generalizations about the exact conditions which will result in normal fertilization are difficult, because the several species vary considerably in such particulars as optimal sperm concentration, degree of washing (if any) of the eggs prior to insemination, etc. However, it is, in general, important to avoid excessive concentrations of sperm. In addition to promoting polyspermy and abnormal development in some species, excess sperm also contribute (after a short time) excess carbon dioxide, decomposing proteins, and a rich culture medium for bacteria which hasten the fouling of the culture. Another general principle is to avoid contaminating the eggs with body fluids (or portions of the shell or test, in the case of echinoderms); as Lillie early demonstrated, such fluids have a deleterious effect on the fertilization-reaction. Mucous secretions of the female (as in the case of *Chaetopterus*) may also constitute a structural barrier to fertilization. A third generalization is that the eggs should never be crowded: one layer on the bottom of a dish gives much better results than multiple layers. For eggs that elevate fertilization membranes or extrude marked quantities of jelly as a part of the fertilization-reaction, the eggs should be even more widely spread.

Raising larvae to later stages of development

After embryos have used up the stored yolk, oil, and other nutritive materials originally present in the egg, they must be supplied with appropriate food if development is to continue. Diatoms are the chief food for marine invertebrate larvae such as trochophores, bipinnaria, etc. Diatom-culture methods are described in the papers by Just (1922, 1939), Galtsoff *et al.* (1937) and Grave (1902). There are several laboratories which maintain one or more strains of *Nitzschia* and other diatoms in pure and in mixed cultures, and it is often possible to obtain inocula from such cultures. Certain marine protozoa are useful for feeding some types of larvae and older stages will often thrive on a diet of mixed plankton. *Fundulus* hatchlings can be fed *Nereis* trochophores.

Methods of observation

In addition to the usual light-field and dark-field microscope, excellent use can be made of several other types of optical equipment. These include:

(1) The phase contrast microscope. This is especially useful for sperm and for very slender structures, such as sperm filaments. However, it is much less useful for larger structures (such as eggs), especially those with jelly-layers and membranes which refract light.

(2) The polarization microscope gives superb visualization of living spindle fibers and of other structures with oriented molecules, such as the spicules of echinoderm larvae. Unfortunately, the birefringence of most organelles (except calcified structures) of eggs and embryos is rather low. A quarter-wave plate greatly enhances the beauty of polarization-images.

(3) The ultraviolet microscope may be used to demonstrate relative concentrations of ultraviolet-absorbing materials (proteins, amino acids, DNA, etc.) in various regions of *thin* structures. By means of a quartz monochromator and the use of special stops, it is possible to obtain a crude absorption spectrum of certain cell-components. Obviously, quartz slides and coverslips must be used with the ultraviolet microscope.

(4) The newly designed interference microscope may eventually be put to interesting uses.

(5) The electron microscope is of no value with living material. Under carefully controlled conditions of fixation and drying of thin material, or by still more carefully controlled embedding and sectioning methods, in the hands of experts, some useful information about the micro-structure of eggs, sperm and embryos may be obtained.

(6) Water-immersion lenses (with all metal parts coated, so that heavy metals cannot affect the eggs) are very useful.

For investigators who are not burdened with excessive and elaborate equipment, but who are capable of making their observations largely with the use of a good compound microscope, there are a few refinements of technique which will greatly aid the perception of microscopic detail:

(1) A good north light, and centered microscope diaphragm (to reduce the illumination and reveal delicate structures).

(2) Critical illumination, if artificial light is to be used.

(3) The use of a Chinese ink suspension to provide a background against which will be revealed the thickness of transparent jelly-layers, etc. This must always be prepared *fresh*, immediately before use: carefully rub the end of a dry stick of Chinese ink on a finely ground glass slide (wet with sea water) until a dark, fine and uniform suspension is obtained.

(4) A very dilute suspension of Janus green (two drops of a 1:1000 solution, made up in distilled water, added to about 20 cc. of sea water) will faintly stain the jelly of certain eggs (*Arbacia*, *Asterias*, etc.), without injuring them appreciably. The membranes, only, of certain other eggs will absorb the dye, and there are also cases where both the jelly and the membranes are stained. For true *intra vitam* staining with Janus green, however, a zinc-free preparation must be obtained.

(5) For quieting larvae, so that details of larval structures (including ciliation) are more easily seen, the following methods can be used:

(a) More concentrated Janus green (one cc. of 1% Janus green in distilled water, added to 10 cc. of sea water).

