REARING OF BIVALVE MOLLUSKS

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I. Introduction

Until recently rearing of larvae and juveniles of marine bivalves, on a basis where repeatable results could be expected, was virtually impossible because of the lack of satisfactory, reliable methods. Thus, although culturing of larvae of bivalves was first attempted in the last century, few workers succeeded in rearing them to metamorphosis and, as a rule, they were rarely grown beyond early straight-hinge stage. Even though, in the twenties, Wells (1927) was able to rear the American oyster, *Crassostrea virginica*, from artificially-fertilized eggs to spat, and Prytherch (1924) raised larvae of the same species in large numbers, their results could not be consistently repeated by other investigators. The failures were usually due to poor culture methods and want of good food for the larvae, especially when they were grown in heavy concentrations. It is also possible that diseases, including those caused by fungi, were responsible for the persistent failures.

Attempts to rear larvae of bivalves were not confined, of course, to *C. virginica*. Cultivation of larvae of several other species was also tried by early workers. For example, Belding (1912) attempted to raise larvae of clams, *Mercenaria mercenaria* (formerly *Venus mercenaria*), but without success. He concluded that there was no practical method for raising clam larvae to straight-hinge stage because of the small size and delicate nature of the egg. Wells (1927), however, was more successful and carried the clam larvae in his cultures until they metamorphosed.

Even in more recent years the situation remained practically the same. This is well demonstrated by the work of Yoshida (1953) who, in his attempts to identify larvae of Japanese bivalves, had to depend upon obtaining the larvae from plankton, instead of trying to grow them from fertilized eggs under controlled laboratory conditions where their identity would be assured. The difficulties experienced as recently as 1953 by Nikitin and Turpaeva (1959), in their attempts to raise larvae of some bivalves of the Black Sea by using old methods, vouch for the inefficiency of these now obsolete approaches.

Obviously, as the general studies of marine organisms progress, the necessity for methods by means of which bivalve larvae can be reared successfully becomes more and more urgent. The availability of such methods would immediately offer the opportunity to study the effects of numerous environmental factors, singly and in combination, upon the growth of larvae, thus helping to determine the physiological requirements of these organisms. It would also offer the means for studying the genetics of bivalves and initiating properly controlled experiments on selective breeding of these mollusks. Moreover, by

growing larvae under different conditions their diseases and parasites could be studied and methods for their control developed. Finally, because the larvae of many species of bivalves are much alike in size and appearance, it was virtually impossible to identify them, with any degree of accuracy, in plankton collections. With the recent development of methods of rearing larvae in the laboratory, however, this difficulty should soon disappear because larvae found in plankton can now be easily and accurately compared with preserved samples and photomicrographs of larvae grown from known parents under controlled conditions.

By using successfully conditioning and rearing methods, many aspects of which were developed at Milford Laboratory (Loosanoff and Davis, 1950; Loosanoff, 1954) and are described in this article, larvae of approximately twenty species of bivalves have been cultured at Milford. Not all of these species are indigenous to New England waters or even to our Atlantic coast. Several are native to the Pacific and one species came from Europe. The non-indigenous forms were representatives of commercially important species in which we were interested. The bivalves, the larvae of which have been reared from fertilization to metamorphosis, included the transverse arc clam, *Arca transversa*; the ribbed mussel, *Modiolus demissus*; the common mussel, *Mytilus edulis*; the bay scallop, *Pecten irradians*; the jingle shell, *Anomia simplex*; the European oyster, *Ostrea edulis*; the native Pacific coast oyster, *Ostrea lurida*; the American oyster, *Crassostrea virginica*; the Japanese oyster, *Crassostrea gigas*; Morton's cockle, *Laevicardium mortoni*; the hard shell clam, *Mercenaria* (*—Venus*) *mercenaria*, and its relative, *Mercenaria* (*— Venus*) *campechiensis*; hybrids of these two species; the Japanese clam, *Tapes semidecussata*; the small clam, *Pitar* (*—Callocardia*) *morrhuana*; the rock borer, *Petricola pholadiformis*; the razor clam, *Ensis directus*; the surf clam, *Mactra* (*—Spisula*) *solidissima*; the soft shell clam, *Mya arenaria*; and the common shipworm, *Teredo navalis*.

Of the above species the larvae of *Crassostrea virginica* and *Mercenaria mercenaria* have been studied most intensively and, as a result, we have accumulated an extensive knowledge of their physiological and ecological requirements (Loosanoff and Davis, 1950; Loosanoff *et al.*, 1951; Loosanoff and Davis, 1952a; Loosanoff and Davis, 1952b; Davis, 1953; Loosanoff, 1954; Loosanoff *et al.*, 1955; Davis and Chanley, 1956a; Davis, 1958; Davis and Guillard, 1958; Loosanoff, 1958a; Loosanoff, 1958b; Loosanoff, 1959; and Davis, 1960). Several other species, such as the European oyster, *Ostrea edulis*, and the Olympia oyster, *Ostrea lurida*, have also received much attention.

Most of the other species, however, were studied less intensively, work on them being confined to culturing their larvae and observing the appearance and general behavior of the latter. Naturally, our knowledge of the requirements of larvae of such species is still fragmentary but, nevertheless, we shall present the information already available even though it is admittedly incomplete.

II. Equipment

The rearing of larval and juvenile bivalves requires an adequate supply of sea water of proper salinity and free of substances that may interfere with their normal development. The water used at Milford Laboratory is pumped from the Wepawaug River at a point about 100 yd from its entrance into Long Island Sound. Because the tidal rise and fall in this area is from 6 to 10 ft, the flushing rate of this comparatively narrow and shallow inlet is relatively high.

The sea water is pumped into a 6 000-gal wooden storage tank located in the laboratory attic. Because pumping normally takes place 1½ hr before and after the high tide stage, the salinity of the water is usually near 27 parts per thousand, which is virtually the same as in Long Island Sound, where the majority of the forms, the larvae of which are described in this article, exists. To assure a supply of water of high salinity the intake of the salt water system is located approximately 4 ft below the mean low water mark; therefore, it is at a safe distance from the surface layers which, after periods of heavy rains, may be greatly diluted.

The main pump providing the laboratory with salt water is rubber-lined. The intake and distribution lines, as well as the check and cutoff valves, are made of lead. The faucets, however, are of hard rubber. The storage tank is of cypress wood and is painted inside with asphalt paint.

We prefer lead pipes because, although pipes made of several new plastics are nontoxic, light and inexpensive, they possess several important disadvantages. One of them is that since it is often necessary to reduce fouling inside of the pipes by treating them with hot water or steam, this treatment, commonly used with lead pipes, cannot be employed in systems containing plastic parts as it may cause damage, especially at the joints of the pipeline.

Another serious disadvantage in using plastics is that they adsorb and absorb many chemicals, including insecticides, and once contaminated can themselves become a source of later contamination of the sea water. Moreover, since some plastics are permeable to insecticides and other compounds, these materials might enter from the sur-

rounding soil into pipes carrying sea water. Finally, some laboratories that have plastic sea water systems have complained that since these pipes are not electrically self-grounded, they present a serious element of danger in laboratories with wet floors.

We are finding an increasing usage for plastic pumps and pipes, especially in our temporary installations. We have also found that tanks made of Fiberglas, instead of wood, can be advantageously used, especially in areas where wood-boring organisms, such as *Teredo*, are common.

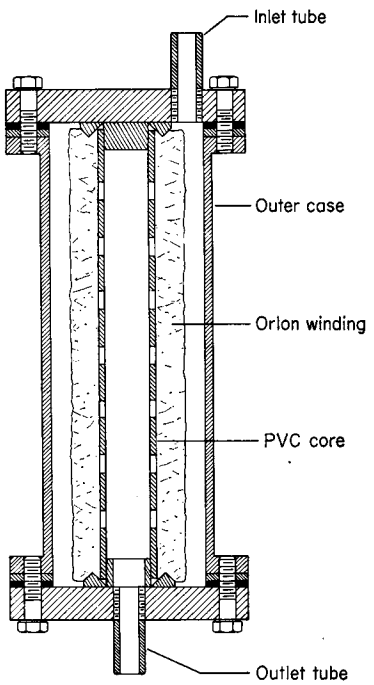


FIG. 1. Diagram of water filter designed to remove all particulate matter larger than 15 μ in diameter. Description in text.

