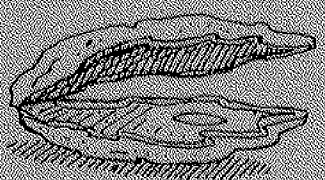
HATCHERY MANUAL
FOR PRODUCING

TRIPLOID OYSTERS



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TRIPLOID OYSTERS

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A related video, "Triploid Oyster Production" (available in 3/4" or VHS format), has been produced at the University of Washington by Washington Sea Grant, Instructional Media Services, and the School of Fisheries. It may be ordered from University of Washington Press, ISBN 0-295-73047-1.

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PREFACE

We have had requests from all over the world for information on how to produce triploid Pacific oysters. In most instances we felt that our response was inadequate: technical reprints seemed too narrow or overly specialized for the kinds of information that commercial companies were requesting, while other, more practical information was inappropriate for scholarly publication. Therefore, we approached the Washington Sea Grant Program with the idea of presenting all the essential elements for the production of triploid oysters in one source manual, as a comprehensive reference of current knowledge.

The manual is organized in the following way. We first present the overall rationale for the production of triploid shellfish as well as a short history of research leading to the activities in the Pacific Northwest. The second chapter is concerned with the biological principles that underlie induced triploidy. These principles can be applied to most animals with externally fertilized eggs that complete meiosis after fertilization.

The third chapter, the heart of the manual, discusses and then outlines the procedures involved in producing triploids using the fungal antibiotic cytochalasin B. Presented in a stepwise, or cookbook, fashion, it describes the procedure that is currently being used. However, we recognize that there is no one method for producing triploids, just as there is no one method for growing larvae, and that individual hatcheries will change or refine the steps we outline.

Chapter 4 explains how to determine whether triploids have been produced by the treatments. Since triploids and diploids look alike, some laboratory analysis is inevitable.

Chapter 5 evaluates adult triploid oysters based on empirical studies of their performance in the field and suggests situations in which triploids may be of value but have not been investigated. Chapter 6 describes two variations on induced triploidy, interspecific triploidy and tetraploidy, that may be useful in commercial settings in the near future. Research in these areas is still ongoing.

Finally, in Chapter 7 we discuss chromosome set manipulation in shellfish research. A grower may find this information valuable in the future.

This manual is not meant to be a guide to growing shellfish larvae. We assume that readers are experienced oyster culturists or have access to this information. Portions of the manual may seem overly simplified to some, but this is only because we are trying to reach a diversity of readers, some of whom may not have a biological background.

In the preparation of this manual we are deeply indebted to the Washington Sea Grant Program: to Louis Echols for awarding discretionary funding to start our work and for continued support for the "little" things; to Trish Peyton and her support staff for putting the published version together; to Fred Lisaius for the artwork. We are also indebted to the Pacific Coast Oyster Growers Association for their financial and moral support during our research. Three commercial collaborators, Coast Oyster Company in Quilcene, Wescott Bay Sea Farms in Friday Harbor, and Ted Kuiper, have been especially helpful in implementing this program.

We thank both the Environmental Protection Agency and the National Marine Fisheries Service for the use of their Manchester facilities. NMFS also helped support our hatchery through Seabeck funding.

We are grateful to our colleagues who spent time evaluating, criticizing, and improving the manuscript: Jonathon Davis, Ken Cooper, Bill Webb, and Mark Billington.

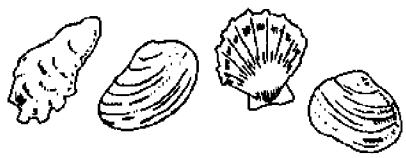
Finally, we appreciate the comments and encouragement of numerous commercial oyster growers while we were pursuing our research. We thank them for their willingness to let us use their oyster beds as grow-out areas for studying the growth and survival of our laboratory-developed triploid oysters.

1. BACKGROUND

Most sexually reproducing organisms are diploid, meaning that they have *two* sets of chromosomes (humans have 23 chromosomes in a set, *Crassostrea* oysters 10). Their offspring inherit *one* set of chromosomes from the maternal gamete (the egg) and *one* set from the paternal gamete (the sperm), and diploidy results when fertilization unites the two gametes.

Triploids Are Different

Triploids are organisms with *three* sets of chromosomes in each cell. Triploidy, therefore, is an aberrant genetic state for oysters and most other organisms; it must be produced artificially. Two features of triploids are pertinent to the discussions in this manual:



(1) The nuclei of triploid cells are larger to accommodate the increase in DNA in the nucleus, and this leads to a concomitant increase in overall cell size.

(2) Triploids have poorly developed gonads and produce far fewer gametes than diploids.

In 1972, triploidy was proposed as a genetic manipulation of potential value in aquaculture (Purdom, 1972). It had been thought that triploid fish, like polyploid plants, would grow larger simply because they had larger cells. It was demonstrated that this was not so: because the number of cells actually decreases when cells are larger, triploid fish tend to be the same size as diploids (see Purdom, 1972).

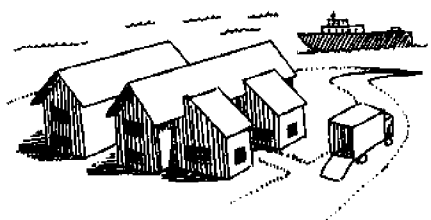
The primary benefits of triploidy derive indirectly from the fact that triploids have poorly developed gonads. Therefore, triploidy can be beneficial to aquaculture when reproductive output of the animal causes a decline in product quality, causes mortality, or impedes growth.

Early Research on the East Coast

Triploid American oysters *(Crassostrea virginica)* were first produced at the Ira C. Darling Center at the University of Maine, Orono (Stanley et al., 1981), using a fungal antibiotic, cytochalasin B. The same research group also produced triploid soft-shell clams *(Mya arenaria)* (Allen et al., 1982), bay scallops *(Argopecten irridians)* (Tabarini, 1984), and hard-shell clams *(Mercenaria mercenaria)* (H. Hidu, pers. comm.) using essentially the same method. At that time the concept of triploid shellfish was new and the utility obscure. Hatcheries in the Northeast were still small, and experimentation of this sort was not easily incorporated into commercial facilities.

Triploids on the West Coast

By contrast, hatcheries were a well-established component of the oyster industry in the Pacific Northwest, an area that grows the nonnative Pacific oyster *(Crassostrea gigas)*. This species produces offspring inconsistently outside its native habitat, and so hatcheries were developed to stabilize the supply (Chew, 1984). Because it is more fecund than the American oyster, it is a less marketable product when sexually mature (Figure 1); according to data provided by the Washington Department of Fisheries, roughly twice as many pounds of *C. gigas* meat are sold in January (a nonbreeding month) as in July. In addition, summer mortality, a phenomenon probably associated with the extraordinary reproductive effort of Pacific oysters (Perdue, 1983), is a problem in some bays in Puget Sound. Triploids seemed a perfect solution.



At the University of Washington School of Fisheries, studies began in 1983 that repeated some of the work done on the East Coast. Soon the concept of triploid culture was embraced by several commercial oyster growers. Research efforts were expanded with financial and logistical backing from Washington Sea Grant and the Pacific Coast Oyster Growers Association. Part of this expanded effort was initiated by Coast Oyster Company in 1984 to apply triploid research done at the University to large-scale production.

The stage was set for the development of triploids for the West Coast industry.

It remained only to demonstrate that (1) triploids could be produced reliably in high numbers, (2) experimental procedures could be scaled up to commercial size, (3) the process was economically feasible, and, most importantly, (4) oyster growers would realize benefits from producing triploids.

The demonstration took four years of research. Triploids now constitute 10 percent of the production at Wescott Bay Sea Farms. In 1987, Coast Oyster Company produced 2.9 billion eyed larvae from triploid spawns, of which more than 70 percent were triploids. This will yield an estimated 125,000 gallons of triploid oysters at market size. Production of triploids in 1988 is projected to be 50%-60% of total production at Coast (K. Cooper, pers. comm.).

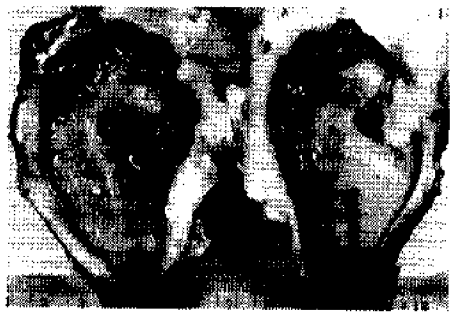


Fig. 1. Comparison of triploid and diploid oysters. Ripe triploid (left) has only local pockets of gonadal differentiation and a larger adductor muscle; diploid cohort (right) is turgid with gametes.

2. PRINCIPLE

Manipulating the Polar Body

Understanding the process by which one produces a triploid oyster from the union of two naturally diploid organisms requires some familiarity with *meiosis*. This is the process whereby a germ cell—male or female—reduces its two chromosome sets to one before sexual union.

At one point during the maturation of the male or female germ cell, the chromosomes in the cell duplicate, so that the cell (at this point called a spermatocyte or an oocyte) temporarily contains four chromosome elements (4N). Two meiotic divisions proceed from this temporary tetraploid condition.

