CTSA Publication #164

Producing Polyploid Pacific Oysters (*Crassostrea gigas* Thunberg)

The story of a private-public partnership to improve Pacific Oyster seed quality and availability

By

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Acknowledgements

We wish to thank the teams that manage the He'eia and Keawanui fishponds for their support for this work. Dr. Ximing Guo served as the Technical Advisor and his advice was invaluable in making these efforts possible. The personnel of the Hawaiian Shellfish LLC hatchery (Brian Koval and Forrest Petersen) were instrumental in this work. Mr. Petersen conducted most of the R&D as a Masters students in Dr. Haws` lab and was supported by the grant from CTSA. Daniel Wilkie assisted in this work while he was an undergraduate student at UHH and was provided financial support for his work. David Nisbet, Owner and CEO of Goosepoint Oyster provided additional financial support to this effort as well as the triploids used for the research. Dr. Anu Frank-Lawale (Geneticist and Breeder) advised on the breeding strategy.

This work is supported by CTSA grant no. 2014-38500-22241from the USDA National Institute of Food and Agriculture.

Introduction

Pacific Oyster (*Crassostrea gigas*) production is a critical industry for small communities on the West Coast of the U.S. and most recently, Hawai`i. Shellfish farming is one of the few viable forms of economic development for many coastal areas where former industries such as fishing and logging, have declined. Oysters in the genus *Crassostrea* are the most commonly farmed oysters on a global basis, with production totaling 4,864 million metric tons in 2016 (FAO 2018). Oysters lead the way for marine shellfish production in the U.S. in terms of volume at 35.2 million pounds valued at $217.17 million in 2016 (NOAA 2017). Oysters are also increasingly are viewed as tools to improve water quality, protect shorelines and provide habitat for other aquatic organisms. This use of oysters for their role as ecosystems engineers is closely tied to the shellfish farming sector since the latter provides hatcheries and skilled personnel to support other uses of bivalves. Thus maintaining and expanding shellfish farming is critical to all coastal states.

The West Coast oyster industry faces numerous challenges such as ocean acidification impacts, rising labor costs, and permitting difficulties. A fundamental underpinning of shellfish farming is the ability to obtain high quality seed in the volume that producers need, and on a schedule that fits their farming practices. Quality in this case means oysters that have high growth rates, good survival and can adapt to the various environments in which oyster farming is conducted. Breeding for disease resistance is also of interest. The shellfish industry is also highly dependent on having triploid oysters available for summer harvest.

Many people know the old saying about not eating oysters in a month with an "R", but the reason for this is not due to food safety, but rather because diploid oysters tend to be of poor quality in these months. Triploid oysterss partition more energy into glycogen accumulation as opposed to producing gametes (eggs and sperm). They therefore grow faster and are also "fat" with glycogen in the warmer months when diploid oysters are either flaccid (i.e. "thin") or are full of gametes. Most U.S. consumers do not like oysters with gametes as they have a milky consistency (Figure 1). Thus, triploid oysters are very important to the oyster industry and constitute about 30% of the annual production, but most of the production during the summer.

Figure 1. Comparison of a "thin" diploid oysters (left) and a "fat" triploid (right). Note that the oysters on the right have so little glycogen that they are nearly transparent. The dark spot is the stomach which is visible. Oysters in this state often have tissue which does not completely fill the available space within the shell. The oyster on the right has a high level of glycogen accumulation that completely envelopes the internal organs. The meat nearly fills the shell. Source: Haws.

Triploid oysters have three sets of chromosomes as opposed to the more common double set of chromosomes that diploid oysters possess. Having more than two sets of chromosomes (polyploidy) occurs naturally in marine invertebrates, but is rare in nature. Many agricultural plants such as bananas and seedless watermelon are also polyploids. In the case of oysters, polyploid oysters must be made in the hatchery and a variety of methods can be used. Without triploid oysters, farmers will not be able to harvest during much of the year, thus threatening the economic viability of the farms, hatcheries, and processors.