Normally, in addition to small algae on which larval and juvenile mollusks feed, sea water contains many large diatoms, free-swimming crustaceans, gastropods, worms, etc., and their eggs and larvae. Many of these forms compete with bivalve larvae for food, prey on them or may even harbor diseases or parasites that could be transmitted to larvae. We prevent undesirable organisms of larger sizes from entering our larval cultures by filtering the water and later killing the smaller forms with ultraviolet light.

The filter element consists of a polyvinylchloride (PVC) core wound with Orlon. The complete unit (Fig. 1) is manufactured by Commercial Filters Corporation, Melrose, Massachusetts (filter no. CFXl-10-5

with an O15-R10X filter element). These filters, designed to remove all particulate matter larger than 15 μ in diameter, are made with a variety of core and winding materials. We chose the PVC core because it is nontoxic, and the Orlon winding because it is inexpensive, nontoxic and does not support bacterial growth.

To prevent fungus diseases in clam larvae and juveniles we began treating sea water with ultraviolet light in 1954 and, within a short time, had some evidence that such treatment, even of running, unfiltered sea

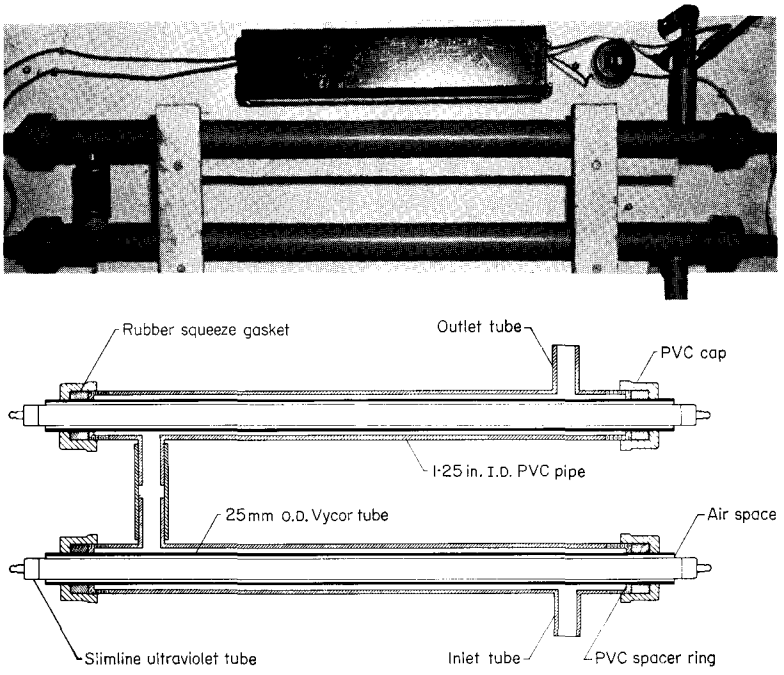


FIG. 2. Photograph (*above*) and drawing (*below*) of ultraviolet water treatment unit used at Milford Biological Laboratory. Description in text.

water, was helpful in preventing mortality of juvenile clams. In the summer of 1955 it was definitely demonstrated that larval cultures, receiving treated water and untreated phytoplankton from the outdoor mass culture, developed fungus, whereas larval cultures in which phytoplankton and sea water were both treated did not. Since that time, it has become a routine practice to treat with ultraviolet light all sea water used for our larval cultures and for keeping recently-set clams and oysters. Moreover, we are attempting to supply ultraviolet-treated running sea water to all containers in which later stages of juvenile clams are grown.

Ultraviolet treatment of sea water for purification of shellfish has been described by several workers in Japan (Sato, 1954; Satoh, 1960) and Wood (1961) in England. As is the practice in our laboratory, Waugh (1958) also used ultraviolet-treated sea water for rearing larvae of the European oyster, *O. edulis*. Several of these authors have described the equipment used but, because of certain considerations, we constructed our own units, a description of which is offered here.

The ultraviolet water treatment unit consists of a $1\frac{1}{4}$-in inside diameter PVC pipe, 30 in long, threaded at each end for caps (Fig. 2). A small ring of PVC is cut to fit inside of each end of this pipe and reamed to act as a spacer for a 25-mm Vycor tube. A squeeze gasket is used to make a water-tight seal between the Vycor tube and the end of the PVC pipe. An inlet tube is located on the side at one end of the PVC pipe and an outlet tube is located on the opposite side at the other end. The 33-in-long, slimline ultraviolet tube lays free in the $32\frac{1}{4}$-in-long Vycor tube and extends slightly beyond at each end.

In practice we use two such units connected in a series so that the water passes the length of both tubes. Since there is only about a $\frac{1}{8}$-in layer of water surrounding the Vycor tube, this apparatus, when used with filtered sea water, should give practically sterile water at the rate of flow of about 10 gal per min. With unfiltered sea water the efficiency is not expected to be as great, but our experience has shown that even then the treatment is of considerable help in reducing mortality of juvenile clams and in preventing fouling by tunicates, worms and bryozoa.

To condition mollusks for out-of-season spawning it is necessary to keep them in running sea water at temperatures of 18° to 20°C or sometimes higher. Warm sea water is also needed for rearing larvae and juveniles during the cold season. Since the water must not contact toxic metals, conventional water heaters cannot be used. Therefore, to heat the water we use a type of heat exchanger (Loosanoff, 1949). The sea water is heated as it passes through a coil of lead pipe immersed in hot fresh water, which fills the tank of a conventional gas water heater that has had the top removed to permit insertion of the lead coil (Fig. 3). However, because the thermostatic controls of a conventional water heater are not sufficiently accurate, the gas flame is controlled through a solenoid gas valve by a Minneapolis-Honeywell thermostat (T415A323XA3). The thermostat-sensing bulb is encased in a lead well in the warm sea water line and maintains the temperature at 37°C $\pm$ 0·5°C.

By mixing varied amounts of cold and heated sea water any temperature between that of the unheated water and 37°C can be main-

tained. In our winter work, when the temperature of the water in Milford Harbor is near freezing, we often simultaneously employ streams of water at 5°, 10°, 15°, 20°, 25°, 30°, and even 35°C. Of course, any other temperature within this range can also be maintained by using constant level jars of cold and warm water and regulating the

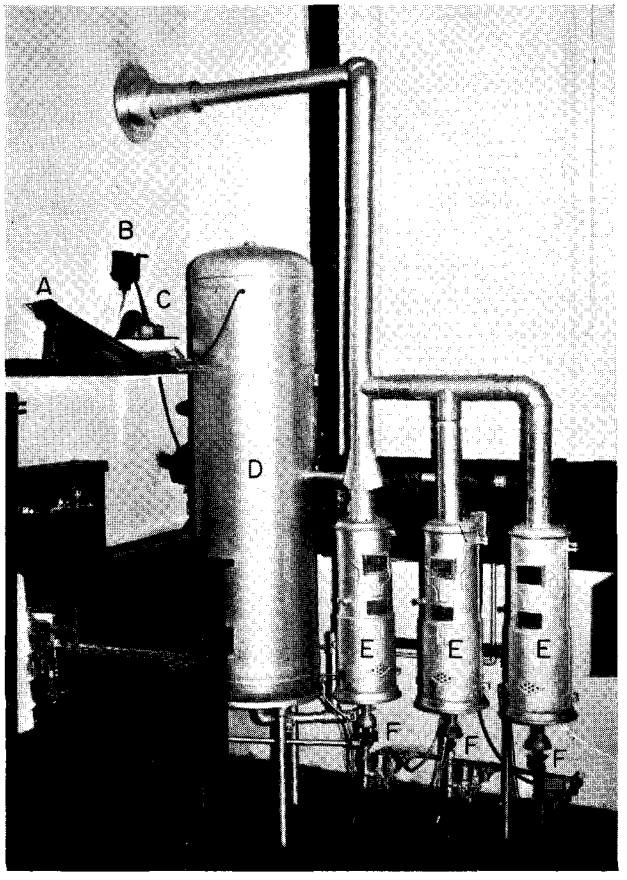


FIG. 3. Heat exchanger to provide laboratory with warm sea water. A, thermostat-sensing bulb; B, thermostat; C, air pump to prevent stratification of fresh water in tank; D, tank; E, gas water heaters; F, solenoid gas valves.

flow from these jars into a mixing chamber from which water of desired temperature flows into trays or aquaria where experimental animals are kept (Fig. 4).