The male cell goes through the two-step division while still in the gonad. It first splits into two spermatocytes each containing half (2N) the chromosomes of the parent cell. Those spermatocytes then divide in two, so that the resulting cells (now called spermatids) contain half the chromosomes (N) of their parent. The spermatids mature into spermatozoa; and it is these haploid sperm that leave the gonad to fertilize an egg.

In the female, the egg remains in a tetraploid state; meiosis does not proceed until the sperm penetrates the egg's outer membrane. It then begins to divide, as the male cell did; but, unlike the male sex cell, the female cell does not ultimately produce four identical cells each with a haploid number of chromosomes. Instead, the oocyte in its first meiotic division extrudes a *first polar body* containing half the chromosomes (2N) of the parent cell, leaving the oocyte with two pairs of chromosomes and most of the cytoplasm. Then, without an interval, the oocyte undergoes a second meiotic division, extruding a *second polar body* that contains one of the two remaining sets of chromosomes.

The egg now has only one (N) set of chromosomes. When that set merges with the haploid set contributed by the sperm, the result is the beginning of a diploid organism (Figure 2).

The meiotic events in the egg of an oyster can be manipulated (by controlled applications of heat, pressure, or a chemical) so that the egg retains rather than extrudes the second polar body. The egg will then contain two sets of chromosomes and the sperm will contribute another, for a total of three (Figure 3). The result is a triploid oyster.

No other special treatment is necessary for growing the triploid. Although there is high mortality initially, oyster larvae grow as well with three sets of chromosomes as they do with two (Downing and Allen, 1987). The trick, then, is to intervene at exactly the right point of development, when the second polar body is vulnerable to manipulation.

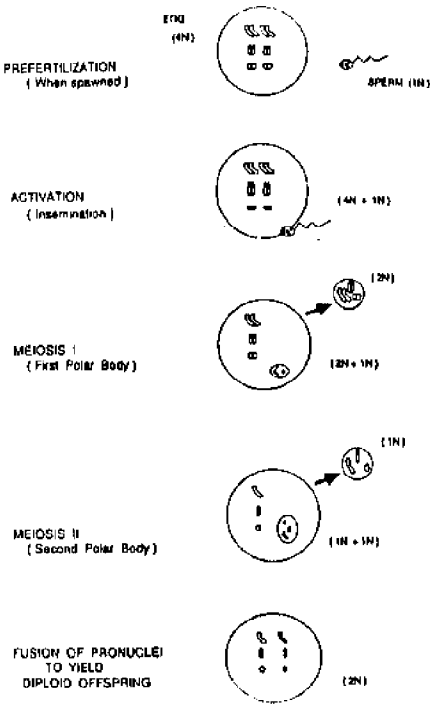


Fig. 2. Normal meiosis in oyster eggs. Sperm has already undergone two meiotic divisions and is fully mature (1N) when released. Insemination activates the egg (at this point a tetraploid because of replication) to undergo two reductive divisons. In the first (meiosis I), a polar body containing two sets of chromosomes is released. In the second (meiosis II)), a polar body containing one set of chromosomes is released, leaving the egg with one set. Diploidy (2N) is restored when the paternal set combines with the maternal set.

Temperature and Salinity

As part of our overall research effort, Lu (1986) examined thousands of newly fertilized oyster eggs at three temperatures to determine the effects of temperature on the timing and duration of meiotic events in Pacific oyster eggs. His results are an excellent demonstration of the correlation between temperature and polar body extrusion.

Two of his conclusions are relevant to inducing triploidy. First, because meiosis speeds up with increasing temperature, the polar body is vulnerable to manipulation sooner at higher temperatures. For example, the second polar is extruded 50 minutes after fertilization at temperatures of 18° C; 43 minutes after at 20° C; and 32 minutes after at 25° C. Second, the meiotic events of the eggs are more synchronous; that is, at higher temperatures (not exceeding physiological limits) the meiotic events transpiring in the eggs are in greater unison. Salinity also affects timing, but less than temperature does and not within the range that oysters would normally experience in a hatchery.

Since we know that temperature affects the rate of meiosis, it follows that,

given the same treatment, eggs treated at different temperatures give different yields of triploids. Downing and Allen (1987) found, in an extensive series of experiments, that cytochalasin B administered at 25° C produced more triploids—and produced them faster—than treatments administered at 18° C or 20° C.

Advantage of Cytochalasin B Treatment

Heat shock and hydrostatic pressure will also induce triploidy in bivalves, but cytochalasin B, or CB, appears to do it better (Downing, 1987a). Heat and pressure dissolve the spindle fibers that separate the chromosomes inside the cells as well as the actin filaments in the cleavage furrow. Once heat or pressure is applied to a fertilized egg, all meiotic events slow to a halt, and only those eggs in stages of meiosis that were vulnerable at the time of treatment—and no others—will become triploid.

Cytochalasin B, on the other hand, acts only upon the actin filaments within the cytoskeleton underlying the cell membrane and prevents formation of the cleavage furrow that partitions the polar body (and its chromosomes) from the egg. But CB has no effect on the movement of the chromosomes. If CB is administered before the polar body forms, meiosis will continue until the polar body begins to bud off (cytokinesis). Only then does CB begin to have an effect. It is therefore a block to polar body extrusion, not an inhibitor of meiosis. Eggs at a vulnerable stage of meiosis will become triploid, as will those that reach the state of vulnerability during the treatment (Downing and Allen, 1987).

Empirical evidence supports this assertion. The highest yield of triploids obtained from application of either heat (Quillet and Panelay, 1986; Downing, unpublished) or pressure (Chaiton and Allen, 1985) was approximately 75-80 percent, but Downing and Allen (1987) have obtained yields of 100 percent using CB. Furthermore, CB yields higher survival than heat or pressure (Downing, 1987a). This manual will describe only the CB procedure.

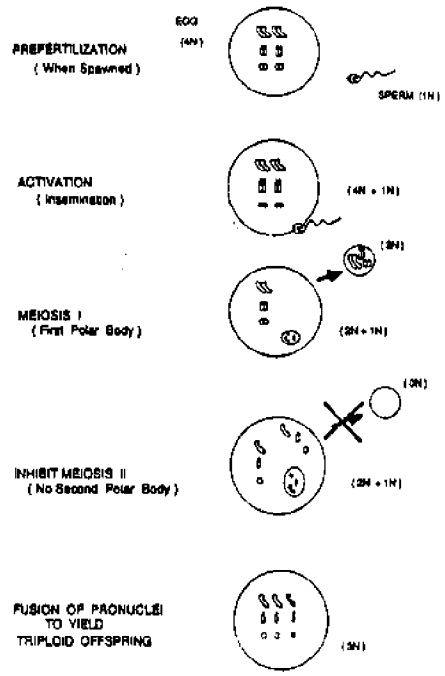
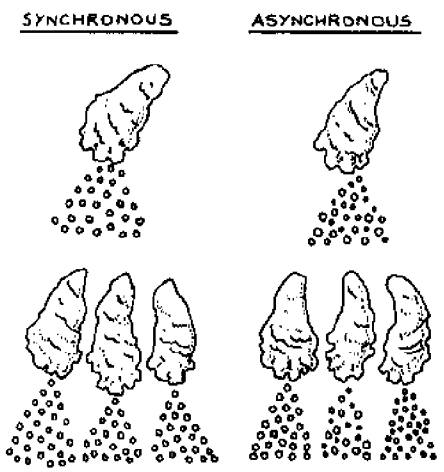
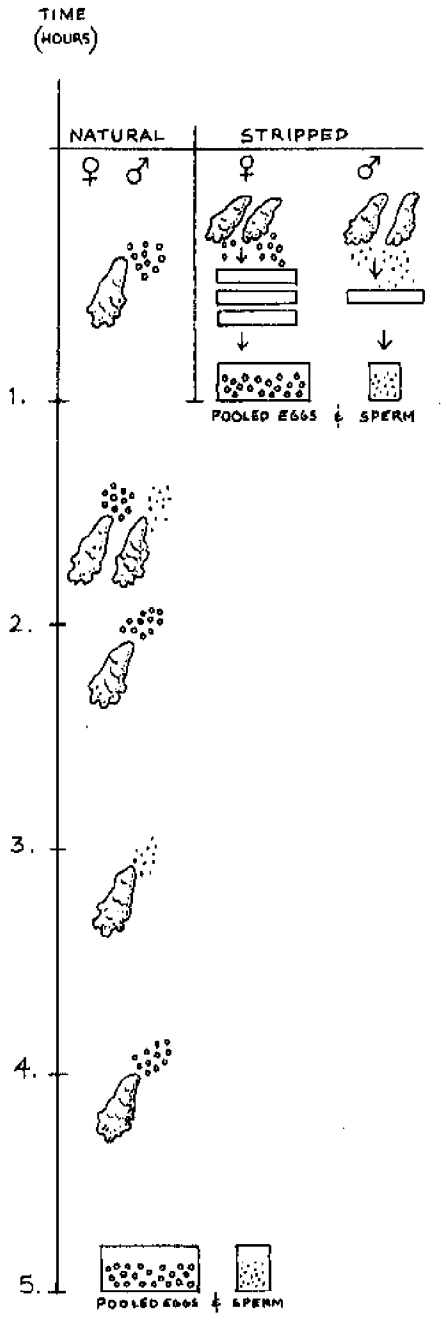


Fig. 3. Interruption of meiosis to yield triploids. Insemination and activation proceed as in Fig. 2, but the second reductive division (meiosis II) is inhibited by either chemical or physical treatment. The egg thus retains two sets of maternal chromosomes, which combine with the paternal set to yield triploid (3N) offspring.