The West Coast oyster industry had triploid oysters first as much of the initial research was conducted at the University of Washington, but it has not had the same availability nor quality of triploid oysters as the East Coast has enjoyed. This is due to the fact that the two co-inventors of the most reliable production method for triploid production established their research programs on the East Coast (Rutgers and the Virginia Institute of

Marine Science). The invention of tetraploid oysters (with 4 sets of chromosomes) can be traced back to Dr. Ximing Guo's dissertation work at University of Washington, where he hypothesized that viable tetraploids may be obtained using eggs from triploids (Guo, 1991). In 1993, Drs. Ximing Guo and Standish Allen tested the hypothesis at Rutgers and obtained viable tetraploid Pacific oysters for the first time (Guo and Allen, 1994). Rutgers received a patent for the tetraploid invention and licensed the tetraploid technology to 4Cs Breeding Technologies Inc. Tetraploid Pacific oysters were first commercialized on the West Coast and then in France, Australia and other countries. In or about 2001, Drs. Guo and Allen developed tetraploid eastern oysters (*C. virginica*) at Rutgers and VIMS, respectively. Triploid eastern oysters have become an important part of the East Coast oyster industry, partly because the tetraploid and triploid eastern oysters are produced from disease-resistant lines.

When tetraploid sperm is used to fertilize diploid eggs, nearly 100% triploid offspring result (Guo et al., 1996). This method is the only truly reliable and commercially feasible method to produce large numbers of triploid oysters. Chemicals can also be used to induce triploidy, but this is less reliable and the use of chemicals for food products entails other complications. The tetraploid oyster was the first animal patented in the U.S., although the process was fraught with difficulties for the inventors. Drs. Allen and Guo maintained vibrant research programs to improve tetraploid and diploid oyster strains, which greatly benefitted the aquaculture industry through the release of disease resistant and high performing diploid and triploid oysters.

The West Coast industry which uses Pacific Oysters (*C. gigas*), lagged in some ways in developing similar high performance strains. The Molluscan Broodstock Program (MBP) at Oregon State University, led by Dr. Chris Langdon, was developing improved oyster strains, but this work focused on diploid oysters. The patent on tetraploid oysters has limited research on polyploidy and the incorporation of polyploids into research and breeding programs. West Coast oyster farmers could obtain triploid seed from several West Coast hatcheries that licensed the use of tetraploids from 4C's, but other issues impeded the ability of farmers and other hatcheries to manage their farms and stock as freely as would have been desired.

Cost was another factor. In part due to the need to pay for a license to use the tetraploids, but also because maintaining tetraploid lines is a costly endeavor. The latter cannot be entirely avoided, but it is hoped that if interested parties thoroughly review the published literature and follows the advice in this manual, the process of producing the first tetraploids will be easier and less costly.

The Rutgers patent (#US5824841 A) on the use of tetraploid oysters expired in January 2015. This opened up possibilities for more flexible use of tetraploid oysters for research, hatchery production and farming. The problem continues that creating "*de novo*" tetraploid oysters is a not a trivial task.

With the expiration of the patent, a team of university and private sector partners came together to conduct research and development to advance the development and use of tetraploid Pacific Oysters, thus making production of triploid oysters more efficient, reliable, and available. The goal was to develop reliable methods of tetraploid production to ensure a readily available supply of higher quality broodstock and thus be able to produce a continuous supply of triploid oysters. This clearly benefits farms that would otherwise have trouble obtaining triploid seed, and moreover, provides some degree of control over the quality.