To keep larval cultures at desired temperatures various constant temperature devices are used. Since a temperature of about 24°C is

FIG. 4. Racks of trays for conditioning bivalves for spawning. Racks are provided with running sea water of different temperatures. Constant level jars for warm and cold water are seen in upper left corner. Water from these two jars is mixed in required proportions in the smaller glass jars located on lower shelf. Ultraviolet unit for treatment of water is located at right.

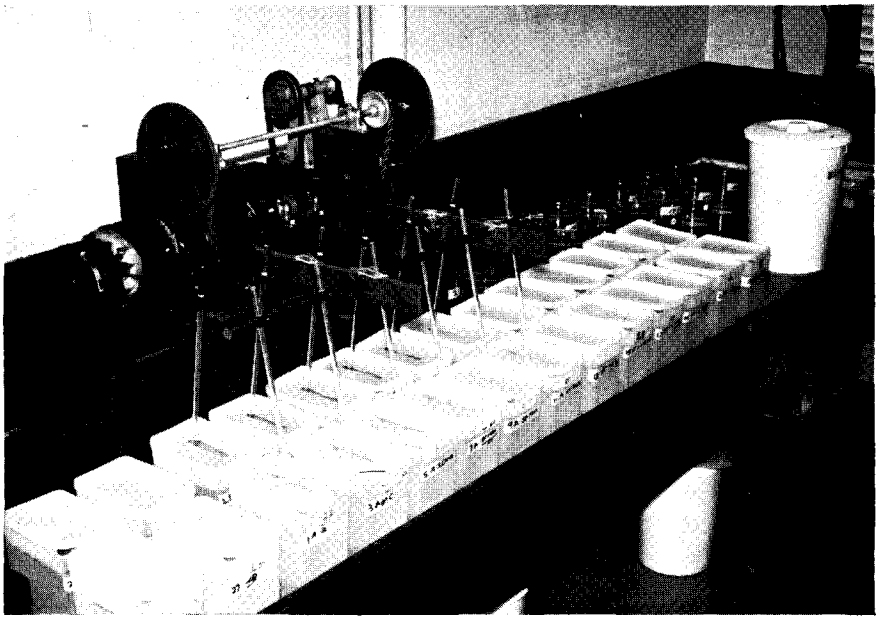


FIG. 5. Constant temperature water bath used to maintain desired temperature in culture vessels, which are partially immersed in water circulating in bath.

near the optimum for growth of algae, such as *Isochrysis galbana* (Ukeles, 1961), which are the best larval foods, this temperature level is often maintained. However, because 24°C is somewhat above normal room temperature, our simplest and most commonly used temperature control devices are lead-lined water tables, 3 ft × 12 ft, that serve as constant temperature water baths (Fig. 5). These tables are filled to a depth of 3 to 4 in with tap water, which is kept in constant circulation by a pump that takes water at one end of the table and discharges it

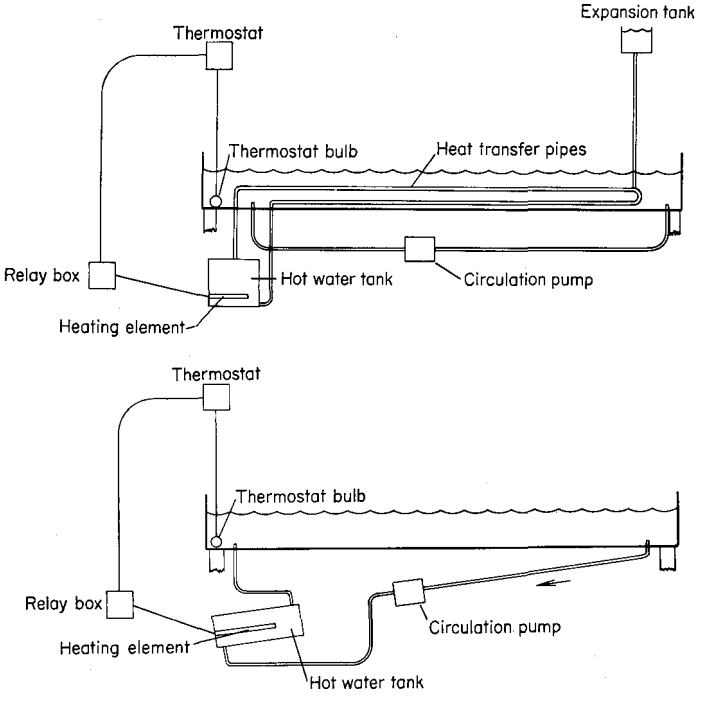


FIG. 6. Two methods of heating water for constant temperature baths. Diagram of closed system above, and open system below.

at the other end. A Minneapolis-Honeywell thermostat (T415A323XA3) with the sensing bulb enclosed in a lead pipe, immersed in the water on the table, controls a 1000- or 1500-W immersion heater. The heater may be either inserted in the pipeline, through which the circulating pump transfers water from one end of the table to the other (open system) or it may be inserted in a small hot water tank and the heat transferred to the water on the table by passage of the water from the tank through a lead coil or loop immersed in the water on the table (closed or heat exchange system) (Fig. 6).

Whenever the larval cultures are to be kept at about 24°C any type of container, the lower part of which is immersed in the bath, will maintain this temperature because convection currents within vessels prevent temperature stratification. Even in containers of

FIG. 7. Constant temperature apparatus consisting of 6 units. Temperature of each unit can be adjusted independently and maintained at any desired level within the range from 5° to 37°C. If necessary, all units may be maintained at the same temperature.

different sizes and shapes the temperature will vary only slightly, while in a series of individual containers of the same type the water will be maintained at almost precisely the same temperatures.

A similar arrangement can be used to maintain temperatures below that of the room by employing a liquid cooler, instead of a

heater. Units that combine a heater and cooler and will maintain temperatures above or below room temperature are also available. However, when cooling devices are used it is necessary to keep the water in cultures continuously agitated to prevent temperature stratification.

To study the effects of different temperatures on development of eggs and larvae of bivalves another apparatus was devised (Fig. 7). This apparatus, which can also be used for studies of many other forms, consists of a series of six lead-lined tanks, each 15 in wide by

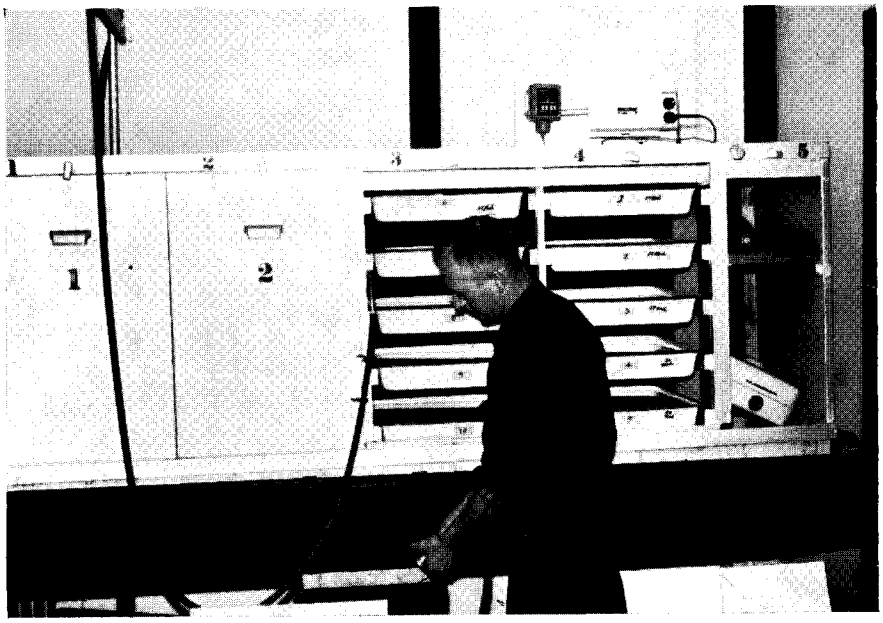


FIG. 8. Constant temperature air chambers in which trays or other containers with larval or juvenile mollusks are held. Electric heater and fan for circulating the air can be seen in right hand corner of unit.