3. PROCEDURE *BEFORE YOU BEGIN*

Broodstock

Broodstock conditioning plays an essential role in producing triploids successfully. An insufficiently ripe female will produce inferior eggs.

This is not a novel concept, but it takes on new meaning in triploid production. Surviving diploid larvae may be obtained from nearly any female that contains eggs, since there are likely to be some ripe eggs present. But for inducing triploidy, uniform development (synchrony) of eggs during meiosis is crucial. Ripe eggs uniformly conditioned for fertilization will go through meiosis synchronously and arrive together at the point in time when CB is adminstered. Unripe eggs will produce an asynchronous "finish."

Synchrony is important not only in the eggs from one female but also in the eggs collected from different females. Females should be at the same stage of maturity so that all the eggs within a female and all the eggs pooled from all the females are as nearly identical as possible.

Males in the broodstock should also be fully mature, so that the sperm are not only active but will fertilize the eggs instantaneously to promote synchronous development.

Work Area

Next to good broodstock, the most important factor in producing triploids is the careful extraction of gametes without cross contamination (i.e., without fertilization). It is better to assume that everything is contaminated with sperm before beginning with spawning than to assume it is not.

Be suspicious! Disinfect everything and keep the spawning area free of extraneous traffic, materials, gear, and oysters.

The Appendix at the end of this chapter suggests guidelines for organizing a work station.

Spawning

There are two approaches to obtaining eggs and sperm for controlled genetic work: natural spawning and strip spawning. The approach one takes depends upon the size of the spawn needed (i.e., number of eggs), the particular species in question, and the importance of keeping the broodstock alive. Generally, the larger and more frequent the spawns, the more favorable stripping becomes, even for diploid crosses. But stripping will not work for all species. And of course it is lethal to the oyster, so maintenance of valuable broodstock (e.g., selected strains) would be ill served by stripping.

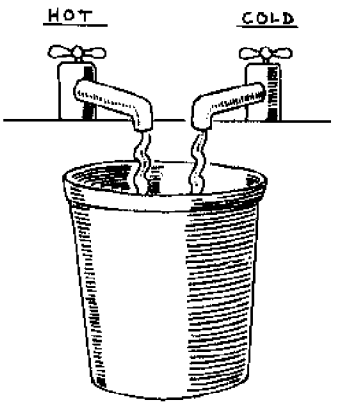
During our research, we investigated the conventional wisdom that stripped eggs of Pacific oysters are inferior to naturally spawned ones. We found nothing to support that belief. Natural spawning did not yield higher survival than strip spawning in diploid crosses. Egg quality seems more defined by the season in which broodstock are conditioned than by how the eggs are obtained from the oyster. Stripping eggs allows one to obtain billions of eggs in the course of an hour instead of waiting for broodstock to spawn naturally.

Obtaining eggs from naturally spawning oysters requires extreme care. First, each animal must be isolated in its own spawning dish. When spawning commences, the gametes of the spawner must be checked for sex. If the spawner is male, the entire dish containing oyster and suspended sperm represents the potential for premature fertilization: one wayward drop can ruin an entire cache of unfertilized eggs. Such wayward drops might come from the valve clapping of a spawning oyster, water dripping from wet hands or utensils, or spillage from the dish into the common water bath in which the spawning dishes are sitting. A dish containing a male must be covered or removed, or both, to prevent inadvertent contamination (fertilization).

Second, it is rare that all oysters respond to thermal or other stimulation at the same time. A considerable time may elapse between the times that the first and the last

oyster spawn. For this reason it is best to have ready in dishes many more animals than would normally be used to yield the needed number of eggs. In our opinion, eggs from different females should be spawned at approximately the same time (e.g., within two hours).

Again, because of the need for synchrony in the events following fertilization, it is imperative that eggs be of uniform quality. Intuitively—and there is some evidence by researchers at the University of Southern California to substantiate our suspicion (D. Manahan, pers. comm.)—it seems that egg quality will decline the longer eggs sit in seawater before they are fertilized.



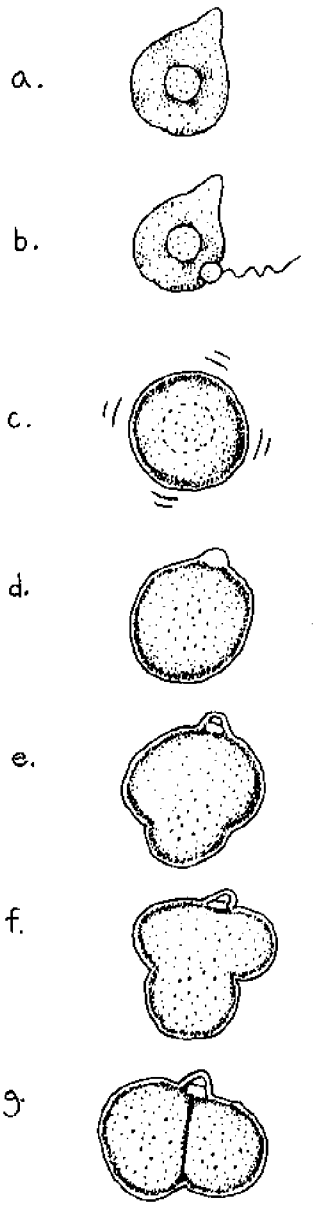
Temperature

Incubation temperature must be carefully controlled from the spawning process on, because the course of early development is so closely correlated with temperature.

We recommend having a source of both cold and hot seawater available during spawning so that it is possible to instantly make up water to any temperature. Lacking that amenity, a large container, such as a plastic trash can, filled with water at the correct temperature will suffice. To maintain temperatures, vessels containing eggs can be placed inside commercial ice chests or into water tables that have water adjusted to maintain proper incubation temperature. (See "Temperature Control" insert in "Doing It.") Temperature should be checked every few minutes.

Egg Density

We have found that the effectiveness of CB treatment diminishes with egg densities of more than 50 million eggs per liter. For example, we have obtained 77% triploidy with 50 million eggs per liter, but only 30% triploidy with 100 million eggs per liter. Therefore, it is desirable to estimate the density of eggs in the final pool, after they have been screened. (See "To Count Eggs" insert in "Doing It.")



Contamination by Fertilization

The object of broodstock management and careful spawning is to obtain a large population of uniformly conditioned and *unfertilized* eggs. Fertilization is easily checked under a microscope by looking for polar body formation or early cleavages. A few such events might be observable in a large pool of eggs, perhaps due to some spontaneous development.

If more than 1% contamination (fertilization) is observed, it is wise to wait for 15 minutes to determine what the actual proportion of fertilization is. If low (say, approximately 10%), it is probably still worth continuing; but if the proportion is much higher than 10%, efforts to produce a reasonable percentage of triploids would probably be futile.

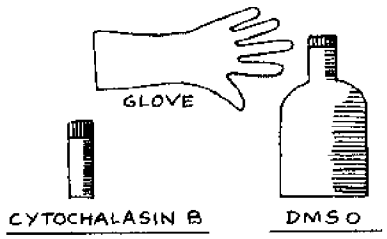
Monitoring Development and Timing

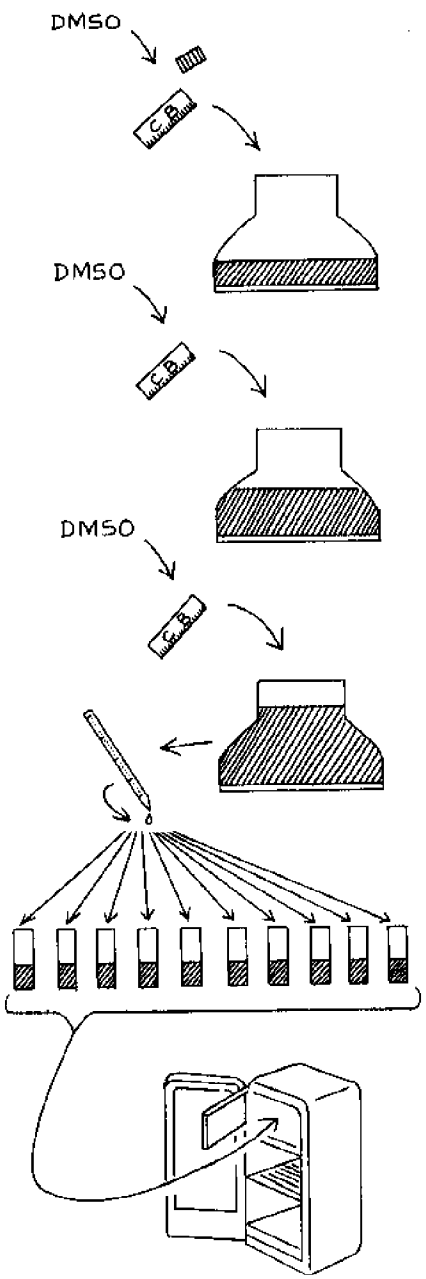
Adding sperm to the eggs activates egg development (meiosis) and starts the clock ticking. It is essential to become familiar with the course of early developmental events—germinal vesicle breakdown, polar body formation, and cleavage—because these events signal the biological time that has elapsed. Monitoring the biological events is more important than watching the clock, because it is probable that different stocks or populations of oysters have different rates for meiotic events. This is a little-studied area.