An additional benefit to developing the capability to produce tetraploids "*de novo*" rather than continuing to inbreed existing stocks is that researchers and hatchery managers can now improve tetraploid lines and integrate tetraploids into breeding programs. This will benefit all stakeholders in the West Coast Shellfish

industry which depend on the availability of triploid seed, but it also improves the resilience of the industry since breeding can help adapt oysters to climate change impacts and disease and optimize performance traits. The approaches described in this manual are based on the methods developed by Drs. Guo and Allen, but have been modified to work in the PACRC and Hawaiian Shellfish LLC hatcheries. Each hatchery may need to modify the methods for their own use. The final result of the research and development efforts supported by the Center for Tropical and Subtropical Aquaculture (CTSA) that there now exists a new stock of tetraploid oysters shared between stakeholders in Hawaii and Washington that will use them for research (i.e. an on-going epigenetics research project) and for production and training. Aquaculture students at the University of Hawaii Hilo were involved in all stages of this research.

Hawaiian Shellfish LLC and Paepae o He`eia partnered with the Pacific Aquaculture and Coastal Resources Center (PACRC) at the University of Hawaii Hilo (UHH) to execute this work. Hawaiian Shellfish also supplies over 20 West Coast and Hawai`i farms with seed, thus multiplying the benefits of the work. The team is fortunate in obtaining the technical assistance of Dr. Ximing Guo, co-inventor of the tetraploid production methods, and Dr. Anu Frank-Lawale, who formerly served as the lead Breeding Manager for the oyster improvement program at VIMS.

Pre-conditions to produce polyploid oysters

Producing polyploid oysters is not a trivial task and the chances of success are influenced by many factors including time, costs and technical expertise. We offer the following considerations to better inform anyone planning to develop a polyploid oyster program.

Time and costs

Any company or research unit which wishes to produce polyploid oysters and subsequently maintain these lines must understand that an initial commitment of at least two years is required. We estimate the R&D process cost nearly $500,000, although it is hoped this manual will allow others to replicate the process more easily. It took two and one-half years to reach the point at which tetraploids could be reliable produced, and this was only due to the tutelage and advice of Dr. Ximing Guo.

Broodstock and broodstock conditioning

One must have experience with broodstock conditioning as good conditioning is required to produce a sufficient number of oysters with viable gametes. One must have ready access to mature triploid female oysters. The triploids used in this work were large (>5 inches in length) and were 2-3 years old. Only about 15% of a given group of triploids will be females with viable eggs. This because most triploid females do not have large numbers of eggs, and at least 15% of each group will be hermaphrodites. The remainder will be males or oysters that do not have gametes. One must also have the ability to condition large number of triploids for prolonged periods since triploids require longer conditioning periods. If one cannot condition diploid oysters well, it is useless to attempt to condition triploids.

Scheduling the work

Broodstock conditioning must start at least 3-4 month in advance (see above). Both diploid and triploid oysters must be conditioned since one uses the triploids to provide eggs and the diploids to provide sperm. Multiple diploid oysters must be conditioned so that enough males are ripe in order to be able to fertilize with more than one male's sperm for the sake of genetic diversity. Triploids take longer to condition than diploids, so extra time must be allowed for this.

We found that the following was the most efficient way to accomplish the induction trials.

Day 1, prepare all materials and clean the conditioned oysters.
Day 2, start the work day as early as possible (at least by 7AM). First shuck the oysters and conduct a microscopic examination to determine the sex and the readiness of the gametes. Discard any males or hermaphrodites. Then tissue samples are taken from the female oysters to validate the stage of polyploidy using flow cytometry. We found that if we started with a pool of 20-30 triploid oysters, we would have enough ripe female triploids to work with for the day. It is best to not attempt to conduct many inductions on the same day when one is starting this work as it may take some practice to be able to conduct several inductions simultaneously. We aimed at conducting the inductions and getting the fertilized eggs in the tanks no later than 2 PM on Day 2 so that the larvae reach the D-stage early enough on Day 3 to complete the work before nightfall.