26 in long and 13 in deep and filled with fresh water to a desired level. Vessels containing experimental animals are immersed in these tanks.

To maintain water in the tanks at desired temperatures each tank is equipped, along its walls, with loops of tubing to circulate cold and hot water. The amount of water passing through each of these tubes is controlled by double-action thermostats which activate solenoid valves so that if the temperature in any tank falls below the thermostat setting, the valve in the line, through which hot water circulates, opens, allowing hot water to flow through the loop. If, on the other

hand, the temperature exceeds that indicated by the thermostat, the solenoid valve in the cold water line opens, allowing circulating cold water to reduce the tank temperature. To have the entire mass of water at uniform temperature a circulator pump is employed. The temperature controls of the entire unit are so arranged that all tanks may be maintained at the same temperature, within the range from 5° to 37°C or, if necessary, at different temperatures.

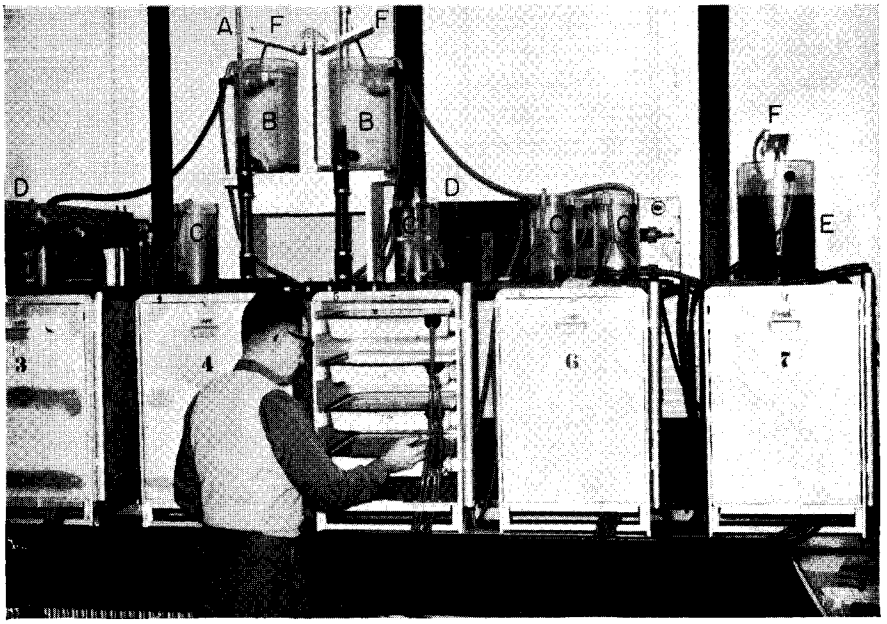


FIG. 9. Temperature apparatus for simultaneous studies of certain aspects of behavior of juvenile mollusks in running water of different but constant temperatures. A, escape tube for air trapped in sea water lines; B, cold and warm water constant level jars from which water in different proportions enters mixing jars (C). Each jar may be maintained at any temperature ranging from 5° (in winter) to 35°C; D, ultraviolet units through which all sea water passes to constant level jars; E, constant level jars from which phytoplankton is added at a definite rate to running sea water; F, floats controlling levels in constant level jars.

To control the temperature of the air chambers in which culture vessels are kept in some experiments thermostatically-controlled electric heaters are used. For example, in experiments, where a series of four banks of five trays each of standing water are used to hold juvenile clams, a uniform constant temperature is maintained by enclosing all twenty trays in a chamber where thermostatically-controlled electric heaters are installed (Fig. 8). When heated air is used, however,

special precautions are necessary to prevent its stratification. To achieve this in the enclosure where our racks of trays are kept a large fan forcing air through has been found sufficient.

Still another temperature apparatus is used at our laboratory for simultaneous studies of growth of juvenile mollusks in running water of different but constant temperatures (Fig. 9). The entire apparatus consists of seven independent units, each insulated so as not to be affected by outside temperatures. As many as five trays may be placed in each chamber. By mixing, in winter, different proportions of warm and cold sea water, temperatures ranging from about 5° to 35°C can be maintained quite accurately.

The amount of water entering each tray can be adjusted to a desired rate and, when necessary, the trays in all seven chambers may receive the same quantity of water and plankton food per hour. As a rule, sea water and food, before entering trays containing juvenile mollusks, are passed through the special unit where they are sterilized by ultraviolet rays.

In addition to various apparatus and devices discussed in this section there are several others that have been used in special studies. A description of these will be given elsewhere.

III. Conditioning Mollusks for Out-of-Season Spawning

Before the present method of providing laboratories with warm water in winter was developed, experiments on most of the bivalves and, especially, their larvae were confined, in New England waters and similar areas, almost exclusively to the short periods of natural propagation, usually lasting for only the $2\frac{1}{2}$ or 3 summer months. However, since it was found that in many bivalves, by using proper conditioning methods, normal development of gonads can be stimulated and spawning induced during late fall, winter and spring, the experimental period has been greatly expanded (Loosanoff, 1945).

Conditioning of bivalves to develop mature gonads during the cold part of the year is relatively simple. It consists of placing mollusks, brought from their natural environment where water temperature may be near freezing, into somewhat warmer water and then gradually increasing the temperature several degrees each day until the desired level is reached (Loosanoff and Davis, 1950). Sometimes, especially towards the spring, instead of a gradual conditioning the mollusks can be placed directly in water of about 20°C. As a rule, the gametes obtained from these mollusks were no less viable than from those conditioned gradually. We have often employed this more rapid

approach, thus shortening by several days the length of the conditioning period which, for oysters kept at 20°C, is approximately 3 to 4 weeks.

The conditioning period can also be shortened by keeping mollusks at temperatures higher than 20°C (Loosanoff and Davis, 1952b). For example, *Crassostrea virginica* kept at 25°C developed ripe spermatozoa and fertilizable eggs by the 5th day, and light spawning could be induced on the 7th day. When kept at 30°C ripe spermatozoa and fertilizable eggs were found in oysters which, only 3 days before, were brought from the ice-covered harbor where they were hibernating. Some oysters of this group were induced to spawn on the 5th day.

Obtaining spawn from another common bivalve, the hard shell clam, *Mercenaria mercenaria*, is also relatively simple in summer. It is often accomplished merely by raising the water temperature a few degrees and by adding a sperm suspension (Loosanoff, 1937a). Previously, as already mentioned, this could be accomplished only during a short period, whereas, using our recently-developed methods, it is now possible to obtain ripe gametes and raise larvae of this species on a year-round basis (Loosanoff and Davis, 1950, 1951).

The method for conditioning clams for spawning in winter is the same as that described for oysters. The entire conditioning period takes approximately 2 to 3 weeks, but can be made even shorter towards or during spring. On several occasions clams brought directly from natural beds during early spring could be induced to spawn without any preliminary conditioning. However, this method often failed and cannot be considered reliable. Usually, only males responded on such occasions. As a rule, some conditioning of clams is necessary, even towards spring, to have a reliable source of eggs and sperm.

Our studies have demonstrated that bivalves can be conditioned for late fall and early winter spawning only after they recover from the natural spawning activities of the preceding summer. This recovery consists of many complex physiological processes leading, in general, to accumulation of reserve materials, of which glycogen is probably the most important (Loosanoff, 1937a, 1942). Since many species of bivalves of Long Island Sound, including oysters and clams, sometimes continue to spawn until late August or even the middle of September and are not completely recovered from these activities until the end of November, they cannot be conditioned for spawning during these months.