Observing development is a good measure of the synchrony of events after fertilization. Observations from spawn to spawn should provide a clue to the major factors affecting synchrony.

Using Cytochalasin B

Safety. When treating the oyster eggs, you will be handling two hazardous substances: cytochalasin B (CB) and dimethyl sulfoxide (DMSO), the substance in which the CB must be dissolved before it can be used. Cytochalasin B is toxic at high concentrations; it is also believed to be carcinogenic. DMSO, although safe itself, is a





universal solvent and passes easily through membranes such as skin and latex gloves. Anything dissolved in DMSO—CB, impurities in the CB, or whatever is on the lab bench—will also be transported through these membranes.

Use extreme caution when handling CB, CB dissolved in DMSO, or DMSO alone. Avoid contact with DMSO by wearing vinyl, neoprene, or nitrile gloves during preparation of aliquots and treatment of oysters.

Preparation and storage of CB. Cytochalasin B is available as a powder in milligram quantities,which makes it impractical to weigh out. Because it is also less expensive in larger quantities, we recommend that you obtain enough for a major portion of your spawning needs, dissolve it in DMSO, and store it in convenient aliquots by the following procedure.

The dose of CB for each treatment should be between 0.5 and 1.0 milligram per liter of seawater, and egg density should not exceed 50 million per liter. Package aliquots of CB according to the number of eggs that you expect to treat each time. To be conservative, aliquot the CB in 1 milligram quantities.

To do this, dissolve the entire quantity of CB in an equal volume of 100% DMSO. For example, dissolve 10 milligrams of CB in 10 milliliters of DMSO (use ACS Reagent grade). Since the CB amounts to little more than a dusting inside the packaging bottle, you will have to repeatedly rinse the inside of the CB bottle with small amounts of DMSO until the final volume is attained.

With a pipette, aliquot the DMSO-CB solution in 1-milliliter quantities (or more, according to anticipated needs) into small glass vials. Stopper the vials and store frozen; the freezer compartment of a conventional refrigerator is fine. Aliquots may be thawed just before they are needed.

In frozen form the solution, now at a concentration of 1 milligram CB to 1 milliliter DMSO, should last for longer than a spawning season and should be stable for up to a year. Contact the CB source (ours is Sigma Chemical Company, St. Louis) to determine absolute storage life.

Treating the eggs with CB. Aliquots of DMSO-CB are easily added to the proper volume of seawater containing the density of eggs at the designated time (see "When to Begin Treatment" insert in "Doing It"). We often add the DMSO-CB solution to some seawater first (e.g., 10% of final volume) before dumping it into the container of eggs, in order to speed dispersal of the chemical and avoid local patches of high CB concentration. Wear gloves here, too.

Rinsing

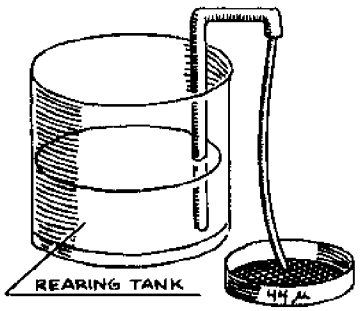
After the eggs have been treated, they should be strained and rinsed with a DMSO solution to remove the CB residue. The straining screen should be small enough to retain all the eggs but not so small that it slows drainage to the point that it significantly increases the time that the eggs are exposed to CB. In our experience, a Nytex screen with a mesh of 20-25 microns is suitable for Pacific oysters. Using a screen with a large surface will also help to decrease the time it takes to drain the egg suspension.

Once drained, the eggs must be considered still exposed to CB since it is probably still bound within the egg membrane. They should therefore be quickly but gently washed with seawater and then resuspended in the rinse solution.

We rinse the treated eggs in a 0.05%-0.1% solution of DMSO in seawater. We have found that survival was significantly less when the DMSO rinse was eliminated. The length of time for the rinse is probably not critical, although we routinely rinse for at least 20 minutes.

We have found no need to eliminate the DMSO from the egg suspension prior to incubation. That is, after rinsing is over, the entire egg-DMSO suspension may be poured into the culture vessel. This also eliminates another handling step for the eggs. We have done controlled tests by incubating eggs with and without DMSO. Our results indicate, if anything, slightly better survival with DMSO than without.

Larval Culture



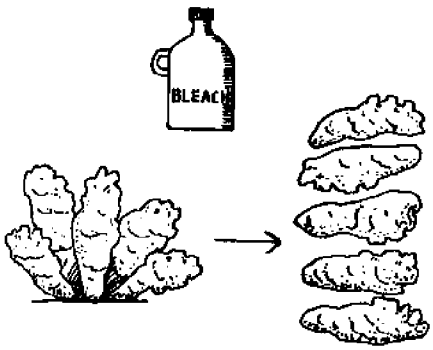
Triploid larval culture is the same as normal diploid culture except in one respect: when to make the first water change. It is more important to triploid than to diploid culture that the water be changed at the straight-hinge stage (by day 2), because more treated larvae are expected to die and therefore produce more extraneous organic matter. Consideration might be given to rearing early larvae at a lower temperature than normal to help control bacterial growth on the organic matter, e.g., 20° C for Pacific oysters.

In the first several days it is impossible to determine whether the small larvae are permanently damaged or are just temporarily retarded by the treatment. Adopt a conservative screening policy in which you maintain small individuals for the first week. We have noticed no difference in growth rate between triploid and normal spawns in the later stages of development.

Early Mortalities

Because CB is essentially a toxin, many of the larvae treated with CB will die—between 50% and 99%, depending on the time of treatment and the quality and synchrony of the eggs. Although it varies, a mortality of approximately 70%-85% means that the treatment was strong enough to have caused an effect but not so intense that it killed everything. Early larval survival of triploids is about one-third that of untreated diploids. This early mortality is the outstanding weakness of the technique.

Fortunately, Pacific oyster broodstock is not difficult to obtain, and mortality is concentrated in the first week, when the investment in larval culture is still small. Obviously, in the production of triploids, many more eggs must be treated than the number of eyed larvae needed.



PROCEDURE *DOING IT*

A. Prepare and disinfect work area. (Guidelines for organizing a work station appear as an Appendix to this chapter.)

1. Prepare several buckets of chlorine solution, approximately 5% household bleach in fresh water.

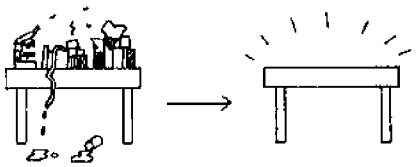
2. Break up clumps into single oysters. Clean oysters of mud and clinging organic matter and rinse them with seawater. Clean outside of oysters with chlorine solution and rinse again.

3. With a sponge, generously apply chlorine solution to all surfaces in the work area. Allow to sit for a few minutes and rinse with seawater.

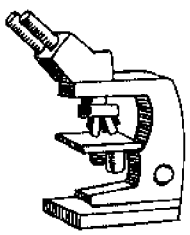
4. Using the chlorine solution, rinse and clean all buckets, dishes, screens, and other apparatus that will contact gametes.

5. Put all shucking knives in their own bucket of chlorine solution and place near shucking area.

B. Spawn the oysters.



If you are strip spawning, the shucking area should be a bench or table with a smooth surface that can be disinfected. Stainless steel is ideal if it is available. Pitted wooden benches should be avoided. A compound microscope should be available so that oysters can be sexed and the quality of the eggs checked. If you are spawning the oysters naturally, make sure spawning table has been disinfected with chlorine solution.



To strip spawn:

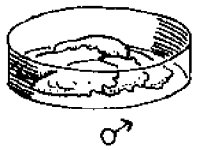
6. Remove one oyster from the pile of broodstock to the shucking table. Open it carefully so as not to puncture its body and release gametes.

7. Sex the oysters. Disposable Pasteur pipettes are ideal for removing small biopsies of gonad for inspection and then discarding. Alternatively, the gonad can be lacerated lightly with the oyster knife and a sample placed on a microscope slide. High-quality eggs have large, well-defined nuclei.