Day 3: by noon or mid-afternoon (depending on the rate of development), the larvae should reach the D-stage and a sample can be removed for testing with flow cytometry (FC). Any batch that does not have at least 70% tetraploidy at this point should be discarded to avoid wasting time and algae by rearing a cohort of larvae that aren't largely tetraploid. Do not be alarmed if one observes a large number of deformed larvae. This is normal and results from use of the chemicals. Most deformed larvae will die although some may end up being apparently normal.

Remainder of the larvae cycle and early spat stages: Larvae can be tested as often as one wishes although this involves extra costs and may affect the final number of larvae produced. At minimum, the larvae should be tested again shortly before they are ready to metamorphose. Generally the percentage of the larvae that are tetraploids will decrease over time. Spat must be tested at regular intervals as the first generation of tetraploids are unstable and tend to revert to a diploid or aneuploid state.

Laboratory and chemicals
This work requires the use of chemicals which can be hazardous to humans, and which could represent environmental risks if not properly disposed of. Once the chemicals are highly diluted, the risks to human health are significantly reduced, but we found that many employees simply refused to work with any chemicals. All personnel must have a good understanding of how to use potentially hazardous chemicals safely and personal safety equipment such as gloves, safety glasses and mask must be provided. Safety gear must be used when preparing the chemicals for use, and thereafter in the process up until the fertilized eggs are transferred to the culture tank.

Additionally, some seafood purchasers have certification requirements which may not allow use of the chemicals despite the fact that no residues will remain in the final product or the progeny. One should be ready to point out that the chemicals are only used on the first set of broodstock oysters to produce the tetraploids. Later generations of tetraploids are produced by crossing male and female tetraploids. Any triploids are produced by using a tetraploid male crossed with a diploid female without using chemicals, so there is no possibility of any chemicals entering the food chain.

The two chemicals commonly used for this are cytochalasin B (CB) and 6-(Dimethylamino) purine (6-DMAP). Both agents inhibit the release of polar body 1 after fertilization. Peachey and Allen (2016) found that 6-DMAP was more effective in producing tetraploids and has lower safety risks than CB. Regardless of which chemical is chosen, the user should familiarize themselves with the safe use of each by reading the Material Safety Data Sheet (MSDS) for each. Chemical suppliers will send the MSDS when a chemical is ordered but an example of a MSDS for CB can be found here: https://www.sigmaaldrich.com/catalog/product/sigma/

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Trained and skilled personnel
This work requires that the staff involved be highly skilled at oyster hatchery production. Also, at least one or two people are required that can handle work that requires precision, accuracy, good record keeping and a great deal of patience. Workers must also be well versed in the developmental processes of oysters, including the gametogenic and embryonic stages. Most skilled hatchery workers will have also gained a well-developed intuitive sense of egg and embryo development.

Flow cytometer
One must have easy access to a flow cytometer and the ability to use it. Many of the problems we initially encountered had to do with having limited access to a flow cytometer, the need to develop methods for sample preparation and the use of the flow cytometer, and the cost of using a flow cytometer that was intended for research. In the end, the private sector partner decided to purchase a FC that is specially designed for detection of polyploid Ploidy Analyzer (Partec) and this instrument is routinely used by many agricultural companies that depend on this process. This instrument costs around $26,000, but is more cost-effective since it runs samples very quickly, requires few reagents, and can be operated by nearly anyone.

Chemicals
The methods for use of 6-dimethylaminopurine (6-DMAP) are described here. Most of the employees at the two hatcheries involved in this research refused to work with cytochlasin B (CB) as it is toxic and carcinogenic in high concentrations. Since Dr. Guo advised that 6-DMAP is equally effective, we opted to use only this chemical for most of the research. Regardless of which chemical is to be used, the reader is advised to use appropriate laboratory and personal safety measures. Arrangements must be made for chemical disposal and any item potentially contaminated with the chemicals.