We solved the problem of supplying ripe mollusks during the period from late August to late November by delaying their gonad development and spawning until late fall (Loosanoff and Davis, 1951). Clams,

M. mercenaria, and oysters, *C. virginica*, are taken from Long Island Sound early in the season, usually late in May, long before the beginning of their natural spawning, and transplanted to the waters of Maine, where the summer temperature averages about 7° lower than in our waters. This temperature, while permitting slow development of gonads, is, nevertheless, low enough to prevent spawning. Thus, when oysters and clams in Long Island Sound are already spent, those transplanted to Maine still retain their spawn. In the fall, small groups of these mollusks are routinely shipped back to our laboratory, where they are easily induced to spawn, providing normal gametes which are unobtainable locally during that time of the year.

By using the above method spawning of *C. virginica* can be postponed only for 6 or 8 weeks after oysters of Long Island Sound are completely spent. After that period the oysters, even if they are still kept in the waters of Maine, begin to resorb undischarged gonad material and, thereafter, become useless as spawners. We overcame this difficulty by developing another useful method, which postpones early gonad resorption. It consists of conditioning oysters early in the spring and spawning them at Milford by early June. After that they are transferred to the colder waters of Maine. Oysters treated in this manner must resorb old gonads and build up glycogen before developing new gonads. Because they are compelled to go through these processes, these oysters reach ripeness much later in the season than those that are planted in Maine without spring conditioning and spawning and, as a result, they do not begin to resorb gonad material as early as do unspawned oysters transferred to Maine at the same time. Taking advantage of this situation we have been obtaining normal larvae from October to January from spawn of oysters so treated.

Because *M. mercenaria* does not resorb undischarged gonad material in the fall, as oysters do, transferring them to the colder waters of Maine in the spring proved to be a highly satisfactory method of delaying spawning. Under these conditions the clams retain sperm or eggs throughout the summer and, as a result, can be induced to spawn throughout the next fall, winter and even during the following spring, always producing gametes which develop into normal larvae.

We have also delayed spawning of clams and oysters by taking these mollusks early in the summer from their natural habitat and keeping them in insulated boxes through which mechanically-cooled sea water flowed. Usually, only a comparatively small number of adult mollusks could be conveniently kept under these conditions and, as a rule, bivalves so treated were in much poorer condition than those kept under natural surroundings in the waters of Maine. More-

over, a failure of the artificial refrigeration system may cause the entire stock to spawn prematurely.

By combining our two methods, one consisting of conditioning mollusks for spawning during the cold periods and the other of delaying gonad development and preventing spawning during their normal reproductive season, ripe bivalves may now be available throughout the entire year.

We have also found (Loosanoff and Davis, 1952a) that *C. virginica* and *M. mercenaria* are able to reproduce several times a year, provided that changes in ecological conditions, especially temperature, are so controlled that these mollusks can rapidly recover from spawning, accumulate in their bodies material needed for gonad development, and begin the cycle again. As a result of the discovery of these new approaches and methods, as much can now be accomplished in one year in certain fields of the biology of bivalves as could formerly be done in three or four.

It should be emphasized that our conditioning methods are not equally successful or applicable to all groups of oysters, *C. virginica*, and, perhaps, certain other species of bivalves of our Atlantic and Gulf coasts. This is probably because populations of these species are not genetically homogeneous, but consist of different physiological races. We began to suspect the existence of such races in *C. virginica* as early as 1937 (Loosanoff and Engle, 1942). Stauber (1950), in reviewing the literature on spawning of the American oyster, also came to the conclusion that oysters from different areas along our Atlantic coast may belong to different geographical races. Our experiments in this field strongly supported this assumption by demonstrating that, even though all these oysters belong to the same species, the temperature requirements for gonad development and spawning of the northern populations are definitely lower than those of the southern groups (Loosanoff and Nomejko, 1951).

The results of our later, more extensive studies, in which several thousand specimens representing populations of different areas of the oyster-producing belt extending from the Gulf of Mexico to Cape Cod were used, fully supported our original conclusions (Loosanoff, 1958a). The oysters used in these experiments and observations were from Florida (Gulf of Mexico), South Carolina, Virginia, New Jersey and New England. They were received in the fall, after they had completely spawned in their native environment, and were kept in Milford Harbor throughout early winter. Some time in January the first groups of these oysters were transferred to the laboratory to be conditioned for spawning.

We employed two criteria to evaluate ripeness of the oysters. The first was to ascertain the number of days needed for 50% of the oysters constituting a sample to develop active spermatozoa or fertilizable eggs. Secondly, we had to determine the length of the conditioning period before spawning in 50% of the oysters could be induced by our usual method. Each sample contained fifty adult individuals.

The experiments showed conclusively that Long Island Sound oysters develop gonads and can be induced to spawn after considerably shorter conditioning periods than those required by southern oysters. When kept at the same temperatures oysters from New Jersey, although slower than those of Long Island Sound, showed, nevertheless, much faster gonad development than oysters of Virginia, South Carolina and Florida. In averaging the results of the experiments it was found that 50% of Long Island Sound oysters, conditioned at 21°, 24° and 27°C, contained mature gametes after only 15, 8 and 5 days, respectively. The corresponding groups of New Jersey oysters reached this stage only after 55, 32 and 22 days, thus requiring three or four times as long at the three above-mentioned temperatures as did the northern race.

In certain experiments we were able to induce spawning in 50% of Long Island Sound oysters after only 18 days of conditioning at 21°C. To achieve the same results with New Jersey oysters 78 days were needed. The more southern groups kept under the same conditions failed, as a rule, to produce 50% spawners.

The most striking differences were noticed when oysters of different geographical regions were kept at relatively low temperatures. For example, after 68 days of conditioning at 12°C, 67% of Long Island Sound oysters contained mature eggs or spermatozoa. In this group we were able to induce spawning in one male and, 10 days later, in one female. Oysters of the other groups kept at the same temperature contained not a single individual with mature gonads, even after 78 days. Moreover, in the majority of New Jersey and Virginia oysters and in all of those from South Carolina and Florida the gonads were so poorly developed that the sexes could not be distinguished, even by microscopic examination of the raw gonad material.

The method of inducing spawning of oysters and clams in summer has already been described in detail (Galtsoff, 1930, 1932; Loosanoff, 1937a, 1954). The same method, as a rule, has also been used to induce spawning in other bivalves. In general, our present method can be described as follows: After the proper conditioning period ripe bivalves are placed in glass spawning dishes, each containing approximately 1 liter of sea water of the same temperature as that at which mollusks

FIG. 10. Ripe oysters in spawning dishes. Male oyster is shown spawning in center dish.

FIG. 11. Inducing spawning of clams, oysters and other bivalves by immersing dishes of sea water containing animals in warm water on spawning table.

were conditioned (Fig. 10). These dishes are partly immersed in a large tray or sink, which is filled with hot water, thus quickly raising the temperature in the dishes to the desired level (Fig. 11).

In some instances thermostimulation alone is sufficient to induce spawning. In other cases, however, mollusks need additional stimulation, which consists of adding to the water small quantities of sperm or egg suspension made from gonadal material of ripe individuals of the same species. Many forms quickly respond to combined thermal and chemical stimuli; others, such as the common mussel, *Mytilus edulis*, do not usually respond to this method but can, nevertheless, be induced to spawn by other means, which will be discussed later.

In a special series of experiments we tried to cause artificial discharge of reproductive elements by injecting weak solutions of Mn_4OH and other chemicals into the bodies of bivalves that could not be spawned by other means. The results were usually not gratifying, except in the case of *M. edulis*, when injection was made in its adductor muscle.

At the time of our experiments to induce spawning in ripe bivalves we were already aware of the success of Japanese workers in inducing spawning in mussels by giving them a mild electric shock. We repeated these experiments but, unfortunately, with indifferent results.

In still other species, for example, *Modiolus demissus*, all our methods, including those that were successful in the case of *Mytilus edulis*, proved to be ineffective in inducing spawning. Therefore, unless ribbed mussels spawn naturally, thus providing normally fertilized eggs, no other means, except perhaps stripping, are left for obtaining their spawn.