8. If the oyster is MALE, the top shell may be replaced during this time and the oyster put into a container. The container should be as far away as possible; keep it well removed from the shucking table and

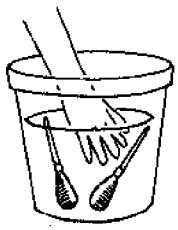
the female containers.

If the oyster is FEMALE, replace the top shell and put it in a female pile or container that is close by but away from the spawning table.

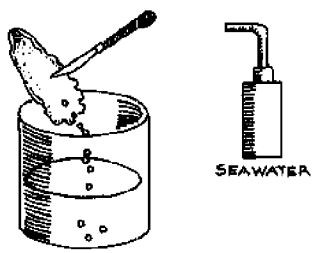


9. Each time you shuck a MALE oyster, throw the shucking knife into the bucket containing disinfectant and disinfect the working surface of the shucking table before you go on to the next oyster (because of spilled oyster juice). Plunge hands into disinfecting bucket, rinse them off, and take another shucking knife for the next oyster.

If the last oyster shucked is a FEMALE, throw the shucking knife into the bucket containing disinfectant. Take another shucking knife and proceed to the next oyster.



10. Start again at step 6, repeating the procedure until you have enough oysters of each sex. For Pacific oysters, three or four of each sex should be enough to produce and fertilize 50-150 million eggs. Other oyster species produce fewer eggs, and so more individuals will be needed. The ideal sex ratio, genetically speaking, is 50:50. (See "Genetic Considerations.")



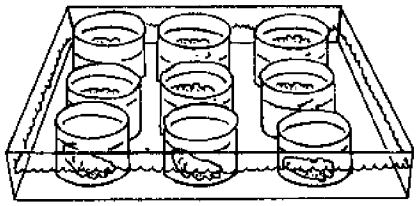
11. Work only with females until all eggs are stripped, screened, counted, and diluted. Females to be stripped may be lacerated on the body surface with a scalpel or razor blade and gently massaged to extract the gametes. Strip each oyster into a separate dish, or several per dish if using more than ten females. In other words, don't put all the eggs into one "basket."

GENETIC CONSIDERATIONS

Normally it is best to maximize the genetic variability during spawning. This is done by several means—using equal numbers of males and females and using as many adult spawners as possible, for example. The rationale for this practice is that it limits the amount of inbreeding that occurs in the stock.

For producing triploids, the argument is somewhat different. Triploids are produced de novo from generation to generation. Therefore, the amount of genetic variation in the production lot seems unimportant in the lineage of the stock. However, genetic variability may be important during the lifetime of a particular generation of triploids.

Consider the extremities and variability of the environment in which the triploids will grow. Although they do not have to reproduce, they still must survive and grow well for one to three years. Limiting the number of broodstock limits the range of genetic combinations, and thus the genetic variability, in the triploid generation. Inadvertently choosing one or two genetically inferior parents will affect a triploid oyster's ability to survive. Therefore, we recommend that genetic considerations be given high priority when producing triploids (as they are with diploids), by maximizing the number of parents and using equal (or nearly equal) numbers of males and females—at least three of each for each 50 million eggs.



If using naturally spawned oysters:

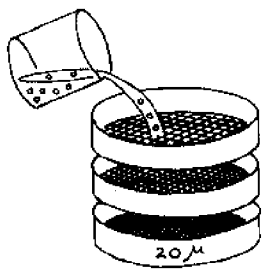
Rather than follow steps 6-11 above, naturally spawn the oysters according to your own protocol, with the following exceptions:

—Keep individual oysters in separate spawning dishes, such as 1-liter beakers or baking dishes. High sides prevent oysters from squirting water from dish to dish.

—Do not allow water from one container to contaminate other containers (as by spilling or dripping water from hands or utensils). Dishes can be put into a common water bath—for example, in a spawning table to stabilize temperature—but take care that the bath water does not contaminate water in the dishes. Assume it is full of sperm.

—Unless eggs are visible, assume that all dishes contain males with the potential to fertilize.

—Do not stimulate spawning with sperm.

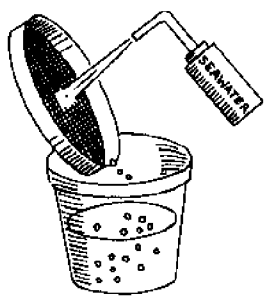


—When a male spawns, isolate or cover, or both, the dish containing the sperm. Leave the oyster in the dish.

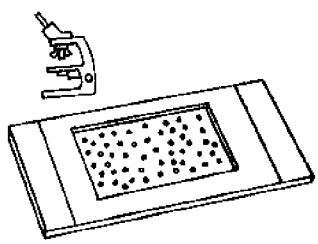
—Rinse hands in chlorine solution between handlings of dishes or oysters.

— When enough females have spawned, collect the eggs on an appropriate sized screen (see below).

C. Prepare gametes.



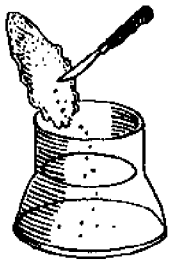
12. When all females have been stripped or have spawned naturally, eggs and other oyster tissue will be contained in one or several vessels. Put this suspension through a series of screens, one on top of the other, to sort the extraneous tissue from the eggs. Depending on the amount of tissue debris, a series of screens consisting of 220, 100, 70, and finally 20 microns (to catch the eggs) should suffice. Pacific oyster eggs average 40 microns in diameter. If the amount of debris is low, 70- and 20-micron screens should do. Eggs can be gently rinsed with seawater. Tilting the 20-micron screen in a circular fashion (similar to panning for gold) facilitates drainage when there are many eggs.



13. Gently wash the eggs off the 20-micron screen into a bucket. Use seawater that has been adjusted to the proper incubation temperature (25° C for Pacific oysters).

14. Count the washed eggs, suspend them in half the correct final

volume of seawater, and set them where the temperature will be maintained. (See "To Count Eggs.") Suggested optimal density in the *final* suspension of eggs from Pacific oysters is 25 million eggs per liter, but lower densities are fine.



15. Strip some sperm from all available males, or pool the spawned sperm so that the contribution of sperm from each male is approximately equal. This can be done by hand since not much sperm will be needed.

TO COUNT EGGS

So that you count only 30 - 50 eggs rather than 50 million, take 1 milliliter of liquid from the egg container (whose volume is about 5 liters) and dilute it with 1 liter of seawater. Take 1 milliliter from that volume and place it in a Sedgewick Rafter slide to count the eggs under a microscope. Repeat the procedure to confirm the accuracy of the first count. If the count is way off, repeat the procedure at least twice.

The number of eggs can also be estimated if the approximate number of eggs per female is known. For example, in commercial treatments with broodstock of fairly uniform size, we have used the estimate of two females per milligram of CB.

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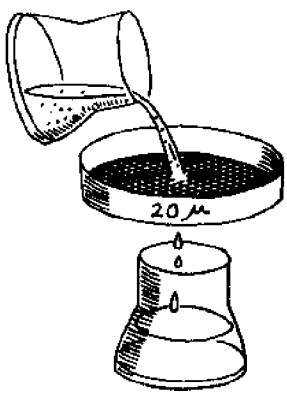
16. Screen all the sperm through a 20-micron screen to separate extraneous tissue. (Keep what drains through, not what is on the screen.)



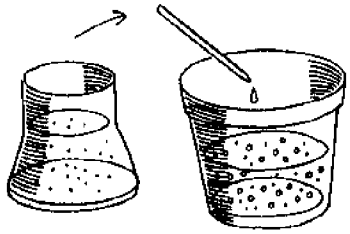
D. Fertilize eggs.

17. At this point, eggs are ready for fertilization. Before fertilizing, check the eggs for contamination, i.e., for evidence of early development. There should be none. Check water temperature and correct it, if necessary, so that it is at 25° C. (See "Temperature Control.")

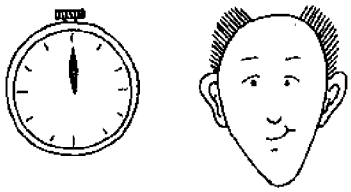
TEMPERATURE CONTROL

It is essential that eggs incubate at a constant temperature so that all the eggs develop consistently. Here is one way to maintain a constant temperature within the spawning containers.

When you know the final density of eggs, and therefore the final volume, place the eggs in a spawning container filled to 50% of that volume. Place the container inside an ice chest or water table containing a water bath at 26° C (which is 1° C over the correct temperature of 25° C for Pacific oysters). Stir the eggs gently and check the temperature of the suspension before adding the sperm. If necessary, correct the temperature by adding warmer or cooler water to the container of eggs. Adjust the temperature of the water bath if needed. After adding the sperm, bring the water in the containers to about 75% of final volume. Check the temperature every few minutes as you stir the eggs and adjust it with seawater. Five minutes before adding CB, bring the level in the container up to 90% of the final volume. Then use the last 10% for mixing with the CB-DMSO aliquot before stirring it into the container.



18. Fertilize eggs by adding sperm. It is better to use a more dilute sperm suspension than is normally used for regular spawns: dilute suspensions will help to disperse sperm throughout the eggs and promote synchronous development. Start a timer set for 20-30 minutes. (See "When to Begin Treatment," to determine what time to use.) Stir the solution for 30-60 seconds after adding sperm.