Dedicated space
Since successful replication of the induction process requires prolonged periods of observation and careful timing, the work is best done in a dedicated space that is quiet and isolated from anything that might cause interruptions. We found that it was nearly impossible to do this work in combination with regular hatchery work. The requirement for additional safety precautions also indicates that many hatchery areas may not be suitable.

Growout

Even once a pool of tetraploid spat has been produced, they must be tested at intervals since there is tendency to revert to the triploid or diploid state. Each cohort will require at least 8 months for the young males to be fertile. We found that some males could be used as early as 6 months post-set, but that sperm is in small volume and may be insufficient for commercial production of triploid seed. Conditioning and allowing the young oysters to grow larger will improve this, but if the goal is commercial production of triploids, the time required for the young oysters to reach the size and condition to serve as broodstock must be taken into consideration.

One must also use the first tetraploids to produce new tetraploid lines. This requires that the young putative tetraploid females become large enough to produce a significant number of eggs. Tetraploid crosses do not need to result in large numbers of progeny since even 200-300 resulting spat are sufficient to constitute a family. Care must be taken to avoid inbreeding over time.

Tetraploid production process

Tetraploids are created using eggs from ripe triploids and sperm from diploids to produce larvae that have a percentage of tetraploids in the population. Ripened triploids are first shucked, sexed under microscope, and then flowed with a Ploidy Analyzer (Partec). Only about 15% of oysters will turn out to be triploids and have a sufficient number of eggs to work with. Thus many more oysters need to be conditioned and tested, which multiplies the costs.

Materials List:

- 5 liter plastic beakers with handles
- 10 liter plastic beakers with handles
- Sieves made with 20 and 75 micron screens (using at least 8" PVC as the frame)
- 200L of 25˚ C filtered seawater. We used seawater from a deep well which had a pH of 7.8 and salinity of 28 ppt which is not standard for seawater. Methods may need to be adjusted according to the water quality of the seawater that is used.
- Graduated cylinders
- Cafeteria trays-style to hold groups of oysters and which are useful for shucking
- Oysters shucking knives
- Scalpels
- Scissors
- Tweezers
- Latex gloves
- Micro capillary tubes or tooth picks
- Microscope
- Counting slides
- Disposable pipettes
- Marker pens
- Notepad
- Oyster knives
- Spray bottles
- Paper towels
- 6-DMAP stock solution
- Make stock solution concentration: 2 mg/mL 6DMAP (Sigma D2629 – 1g)
- Add 1 g of room temperature 6DMAP to 50 ml of DI water along with 2ml of DMSO and mix well. Add additional 450ml of DI water. Mix well until completely dissolved. Although caution should be taken during all stages of this work, be advised that once the 6DMAP (or CB) is mixed with the DMSO, the latter can act as to transport other chemicals through human skin. Therefore care must be taken at all stages not to have the solution touch one's skin.
    - Eggs are treated with 6-DMAP at 50-70mg / liter (25-35ml of stock solution per liter). Store the stock solution at 4˚C and it will last about 1 year in the freezer. If it re-crystalizes, make sure it re-dissolves before use.

Tetraploid Induction Process

The methods developed here for tetraploid induction are similar to the methods described previously by researchers (Guo and Allen, 1994; Eudeline et al., 2000), but a great deal of practice, precision and repetition is required to be able to accomplish high rates of induction with some reliability . It is critical that users be very

familiar and practiced with standard oyster hatchery methods, particularly for spawning and early larval rearing. Great care must be taken to perform each step carefully to obtain optimal results. Keeping records of induction conditions and their outcomes is essential in order to replicate them. The usual "quick and dirty" hatchery spawning methods will not suffice in this case. We do not provide detail on the flow cytometry methods since each user will have access to different instrumentation. It is important however, that the flow cytometer (FC) be equipped to detects polyploidy. Partec makes a model that is specifically equipped for detection of polyploidy. Barring access to this, other FC's that are designed for measuring polyploidy will work, but the user should become conversant with their use prior to attempting induction. Failure to understand and be able to practice standard FC methods will lead to confusion and wasted time if novices make their first attempts in conjunction with the induction trials.