Fertilizable eggs of many species, including those of *C. virginica* (Brooks, 1880), can be obtained by stripping mature females but, since many of these forms spawn so readily in response to chemical and thermal stimulations, it is seldom necessary to resort to this means. However, when working with other species, especially those that cannot be spawned by conventional methods, stripping may be the only way to obtain ripe eggs. It is a simple process and is carried on as follows: After removing the outer membrane that covers the gonads, the mollusk is gently rinsed in sea water. This action separates from the gonad large numbers of eggs without serious injury to them. Using a series of sieves of proper size mesh the eggs are later freed of blood cells, pieces of tissue, etc., and then placed in sea water to which sperm is added. The fertilized eggs can then be placed in culture vessels.

This approach is possible only for eggs of those forms in which the germinal vesicle dissolves after stripping. In many species, however,

including *Mercenaria mercenaria* and *Pitar morrhuana*, attempts to fertilize stripped eggs usually fail because in these eggs the germinal vesicles remain intact and, as a result, fertilization does not occur. Under normal conditions the germinal vesicles in eggs of such species dissolve while they are still in the ovaries of the female, just before they are discharged in the process of spawning. Upon dissolution of the germinal vesicle the germinal spindle is formed and the discharged egg is ready for fertilization (Loosanoff, 1953).

Recently, following the suggestion of Mr. David Tranter of Australia, we used a weak solution of ammonium hydroxide to break the germinal vesicle of eggs of certain bivalves. By employing this method we succeeded in raising normal larvae from eggs stripped from *Mercenaria mercenaria*, *Tapes semidecussata* and several other species.

After the eggs were washed from a gonad they were passed through a coarse, 50-mesh screen to remove debris, large pieces of tissue, etc. Later, they were washed on a 325-mesh screen which retained the eggs but let pass the body fluids that might pollute the water in culture vessels. After that, 3 ml of 0·1 normal solution of ammonium hydroxide were added to every 100 ml of the prepared suspension of eggs in sea water. After the eggs were in this solution for some time they were washed again on a 325-mesh screen, being finally ready for fertilization. A more detailed description of handling fertilized eggs will be given in the section on methods of cultivation of eggs and larvae.

The length of exposure to the solution of ammonium hydroxide may vary somewhat from species to species. The following table shows the ratio between length of exposure and percentage of normally developing eggs of *M. mercenaria*:

15 minutes—32%
30 minutes—16%
45 minutes— 9%
60 minutes— 3%
75 minutes— 2%
90 minutes— 0%

Even after 90 minutes of exposure to the solution of ammonium hydroxide some eggs became fertilized, but their development was not normal.

The percentage of normal larvae obtained from chemically-treated eggs was low compared to that of naturally spawned eggs but, nevertheless, it was high enough to permit successful culturing of larvae of those species in which we were not able to induce spawning. Perhaps by changing the concentration of ammonium hydroxide, using other

chemical agents, or by improving the methods of stripping even better results may be obtained.

Finally, there were several species of bivalves which we could neither spawn artificially nor collect their normally discharged eggs. Moreover, eggs stripped from some of these forms could not be fertilized regardless of various preparatory measures, which included the chemical treatment described above.

Fecundity of many lamellibranchs, especially those of commercial importance, has been speculated upon for a long time. Brooks (1880) estimated that *C. virginica* could produce between 18 750 000 and 125 000 000 eggs. He based his estimate upon volume of material removed from the ripe female, but stated that this figure should be reduced by approximately 50% because of other matter that was measured together with eggs. Churchill (1920) stated that a large oyster may discharge 60 million eggs, while Galtsoff (1930) estimated that the number of eggs released in a single spawning may range between 15 and 115 million. He concluded that the maximum number of eggs that can be released by a single female during the entire spawning season is approximately half a billion. Burkenroad (1947), without offering any experimental observations of his own, suggested that Galtsoff's estimate was approximately ten times too high. Belding (1912) estimated that *M. mercenaria*, $2\frac{1}{2}$ in long, produces an average of 2 million eggs, a figure not substantiated by experimental studies.

Since reliable information on the fecundity of even the most commonly studied pelecypods was unavailable, experiments were undertaken by Davis and Chanley (1956b) to determine total numbers of eggs actually produced by individual oysters, *C. virginica*, and clams, *M. mercenaria*, under natural and artificial conditions.

The first series of observations was made on seventy-five oysters, measuring from $3\frac{1}{2}$ to $4\frac{1}{2}$ in long, and on the same number of clams approximately 3 to 4 in long.

The experiments were conducted in the laboratory during the winter, a most convenient period for proper conditioning of both clams and oysters. Each bivalve was individually numbered and a complete record was kept of its behavior during the entire experiment. The first group of oysters composed of twenty-five individuals was spawned at 3-day intervals, the second group at 5-day intervals and the third at 7-day intervals. In clams, which were also divided into three groups of twenty-five individuals each, spawning was induced at 3-, 7- and 14-day intervals. Spawning of these groups of clams and oysters was continued at the specified intervals for more than 2 months.

Experiments have shown that, as a rule, an individual oyster or clam does not discharge all its eggs or sperm in a single spawning, but will continue to spawn at intervals over extended periods. One female oyster spawned on sixteen occasions and a clam, eleven times. The number of spawnings per female oyster ranged from two to sixteen. The highest number of eggs was produced by an oyster that spawned nine times, while a female that spawned sixteen times ranked second. The lowest total number of eggs released by an oyster was by an individual that was induced to spawn seven times.

No significant difference was observed in the average number of eggs released during the entire experimental period, whether the oysters were induced to spawn at 3-, 5- or 7-day intervals, although the average number of spawnings per female oyster decreased progressively as the intervals between spawnings were increased. It was also determined that female oysters having larger numbers of eggs tended to spawn more frequently than did females with smaller numbers.

The highest number of eggs released by any female clam in a single spawning was 24·3 million and the total number released by individual clams during the entire experimental period of about 2 months ranged from 8 million to 39·5 million, with an average of about 24·6 million.

There was no significant difference in the average number of eggs released in a season, whether the clams were spawned at 3-, 7- or 14-day intervals. It was also found that correlation between the number of times a female clam spawned and the number of eggs produced was not significantly different from zero.

An auxiliary experiment consisted of observations on spawning of fifty oysters taken from Milford Harbor early in April, brought into the laboratory and placed in conditioning trays at temperatures of about 20°C. Three weeks later these oysters were induced to spawn daily for 5 consecutive days, and seventeen females and twenty-four males responded during the first day. Altogether, this group contained twenty-four females and twenty-six males. Of the twenty-four females, fourteen spawned on 2 or more consecutive days, eight spawned on 3 or more consecutive days, five spawned on 4 or more consecutive days, and three females spawned on each of the 5 days of the experiment. Eight males spawned each day.

The important contribution of this experiment was the clear-cut demonstration that there is no 2- to 5-day refractory period during which female oysters cannot be induced to spawn, as maintained by Galtsoff (1930). On the contrary, the results suggest that upon proper

stimulation both male and female oysters can spawn any time they have physiologically-ripe sex cells to discharge.

The final experiment consisted of observations on nine female oysters developing gonads under normal conditions in Long Island Sound and induced to spawn at the end of June. The total number of eggs discharged by these oysters ranged from 23·2 million to 85·8 million and averaged 54·1 million eggs per female. Thus, both average number of eggs and maximum number per female of the summer

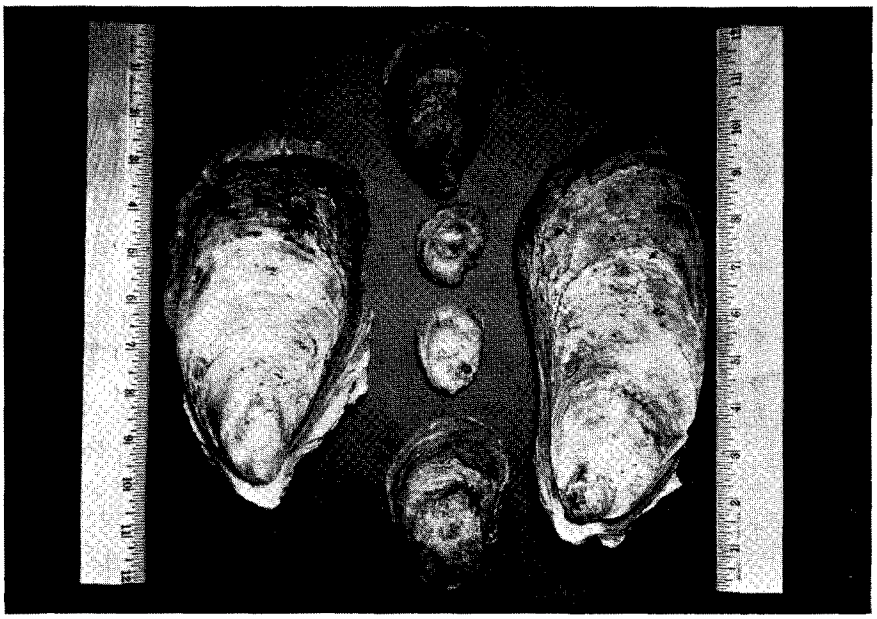


FIG. 12. Representatives of three groups of oysters of different ages and sizes used in studies to determine viability of their gametes. Members of oldest group were estimated to be between 30 and 40 years.