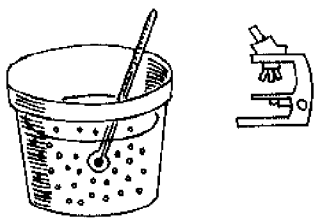


WHEN TO BEGIN TREATMENT

The best time to add CB varies from hatchery to hatchery. We found that 30 minutes after fertilization worked well in our research facility and at one hatchery; but at two other hatcheries, 30 minutes yielded few survivors. Survival there was improved by starting the treatment at 20 minutes.

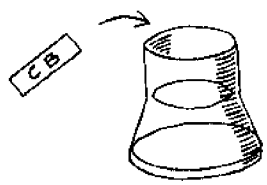
We stress that biological time is more important than elapsed time: slight variations in incubation temperatures and possible stock differences lead to variable times of polar body extrusion. You should start the treatment when 50% of the first polar bodies are being extruded, which occurs between 20 and 30 minutes after fertilization.

A hatchery can experiment with treatment time by subdividing a spawn into two or three groups, adding CB at 5-minute intervals, and then noting survival rate, as in the following table, which shows absolute survival (in percent) when CB is administered 20 and 30 minutes after fertilization.



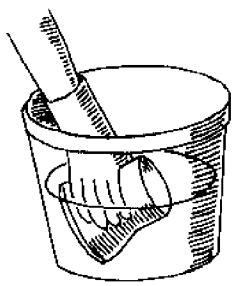
Days	20 min.	30 min.	Control
0	100	100	100
2	26.2	26.2	78.1
5	25.2	23.1	78.0

E. Stir and check.



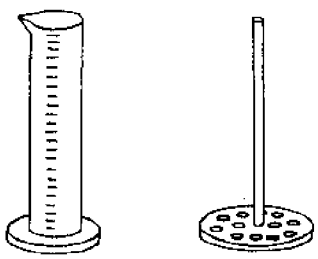
19. Stir and check temperature every 3-5 minutes. Adjust water temperature if necessary (see "Temperature Control").

20. Check development with a microscope to note biological development.



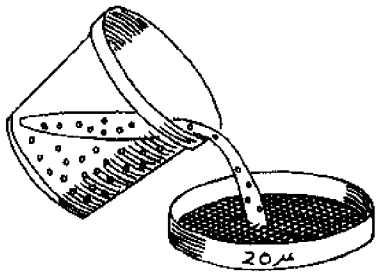
F. Treat the eggs with CB.

21. At the buzzer (20-30 minutes after fertilization), add 1 mg CB/ 1 ml DMSO stock solution to every 1-2 liters of fertilized egg suspension. Wear gloves when you handle CB. We recommend diluting the CB-DMSO solution in seawater first, at approximately 10% of the final volume. At this time, bring the solution up to its final volume by adding seawater of the appropriate temperature.



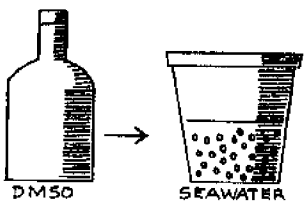
22. Set the timer again for 20 minutes. Using a plunger (a graduated cylinder works well), keep the suspension roiling *gently* throughout the 20-minute treatment period.

G. Strain the eggs.



23. After 20 minutes of exposure to CB, separate the eggs from the chemical by screening them onto the 20-micron screen. If screening takes an inordinate amount of time, treatment could be shortened, but screens with larger surface areas are a better alternative.

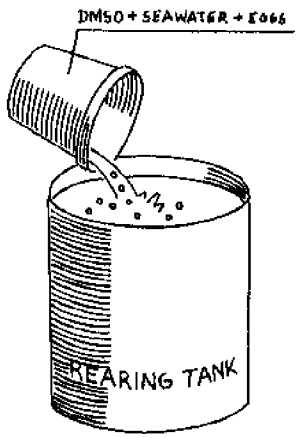
H. Rinse eggs.



24. Gently rinse the eggs with 25° C seawater and resuspend in fresh seawater at 25° C. Add 1 milliliter DMSO for every 2 liters of seawater. Stir the solution, and set timer again for 20 minutes. Stir the solution occasionally.

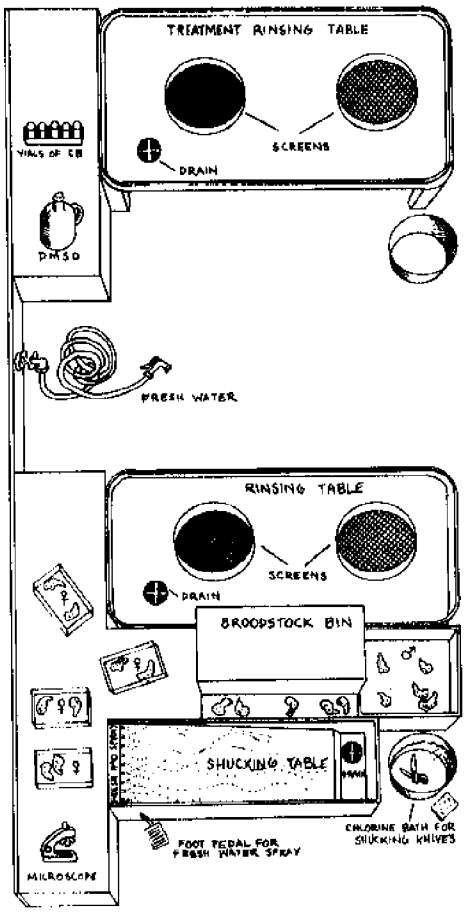
25. After approximately 20 minutes, pour the egg-DMSO suspension into a culture tank.

I. Incubate the eggs.



26. Culture the triploid larvae by the same procedure as used for culturing diploid oysters, but be sure to make the first water change by the straight-hinge stage (day 2).

During the second water change, continue to use small screens (e.g., 44 microns) to keep small individuals that may develop into normal larvae.



APPENDIX *WORK STATION FOR TRIPLOID PRODUCTION*

The accompanying sketch represents an idealized work station for strip spawning and subsequent genetic techniques such as chromosome set manipulation. It is meant as a guide to setting up a work area, not as a blueprint required for this type of work.

At bottom is the shucking side of the table. Oysters kept in the broodstock are reached by a slot at the bottom of the bin, as in a shucking house. Oysters are opened one at a time on the stainless steel working table, which is equipped with a freshwater spray at left. The spray can be controlled by a foot pedal to keep hands free.

To the right of the opening table is a chlorine bath for disinfecting oyster knives and holding a sponge to wipe the table. At the left of the opening table is a microscope for sexing the oysters.

When a male is opened, it goes into a sink or basin that will hold only males. Note that this sink for males is separated from the general work surface by the chlorine bath on one side and partitions on the other three sides.

When females are shucked, they are placed in dishes, one or several per dish, and placed to the left, where they can be passed to the egg rinsing side of the table. *No sperm is allowed on the egg rinsing side while females are being handled.* Eggs are stripped here and cleaned on screens. When cleaned, eggs are counted, then pooled into one or more 10-liter buckets and set aside in a water bath (for temperature control) near the treatment rinsing table. The eggs are not yet fertilized. When all females have been stripped, males may then be stripped and cleaned at the egg rinsing table.

Fertilization and treatment occur at the treatment rinsing table. Eggs should *never* be placed on this table before they are ready for fertilization.

Items to have on hand by the treatment table include thermometer, CB, DMSO, pipettes, and a graduated cylinder or plunger of some sort.

Items to have on hand in the work station include (besides the oysters) knives, pipettes, slides, microscope, CB, DMSO, screens, 1-liter beakers, buckets, bleach, and a plunger.

4. HOW DO I KNOW THEY ARE TRIPLOID?

Asking the question "Did the procedure work?" is actually asking two questions: "Were triploids produced?" and "How many?" In other words, what proportion of the living spat are triploid?

As adults, triploids can be visually distinguished from diploids during the reproductive season, when diploids are ripe and triploids have poorly developed gonads. Unfortunately, this distinction does not exist in oyster spat. Determining whether treated oyster spat are triploid therefore requires some kind of cytological analysis.

Four such analyses are discussed below (and their protocols are detailed in the Appendix at the end of this chapter). Since none of them is particularly amenable to the lab facilities in most hatcheries, it is probably wise for the commercial producer to anticipate this need either by assigning a small area to rudimentary laboratory space or by reaching some sort of cooperative agreement with extension or University personnel.

Chromosome Preparation

Chromosome preparation is the most direct method of distinguishing diploids from triploids (see Figure 4). The technique involves preparing the cells of spat on glass slides so that their chromosomes can be visualized through a compound microscope. Although it sounds difficult, the technique is quite straightforward, requiring only a little practice to learn.

Chromosome preparations are time consuming—about half an hour per spat. If triploids are to be made infrequently, e.g., once or twice a month, an extra day of labor to estimate the effectiveness of the treatment is not overwhelming. But if they are produced more often than that, evaluating spat via chromosome preparations becomes laborious.