The user must obtain a source of large triploid oyster specimens. Expect to spend most of one day conducting the induction trials. Some diploids may be mixed with the triploids, hence, each putative triploid female must have a tissue sample removed and the ploidy level validated using FC. Any diploid oysters are discarded. Any males or hermaphrodites detected in the pool of triploids are also discarded. Care must be taken to avoid cross-contamination between oysters when making the microscopic assessment of sex.

Triploid females are strip spawned individually. Triploid eggs have been observed to exhibit more variability in their rate of development and diploid eggs (Eudeline et al., 2000). We found that this observation was true and when we attempted to combine the eggs of several females, the results were always poor. This is because the fertilized eggs will develop at widely varying rates, so timing of the addition of the 6-DMAP is nearly impossible.

Strip spawning is conducted using a clean scalpel blade. The eggs are delicately scraped from the gonad of the oyster taking care not to puncture the gut (the enzymes in the gut can decrease fertilization rates of the eggs and damage sperm). The eggs in triploids are often in little "pockets" that have a "popcorn" appearance (Figure 2) throughout the region normally containing gametes in diploid oyster. A clean spray bottle filled with 25˚ C salt water can be used to irrigate the scraped eggs from the oyster. The eggs are passed through a 75-um screen to remove large pieces of tissue and the eggs are then caught on a 20um screen. The eggs are then placed in a container and filled with 25˚ C salt water to a density no greater than 2 million eggs/Liter and allowed to hydrate for 40-60 minutes.

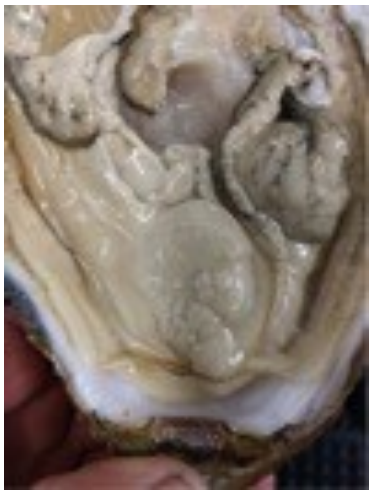

Figure 2. Triploid gonad exhibiting the concentration of gametes in discrete areas producing "popcorn" appearance.

Fertilize eggs from the triploids with haploid sperm from normal diploids at a density of at least 10-15 sperm around each egg. A timer is then started immediately upon initial fertilization. At 1-5 minutes post fertilization, treat the fertilized eggs with 50-70 mg/liter previously prepped stock 6-DMAP solution. The goal is to treat the eggs before the expulsion of the first polar body. Mix egg, sperm, and 6-DMAP solution well and let sit with gentile additional mixing every 2 minutes. Treat eggs for 15 to 20 minutes and stop the induction processes by rinsing the eggs gently on a 20-um screen. Immediately after rinsing the fertilized eggs onto the sieve, stock the eggs into an incubation tank at density no greater than 50 eggs/ml. Reserve an aliquot of the fertilized eggs to observe development. Do not add algae initially to the tank as the trochophores and young larvae will not feed.

When the larvae reach the D-stage, which will be on the day after the induction trials are conducted, a sample is removed and will be tested using FC to determine the percentage of tetraploids. Be sure to use the appropriate standard (i.e. larvae standard for larvae tests). Cohorts which do not exhibit at least 70% tetraploidy should be discarded to avoid wasting time in raising larvae which are destined to have increasingly lower levels of tetraploidy over the development period. Larvae should be tested at the D-stage and just prior to metamorphosis. Spat should be tested once they reach 1 mm and about every other month during the growout period. Again, cohorts with low levels of tetraploidy should be discarded. Any cohort with 70% tetraploidy should be kept. Putative adult tetraploids should be tested prior to using as broodstock.

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