spawning group were about 20 million higher than found in the winter experiment. Nevertheless, none of these oysters discharged as many as half a billion eggs, as suggested by Galtsoff (1930). The larger number of eggs developed by oysters of this group, as compared with production of eggs by oysters conditioned in the laboratory during early winter, may be ascribed to larger reserves of glycogen possessed by oysters developing gonads under natural conditions.

A question that had long been of interest to biologists was, At what age do oysters and clams produce the best, most viable sexual products? Until recently, no answer could be given because no reliable methods

were available to conduct critical experiments on development of eggs and growth of larvae to setting or post-setting stage. Since development of these methods, such studies have become possible and recently were undertaken at our laboratory.

Three groups of oysters of different ages and sizes were conditioned for spawning (Fig. 12). The average age of individuals of the oldest group was estimated to be between 30 and 40 years, some of them being over 9 in long and over 4 in wide. The intermediate group of oysters of marketable size was from 5 to 7 years old, while the youngest group was composed of small oysters approximately 2 years old. These groups were conditioned and induced to spawn under controlled conditions, their larvae grown to setting stage, and rates of survival and growth of larvae from the three size groups compared.

The results showed no significant difference between oysters of the different age groups in the time needed to develop ripe gonads. We were somewhat surprised, however, to find that oysters of the oldest group responded to spawning stimuli more rapidly than individuals of the two younger groups.

There was also no significant difference in percentage of fertilizable eggs because almost 100% of the eggs of all three groups became fertilized. Furthermore, the percentage of fertilized eggs developing to straight-hinge larval stage showed no consistent variation that could be ascribed to size or age of parent oysters. Finally, no consistent difference was found either in the sizes of the early straight-hinge larvae originating from eggs of different age-group oysters or in survival and rate of growth of their larvae.

Similar studies on hard shell clams, *M. mercenaria*, measuring from 37 to 110 mm in length, also showed that there was no significant difference in viability of spawn produced by clams of different sizes and ages. Often the differences between the progeny of individuals of the same size groups were as great as the differences between those of different ages and sizes. Larvae grown from eggs of clams of all three sizes were successfully carried to setting stage.

On the basis of the above-described experiments we came to the conclusion that since there was no significant difference in the quality of spawn developed by individuals of different ages or sizes, mature oysters and clams of all age groups may be safely used as spawners.

Of special biological interest was the observation that the sexes among the oldest oysters were about evenly divided. This discovery was contrary to the old conception that in the oldest groups females should decidedly predominate in numbers. We also noticed that many of the largest and oldest oysters, while kept in the laboratory to be

conditioned for spawning, formed normal, new shell growth, thus indicating that even at that age and size the oysters did not lose their ability to grow (Fig. 12).

IV. Cultivation of Eggs and Larvae of Bivalves

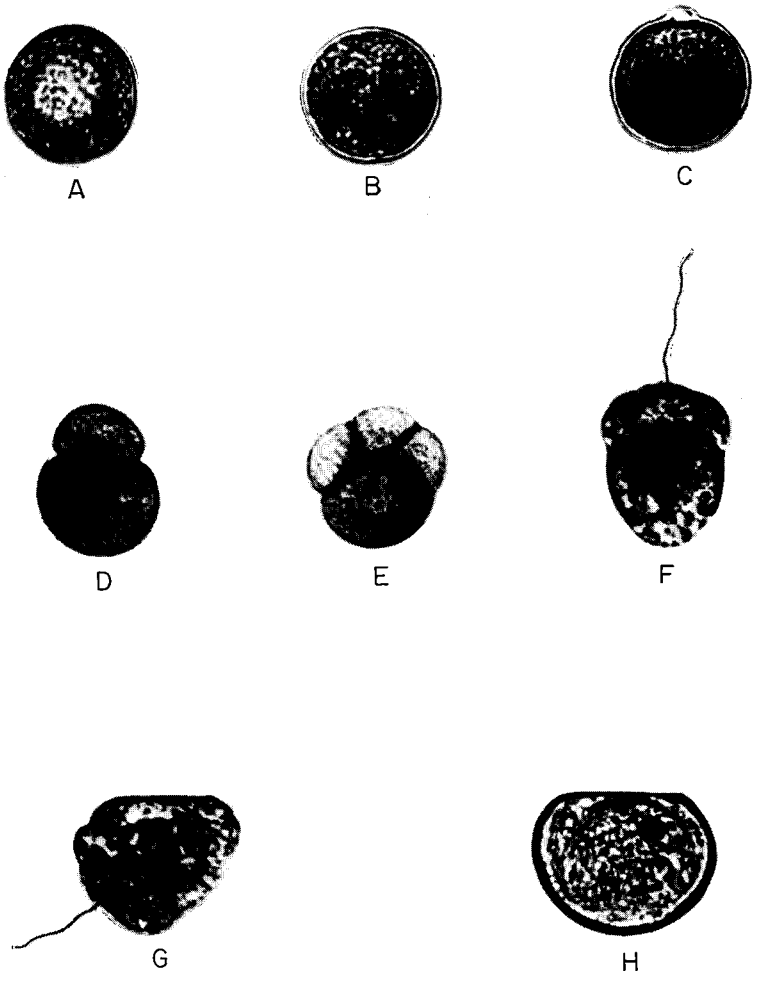
A. *General description of the development*

Eggs of bivalves differ in many respects, including their size, color and specific gravity. They also differ in thickness of the membrane surrounding them (Costello *et al.*, 1957). In oysters and certain other forms this membrane is only a few microns thick. In others, however, such as *M. mercenaria*, the egg proper measures only 70 to 73 μ, while the total diameter of the egg and surrounding gelatinous membrane is about 170 μ. This membrane, in many instances, continues to surround the embryo past blastula stage and, on some occasions, until late trochophore stage is reached (Loosanoff and Davis, 1950).

We shall describe specific characteristics of the eggs later on, when discussing each of the species studied. Here, because the description of a typical bivalve egg and its development to straight-hinge stage or, as it is often called, early veliger has been given on many occasions, including Brooks (1880), MacBride (1914) and others, we shall present only a general picture of changes occurring from the moment the egg is discharged, or stripped, until it becomes a straight-hinge larva. This description is based upon observations made on eggs and early embryos of *Mactra* (= *Spisula*) *solidissima*, the surf clam, which is the largest bivalve of our Atlantic coast. It measures up to $7\frac{1}{2}$ in long and can be found in considerable numbers from Labrador to Cape Hatteras. Additional information on spawning of these clams and rearing of their larvae is included in the section dealing with rearing of larvae of different species.

The diameter of a mature egg of *M. solidissima* averages 56·5 μ (Fig. 13*a*). Costello *et al.* (1957) give the diameter of the unfertilized ovum of the same species as ranging between 53 and 56 μ, thus agreeing with our measurements. According to Cahn (1951), who bases his conclusions on the work of the Japanese investigators, Kinoshita and Hirano (1934), whose paper was not available to us for consultation, the diameter of the egg of a closely related form, *Spisula sachalinensis*, is only 50 μ. Another group of Japanese workers (Imai *et al.*, 1953), studying the same species reports that the diameter of the mature egg of this clam varies from 70 to 75 μ, thus being considerably larger than the size given by Cahn. Jørgensen (1946) states that eggs of *Spisula subtruncata* of European waters vary in diameter from 50 to 55 μ.