Fig. 4. Chromosomes of Crassostrea gigas, *visualized through a compound microscope. Diploid (top) has two sets of 10 chromosomes; triploid (bottom) has three sets.*

Flow Cytometry

The method we used for most of our research is a technique called *flow cytometry*. Flow cytometers are usually found at a university or in a hospital (especially a research hospital). They are highly specialized instruments designed to address sophisticated questions about DNA content, and using one to answer our straightforward question "Is it diploid or triploid?" is akin to using a hydraulic press to open a walnut. The analyses, however, are fairly easy.

The two major problems are access to the equipment and cost of running the samples. Usually a flow cytometer is run by one or several technicians who are solely responsible for its care and maintenance. When the equipment is hired, so are the technicians. In most cases they are generous with expert advice on improving the analysis, but their time increases the cost of the service.

However, since the question of diploid versus triploid calls for a straightforward analysis, many samples can be performed in only an hour. Thirty samples an hour is probably reasonable to expect; that is, a sample of thirty spat can be analyzed from the total population of possible triploids. Sample preparation requires only about 2-4 minutes per sample—considerably less than for chromosome analysis. So even at rates between $35 and $100 per hour, flow cytometry can be economical.

Microfluorimetry

A third technique, related to flow cytometry, is *microfluorimetry*. In microfluorimetry, nuclei are stained with fluorescent dyes just as they are in flow cytometry. However, instead of hundreds or thousands of cells per second being measured as they flow by a sensor, the fluorescence from individual nuclei is measured one by one with the aid of a microscope-mounted photometer.

This is a more appropriate technique for a straightforward task, like using a nutcracker to open a walnut. A substantial investment is still involved in obtaining a high-quality microscope with an ultraviolet light source and a photometer, but such equipment is more likely than flow cytometers to be found in laboratory facilities.

Determination of Nuclear Size

A fourth technique that might be useful in hatchery situations is *determination of nuclear size*. The nuclei of triploids have 1.5 times the *volume* of diploid nuclei (in order to maintain a constant nucleus-cytoplasm ratio). It is possible to measure nuclear volume directly with a Coulter counter, another piece of specialized equipment but one that is both cheaper to use and more accessible than the flow cytometer. Not much work has been done to adapt this technique for shellfish, and the reader is referred to Benfey et al. (1984), Johnson et al. (1984), and Wattendorf (1986) for discussions of this method with fish cells.

It is equally possible to discriminate between diploids and triploids by the *difference in diameters* of their nuclei as measured from smears of leucocytes. We have not given this technique much attention because we can use flow cytometry, but it was investigated by Mark Sellers at the University of Maine (see Appendix). Least expensive of all the techniques, it is probably also the most time consuming.

APPENDIX *PROTOCOLS FOR PLOIDY ASSAYS*

Chromosome counting (Allen et al., 1982)

1. Arrest metaphases. — Try to use spat that are 3-5 mm in diameter. Take several shells on which spat have set or, if cultchless, take about two dozen spat, and place them in a beaker containing seawater, some algae to keep oysters pumping, and 0.05% colchicine. Colchicine should be made up fresh from a more concentrated stock solution (kept frozen between uses). Leave them in colchicine for 4-8 hours at the warmest temperature that keeps them healthy, e.g., 25°-30° C.

2. Swell the cells with hypotonic solution . — Remove oyster tissue from shell in one piece and place all the tissues together in cold 0.9% sodium citrate (about 50-100 times the volume of tissue) for an hour. Agitate the oysters in the hypotonic solution several times during the hour.

3. Fix the tissue. — Drain off the citrate solution and add about 10 times volume of freshly made Carnoy's solution (3:1 absolute methanol: glacial acetic acid). Agitate tissues in fixative. After a few minutes, drain Carnoy's solution and add the same volume of fresh Carnoy's solution again. Fix for one hour.

4. Store the tissue. — Drain the Carnoy's solution and add about 5 times volume of absolute methanol. Drain and repeat. Oyster tissues may be stored in methanol at 4° C for at least six months.

5. Make cell suspensions. — Remove one oyster from methanol and place in watch glass or other small container. Dab with tissue to remove excess methanol. Add 1 drop of 50% acetic acid and mince the tissue with scalpels. It should fall apart easily. Using a pipette, place the minced tissue suspension into a test tube and suspend in about 1-2 milliliters of 50% acetic acid.

6. Prepare slides. — Allow cells to stand in 50% acetic acid 10-15 minutes. (Several more tissues may be minced during this time.) Using a Pasteur pipette, drop single drops of the cell suspension onto a glass slide that has been warmed to 45°-50° C. Immediately aspirate the drops off the slide by taking up the drops into a pipette. There should be a ring of cells where the edge of the drop was. Repeat until 10-20 individual rings are present.

7. Stain cells. — Remove slides from slide warmer and allow to air dry overnight. Stain in 5% Giemsa in pH 6.5 phosphate buffer for 10 minutes. Giemsa should be made fresh for every use from a stock solution.

8. Scan the slides under a compound microscope for chromosome spreads. Diploid Pacific oysters have 20 chromosomes, triploids 30.

Flow cytometry preparations

1. Remove spat or biopsy. — Spat as small as 1 mm can be used. Pry off one shell and place the whole tissue into a test tube containing 0.5-1 milliliter of fluorescent dye with 1% detergent (e.g., NP-40). If the oyster is larger than 5 mm, use only a piece of the adductor muscle.

2. Dissociate cells. — Leave spat or biopsy in fluorochrome solution at 4° C for 6 hours or overnight. Or, for immediate use, syringe and expel 3-5 times in test tubes. Samples may be frozen in 10% DMSO at this point.

3. Filter cells. — Syringe and expel the suspension one or two times through a syringe fitted with a 23 G needle to isolate individual nuclei to run through the machine. Then filter the cell suspension directly into the flow cytometer containers, using a Nytex screen with a pore size of 20-50 microns (the smaller the better).

4. Note that, depending on what kind of flow cytometer is used, different dyes may be used. Discuss this with the technician.

Microfluorimetry (Komaru et al., in press)

1. Obtain biopsy. — From large spat or juveniles, dissect a small piece (2-5 cubic millimeters) of gill and fix it in fresh Carnoy's fixative (3:1 absolute methanol:glacial acetic acid).

2. Prepare cells. — Mince fixed gill with scissors or scalpel in 50% acetic acid. Drop the cell suspension on a warm (50° C) slide. As a reference, drop cells from a known diploid onto another region of the same slide and air dry.

3. Prepare slides. — Stain with DAPI solution for one hour at 4° C. Cover slides with coverslips and seal with nail polish.

DAPI solution:

50 ng/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI)

10 mM 2-mercaptoethylamine hydrochloride in Tris buffer

Tris buffer, pH 7.4:

10 mM Tris

10 mM EDTA

100 mM NaCl

4. Measure fluorescence. — Excite the DAPI-stained nuclei with an ultraviolet light source (365 nm) on a fluorescent microscope equipped with a photometer. Measure 10-20 nuclei, comparing the mean intensity of fluorescence of the putative triploid nuclei with that of the diploid cells on the same slide.

Nuclear size determination (Sellers, 1982)

1. Remove cells for analysis. — Using a hypodermic syringe, withdraw cells from the adductor muscle sinus. Only 0.1 cc is needed. Place fluid containing cells on a clean glass slide and allow cells to settle for a few minutes. In small spat, cells from a particular tissue might be used for the same comparison as granulocytes. In that case a sample of tissue, e.g., gill, would be cut away and minced to form a cell suspension.

2. Fix cells. — Flood slides with absolute methanol for 5 minutes and then rinse with fresh absolute methanol.

3. Stain cells. — Remove methanol and flood slides with certified Wright's stain (Fisher Co.) for 2 minutes, then dilute with an equal amount of Bohorfoush diluent (0.1-0.2 grams of sodium thiosulfate per liter of distilled water) for another 2 minutes.

4. Dry slides. — Remove stain, wipe the back of the slide, and allow to dry. Slides may be mounted in Euparol, but this is not necessary. Two or more types of granulocytes may be observed. They are either basophilic (granules stain blue, nuclei red) or acidophilic (granules stain pink, nuclei blue). Measure only one kind.

5. Measure nuclei. — Using a compound microscope, measure the maximum diameter of granulocyte nuclei with an ocular micrometer under oil immersion.

6. Calculate means, differentiate ploidy . — The greater the number of cells measured, the more confidence can be attributed to the differences between the observed mean diameters. For a thorough discussion of the statistical power of various sample sizes, consult Wilbur (1976). Triploid maximum diameters should be approximately 1.33 times as long as those of diploids.

5. TRIPLOIDS VERSUS DIPLOIDS IN COMMERCIAL CULTURE

The outstanding characteristic of triploid Pacific oysters, as we have said, is poor gonadal development, a trait they share with triploid American oysters, soft-shell clams, and bay scallops and one that easily distinguishes them from diploids during the reproductive season (Figure 1). We have grown diploids and triploids in parallel in California and Washington State bays to monitor their adult performance and to compare them as marketable products.