(b) Squid egg-string extract, especially for echinoderm larvae (see the section on *Asterias*, p. 172).

(c) Shredded lens paper (usually not very satisfactory).

(d) Chloral hydrate or magnesium sulfate (the concentration for each species must be ascertained by trial and error).

Glassware

It is essential that the glassware used for living material be completely free of all toxic substances. Washed new glassware (which should then be reserved specifically for living material) is much safer than glassware previously used for various unknown chemical procedures. Just (1939) discusses this problem in detail. He has given excellent advice, also, on utilization of sea water, and on the uses of thermometers and stop-watches in embryological research.

Moist-chambers, for keeping embryos or larvae so that there is relatively little evaporation of the sea water medium in which they are being raised, can be made from crystallization-dishes with covers. Large fingerbowls (with provision made for covering them, either with large glass plates or with other fingerbowls) may be used if crystallization-dishes are not available. The culture dishes (Columbia watch glasses, depression slides, small stender dishes, etc.) are supported on a platform composed of a round glass plate cut to slightly less than the diameter of the moist-chamber; this platform can conveniently be arranged to rest on four square embryological watch glasses ("salt cellars"). The bottom of the moist-chamber is filled to a depth of about one-fourth inch with slightly diluted (about 90%) sea water, to maintain the proper humidity. Some workers prefer a paraffin-coated low hardware-cloth rack to the round glass platform.

Instruments and miscellaneous equipment

The experimental embryologist who works with marine material makes use of many of the instruments originally devised for operative techniques in vertebrate experimental embryology. (See the book by Hamburger, 1942.) These include iridectomy scissors, watchmaker's forceps, hair loops, sharp steel beading needles (which can be fused into handles made of glass tubing), glass needles, etc. Another most useful implement for moving eggs without injury from one container to another is the mouth pipette. This consists of a pipette drawn out to the requisite bore, attached to a piece of clear gun-rubber tubing (about 3 mm. in outside diameter and about 36 inches long); a mouthpiece made of fire-polished glass tubing is provided. Wooden blocks about two inches in thickness, with a series of holes drilled in them, are useful as racks for glass needles, hair loops, etc. They should be provided with glass or rigid plastic bell-jar covers.

Since most of the marine invertebrate eggs are very much smaller than the traditional amphibian and chick material, the hair loops should be smaller in diameter and made of finer hair than those used by the worker with vertebrate eggs and embryos. The steel forceps and needles must be sharpened to very fine points

(utilizing an Arkansas stone and a dissecting binocular microscope for the sharpening process), and the glass needles drawn out to points of the requisite diameter, length, sharpness and flexibility for particular purposes on a given material. It goes without saying that all precautions should be taken to protect expensive steel instruments from the corrosive effects of sea water.

Operating dishes for the small invertebrate eggs can be readily made by filtering a few drops of hot, freshly prepared 2% agar in sea water into Columbia watch glasses. (It is convenient to use a metal, water-jacketed funnel for filtering the agar, so that the material remains liquid until it touches the glass dish.) Hardening of the agar occurs as it cools, and it makes a resilient surface on which eggs can be cut with glass needles. Such dishes are used, also, for raising embryos denuded of their membranes, for isolated blastomeres, and for other delicate embryos or embryo-parts; the agar prevents cells from attaching and sticking to the glass bottoms of the dishes. Hörstadius (1937) uses washed photographic film for an operating surface.

At most marine laboratories, high atmospheric humidity presents a number of problems to workers, especially when histological or cytological preparations are being made. We have found it most helpful to use an ordinary commercial infrared lamp (either red, with a "built-in filter," or plain white) in a goose-neck desk lamp from which the shade has been removed so that the large bulb will fit. The beam from such a set-up can be directed toward the area where one is working, to facilitate the final steps of dehydration and clearing. Sometimes, it will also prove helpful to pre-heat the slides and/or coverslips to be used; an ordinary slide-warmer is convenient for this purpose.

Other useful but more specialized items of miscellaneous equipment are described in connection with the forms for which they are most often utilized.

Formulae

Formulae for artificial sea water, calcium-free sea water, fixatives, stains, etc., have, for the most part, been omitted from this manual. The Chemical Room of the Marine Biological Laboratory at Woods Hole publishes, from time to time, a revision of its compilation, "Formulae and Methods," which includes such information.

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