Early development of the egg of *M. solidissima* is basically the same as that of many other bivalves. After dissolution of the germinal vesicle (Fig. 13*b*) the size and shape of the egg remain the same. If



Mactra (Spisula) solidissima

FIG. 13. Development of *Mactra* (= *Spisula*) *solidissima* from unfertilized egg (A) to straight-hinge larva (H). Diameter of egg is about 56 μ, while length of early straight-hinge larva is about 79 μ. Detailed description in text.

the fertilized egg is kept in water of about 20°C, the polar body is formed in about 45 min (Fig. 13*c*) and the two-cell stage, measuring about 65 μ along the longest axis, is reached in 90 min (Fig. 13*d*).

Development of the egg of a bivalve, as described above, is typical only of a group in which the germinal vesicle breaks upon discharge

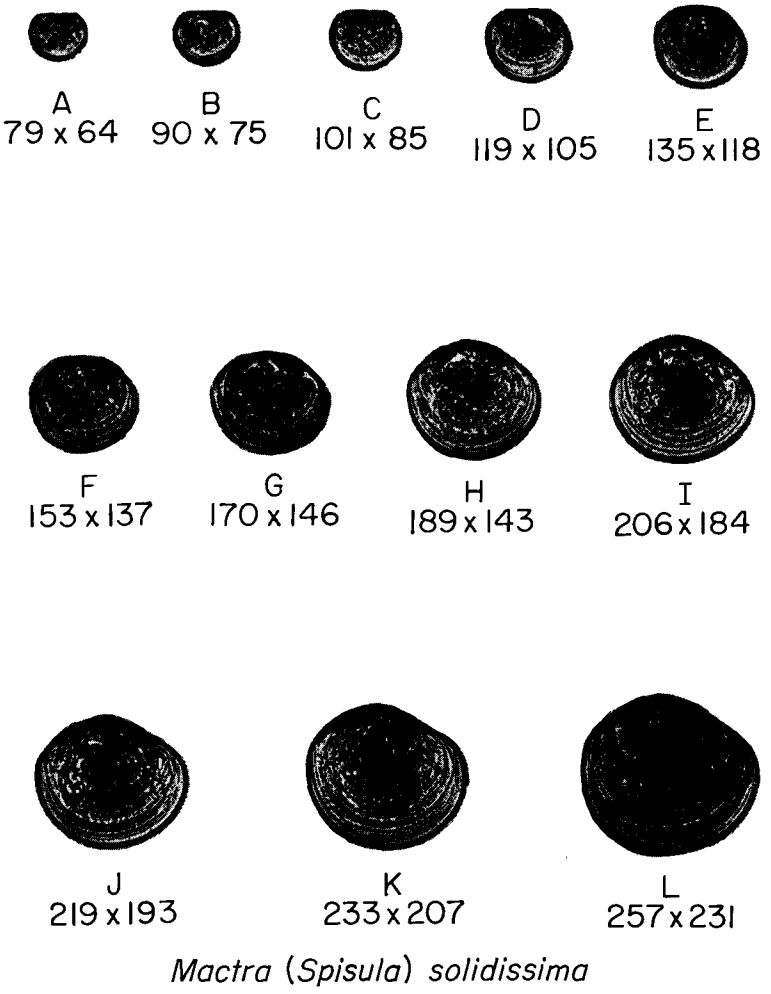


FIG. 14. Development of *Mactra* (= *Spisula*) *solidissima* from straight-hinge stage (A) to metamorphosis (L). Measurements of length and width of larvae of different stages are given in microns.

or upon stripping, thus rendering the egg ready for fertilization. Eggs of *Crassostrea virginica* and many other species belong to this category. In the other group stripped eggs continue to retain their

germinal vesicles intact and fertilization does not occur. However, as already mentioned, eggs of such species can be chemically treated and thereby become ready for fertilization.

As cleavage progresses and more micromeres are formed (Fig. 13*e*), the embryo gradually develops into a swimming, ciliated larva which eventually reaches trochophore stage (Fig. 13*f*). Under favorable conditions this stage may be reached in 12 to 16 hr, depending upon the original condition of the eggs, culturing methods and, of course, water temperature.

During late trochophore stage (Fig. 13*g*) the cell gland begins to secrete the shell. When the shell completely encloses the soft parts the larva has reached early straight-hinge stage (Fig. 13*h*).

Development of larvae of *Mactra solidissima*, from early straight-hinge stage until metamorphosis, and their length—width measurements during this entire period are shown in Fig. 14. Very early, normal straight-hinge larvae measure only about 79 μ in length and usually between 63 and 65 μ in width. In some cultures, composed mostly of abnormal individuals, somewhat smaller, slightly deformed straight-hinge larvae can be seen occasionally, but it is doubtful that they survive to metamorphosis.

Individual larvae of *M. solidissima* display considerable variations as to the size at which certain organs of their bodies begin to develop and at which metamorphosis occurs. For example, in some individuals the foot can be seen when they are only about 160 μ long. Approximately 80% of the larvae show a well-developed foot by the time they are 215 μ long, and at a length of 240 μ practically all possess this organ.

Disappearance of the velum is another step in larval development that is not strictly correlated with a definite size. In some larvae measuring only 219 μ in length the velum was already completely resorbed, while in extreme cases a diminishing, but still functional velum was seen in larvae about 257 μ long.

A few larvae begin to metamorphose when they are about 220 μ long, but the majority are between 230 and 250 μ before metamorphosis occurs. At this time the velum is resorbed, rudimentary gills develop, and a powerful ciliated foot, which when expanded is as long as the young clam itself, serves as the only means of locomotion. Individuals measuring 262 μ in length were the largest true larvae recorded. In this respect our observations are in agreement with those of Imai *et al.* (1953), who found that in *Mactra sachalinensis* the foot begins to develop at a length of about 200 μ and that larvae set at about 270 μ.

B. *Abnormal eggs and larvae*

Abnormal development of eggs and larvae of bivalve mollusks may be due to any one of a variety of factors or to a combination of such factors. It is our practice, however, to discard cultures in which less than 50% of the eggs develop into normal straight-hinge larvae. This is done because batches of eggs giving a low percentage of larvae may be abnormal in some respects, and these abnormalities may lead to aberrant experimental results and wrong conclusions. We do not know, as yet, what factors are responsible for poor eggs and feeble embryos. In some instances abnormal larvae, or failure of eggs to develop to straight-hinge larval stage, may be the result of incompatible genetic combinations. Our experience indicates, however, that such combinations are comparatively rare.

Some abnormalities of larvae may be ascribed, no doubt, to the poor physical condition of spawners. Several investigators have believed that eggs released late in the season were less viable and produced less vigorous larvae than those from earlier spawnings. Loosanoff and Davis (1950) were under the impression that the last batches of eggs discharged by virtually spent females gave feeble larvae that grew slowly and showed high mortality. Cole (1941) offered evidence that the brood strength of *Ostrea edulis* may decline during the course of a breeding season and he believed that this was due to a depletion of food reserves in the bodies of parent mollusks. Walne (1956) thinks that the lack of " vigour " in larvae may result from poor condition of the parent oyster and believes it possible that the vigour of larvae may be affected by the quantity of food reserves laid down in the eggs.

More recently, Davis and Chanley (1956b) have shown conclusively that the last batches of eggs of both clams, *M. mercenaria*, and oysters, *C. virginica*, discharged by virtually spent females were cultured with no apparent diminution of either percentage of eggs developing into straight-hinge larvae or rate of growth of these larvae. Accordingly, we now believe that abnormal or feeble larvae do not occur more frequently in later spawnings than in other spawnings throughout the season. As has already been mentioned, our experiments have shown that there is no correlation between viability of spawn and age of parents.

There is evidence that bivalves can be induced, by strong chemical and thermal stimulations, to abort eggs even though they are not fully ripe (Fig. 15). In some cases such spawnings appear to be quite normal and a large number of eggs may be released. More often, however, comparatively few eggs are shed. In the case of *C. virginica* immature oyster eggs usually develop only to late gastrula or early