Maturity (Reproductive Effort)

During winter and early spring, both triploids and diploids are inactive sexually (Allen and Downing, 1988). At that time, diploids can often be identified as male or female by the germ cells present. In late spring, the diploids start their annual gametogenetic cycle as they go through the active stages and become ripe by midsummer.

Triploids also start the gametogenetic cycle, but they do not complete it. Triploid males proceed as far as the late active stage, while females mature only to the midactive stage. Triploid males devote more effort to reproduction than triploid females do, but neither sex devotes as much as diploids. The sex ratio of males to females (1:1) is identical for diploids and triploids, but triploids have a significantly higher proportion of hermaphrodites (individuals with gametes of *both* sexes): 30% in triploids versus 1%-2% in diploids.

Growth

Our data indicate that triploids and diploids have equivalent growth rates until they reach first sexual maturity. Diploid growth then slows with gonad development, while triploids use their extra glycogen to grow faster. In terms of *rate* of growth to market size, triploids are either faster or equivalent.

A closer look at summer growth patterns in productive temperate environments shows that, at the peak of gametogenesis, diploids cease to grow as they convert glycogen reserves into lipid (eggs) and protein (sperm) (Downing et al., 1988). Since triploids go through less gametogenesis than diploids, they continue growing throughout the summer. In some instances, subsequent growth in diploids does not compensate, so the triploids are larger after the summer. Data suggest that diploids in unproductive bays may have an advantage because they have a lower metabolic basal rate than triploids. Although it will depend on the rearing environment, producing triploids for larger size alone would appear to be ill advised.

Survival

Survival also depends on the environment, but in a less predictable manner than growth. When growing triploids and diploids in parallel, we found that in some cases triploids survived better than diploids. For example, in 1986 in Humboldt Bay, California, spawning stress caused significant mortality among the diploids: before spawning, approximately 40% of the total population were triploids; after spawning, the proportion had risen to approximately 60%. Therefore, triploids must have outsurvived diploids.

However, in a Washington state study we designed specifically to investigate comparative survival, mortality occurred randomly and equally in both diploids and triploids in three bays during 1987, although triploids in one of the bays had suffered a higher mortality two years earlier. Overall, adult survival should be about equal in diploids and triploids.

Marketability

Because they use far less of their glycogen reserves than diploids during the reproductive period, triploids in that season constitute a more nutritious product, higher in protein and glycogen and lower in lipid.

For the same reason, they are also a firmer, sweeter, tastier product. In two taste tests we ran to compare triploids and diploids at the peak of gametogenesis, consumers and oyster growers preferred the triploid oyster's flavor and texture and ranked it higher in overall preference. We assume that diploids and triploids taste the same during the nonreproductive months.

Growth of Continuous Spawners

In regions where gametogenesis is more or less continuous throughout the year, triploid culture may hold great promise. Triploids have not been tested in tropical or subtropical environments, but they may fare much better there than diploids, which, during a protracted spawning period, must devote a constant proportion of their energy budget to gametogenesis.

6. OTHER APPLICATIONS OF CHROMOSOME SET MANIPULATIONS

Interspecific Triploids

Chromosome manipulation might also be used to produce triploid hybrids that would combine the market qualities of both species, e.g., the vigor of *Crassostrea gigas* and the high glycogen content of *C. rivularis*. The reciprocal crosses between *C. gigas* and *C. rivularis* have been accomplished (Downing, 1987b). What market characteristics and additional robustness they may have as a culture species are yet to be determined. But in the case of the *Crassostrea* genus, different species and physiological races have a vast range of characteristics that could be combined to improve the marketability of the final product. One or more combinations of triploid hybrid may find their way into cultivation.

Tetraploidy

Tetraploid shellfish—organisms that possess *four* sets of chromosomes—have not yet been produced, but tetraploid fish are a reality. Tetraploidy is induced in a slightly different way from triploidy.

One way is to fertilize the egg and let development proceed normally until the first mitotic division (Figure 5). At this time the one-celled zygote duplicates its two sets of chromosomes mitotically into four sets that normally will divide into two daughter cells, each with two sets of chromosomes. A tetraploid would be produced by inhibiting this first cleavage division so that the zygote returns to the one-cell stage.

Another way to induce tetraploidy is to inactivate the paternal DNA while preserving sperm motility (for techniques, see section on gynogenesis in next chapter). Chromosome manipulation may be used to prevent both polar bodies from being extruded, thereby maintaining the entire tetraploid complement of maternal chromosomes. In theory, normal development would follow.

Tetraploids would not be useful as production animals, but they might be valuable as broodstock to produce triploids, the assumption being that tetraploid oysters would produce *diploid* gametes, i.e., eggs and sperm that naturally contain two sets of chromosomes (Chourrout, 1984). Crossing these gametes with haploid gametes produced from diploids should result in 100% triploidy.

This method of producing triploids, if it works, would be far more efficient than chemical treatments. Larval survival might improve, and selection for important traits could be made.

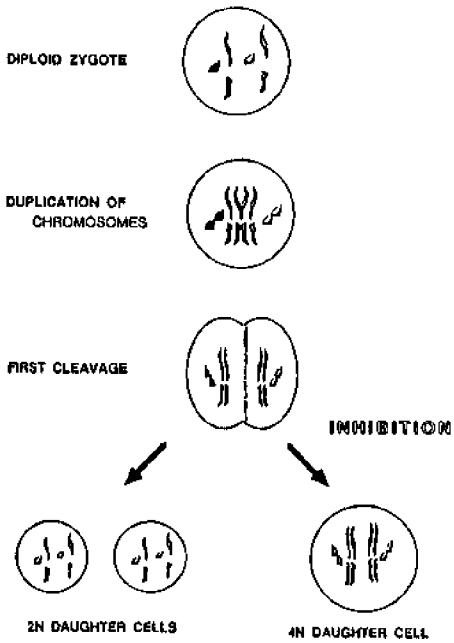


Fig. 5. Theoretical production of tetraploidy by inhibiting first cleavage division.

7. CHROMOSOME SET MANIPULATION IN RESEARCH

Physiological Energetics

Triploids represent a useful tool for estimating the increased metabolic costs associated with reproduction. Preliminary work undertaken at the University of Washington by Jonathan Davis (1986) indicates that triploid oysters have lower metabolic energy requirements than diploid siblings during the period corresponding to peak gametogenesis in diploids. By using triploids to measure somatic tissue costs and diploids to measure both somatic and reproductive costs, estimates of reproductive cost may be obtained. This approach is like no other now available and is of value for any species in which triploids can be produced.

Sex Determination and Hermaphroditism

In a study of diploid and triploid soft-shell clams during the reproductive season, triploids did not mature and most had undeveloped gonads (Allen et al., 1986). Of the triploids, 77% were female, 16% were femalelike, and 6% could not be sexed because of lack of development. Assuming that triploids are all female, the sex-determining mechanism that best fits these data is the model of an X:autosome balance mechanism. The triploid Pacific oysters did not differ in sex ratio from the diploids, but they were extraordinary because so many of them were hermaphrodites (Allen, 1987).

What aspects of sex determination can be deduced from the way that triploids mature sexually? A systematic survey of gametogenesis in triploid shellfish might be a valuable study.

Gynogenesis

Gynogenesis (gyno- "female," -genesis "creation") is the production of embryos from the maternal gamete only. Gynogenetic oysters must have two sets of chromosomes to become viable larvae, and chromosome manipulation can produce this condition.

It is done by inactivating the paternal gamete—by exposing the sperm to ultraviolet light, gamma rays, or mutagenic chemicals— so that its DNA does not take part in development. The sperm can still activate the egg and thereby begin meiosis. As in the case of producing triploids, treatments are used to inhibit the extra set of chromosomes usually given off with the polar body. If this inhibition is successful, the zygote then has two sets of maternal chromosomes—and one inactivated paternal set, which will subsequently be lost. Although it is diploid, the zygote has inherited only maternal chromosomes and, therefore, only maternal traits.

Unfortunately for the study of genetics, efforts to produce gynogenetic shellfish have yet to succeed. Exposure of lethal recessive genes is at least one plausible explanation for the mortalities. There may also be some incompatibility in the wholly maternally derived chromosomes, or perhaps some essential factor normally contributed by the sperm is missing.

Why make gynogenetic shellfish? The major reason is to create highly inbred individuals that, by cross-breeding, could be used to produce vigorous and valuable progeny. Gynogenetic oysters (shellfish) will probably not be commercially feasible for some time. One difficulty is that inbreeding occurs randomly with regard to commercially valuable traits. That is, it is as likely that a gynogenetic oyster will be inbred for a bad growth gene as for a good one. In contrast, agricultural species were inbred using classical selection procedures for valuable traits (so inbreeding was not random).

As a research tool, gynogenetic shellfish would be particularly valuable for studying and mapping the genes of highly inbred individuals and their offspring. With such information, questions regarding the genetic control of development, growth, sexual maturation, and disease resistance would become more tractable. Such studies are always valuable to the grower in the end.

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