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Transforming Lives in Coastal Communities Through Economic Development: Breeding Crassostrea virginica for Alternative Oyster Culture

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TRANSFORMING LIVES IN COASTAL COMMUNITIES THROUGH ECONOMIC DEVELOPMENT: BREEDING CRASSOSTREA VIRGINICA FOR ALTERNATIVE OYSTER CULTURE

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Abstract

This dissertation helped address the commercialization of triploid *Crassostrea virginica* production and develop tetraploid *C. virginica* broodstock in Louisiana by addressing triploid commercialization and tetraploid broodstock development. This was done by: 1) developing regional breeding plans for diploid and tetraploid *C. virginica* targeted at the Gulf Coastal Region, 2) assessing the effect of chromosome set manipulation on gonadal development in diploid, triploid, and tetraploid *C. virginica*, 3) quantifying the variation of commercially important hatchery traits in triploid *C. virginica* larvae from different tetraploid parents, 4) developing an up-to-date remote setting manual for the Gulf Coastal Region for industry use, and 5) performing a basic economic analysis to produce an enterprise budget for floating cage oyster farming in the Gulf Coastal Region.

I designed two breeding programs for oysters in the Gulf of Mexico Region: a diploid oyster breeding program and a tetraploid oyster breeding program. These breeding programs were designed to exploit genotype-by-environment interactions and combining abilities. To improve breeding, an analysis of gonadal development revealed 76% triploids exhibited abnormal and retarded gonadal development, whereas tetraploids followed diploid gonadal development with the only exception being a temporal delay. Investigating differences in larval traits attributable to the tetraploid parent suggested potential routes of selective breeding to add value to triploid offspring and make the hatchery production of triploid oyster larvae more efficient. These traits were hatching rate, survival, time to pediveliger development, and duration of pediveliger harvest. Improvements in these traits would increase hatchery production capacity and efficiency.

The final chapters provide two tools aimed at lowering entry or expansion barriers for oyster aquaculture activities: a remote setting manual and enterprise budget for off-bottom oyster farms. The aim of these chapters was to deliver tools for industry that are current, applicable, and useful. Both manual and budget worksheet will be available through Louisiana Sea Grant College Program.

Chapter 1
Introduction

Extensive oyster culture is the traditional method of farming oysters in the United States and has historically involved transplanting oyster spat, or seed, from public reefs to private areas (MacKenzie, 1997; Supan, 2002). Extensive oyster culture describes culturing oysters directly on the substratum (i.e., *on-bottom*). Intensive oyster culture describes the use of enclosures for rearing primarily hatchery-produced oysters (e.g., with tanks in a hatchery, raceways, cages, and bags).

Louisiana has a long history of extensive culture of the eastern oyster (*Crassostrea virginica*) with practices that have remained remarkably unchanged for over 200 years (Wirth and Minton, 2004). These manipulations of oyster populations began in the early 19th century and were adapted from traditional methods used by Yugoslavian fishermen that had immigrated to Louisiana. Once the adapted methods proved successful, they were then adopted by local fishermen (MacKenzie, 1997). It was because of these extensive culture practices that the regional oyster market potential would eventually be realized.

The extensive oyster culture practiced by early oyster cultivators consisted of removing oysters from abundant and dense oyster reefs found east of the Mississippi River and transplanting seed oysters to private waterbottom leases west of the Mississippi River. By transplanting seed oysters to more productive waters, fishermen were, and are still, able to improve growth and flavor of oysters destined for market. Oysters thrived west of the Mississippi River because of increased salinity and planting practices that spread oysters in a way that reduced density and reduced competition for food. East of the Mississippi River, where salinity was lower, oysters thrived from a lack of disease pressure and predators which are kept low by lower salinity conditions. In addition to improving growth and flavor, planting seed

oysters on private leases allows farmers to adjust harvest practices based on predicted market performance (Supan, 2002; Wirth and Minton, 2004).

Challenges to oyster culture, both extensive and intensive, include predation, disease, and permitting, among others. Predation comes from a variety of organisms including fishes (sheepshead [*Archosargus probatocephalus*], skates [*Raja* spp.], and drumfish [*Pogonias cromis*]), crabs (stone crab [*Menippe mercenary*], mud crab [*Panopeus herbstii*], rock crab [*Cancer irroratus*], and blue crab [*Callinectes sapidus*]), and other molluscs, specifically gastropods like *Urosalpinx cinerea*, *Eupleura caudata*, and *Strominata haemastoma* (White and Wilson, 1996). Predation threats can be eliminated by the adoption of intensive culture techniques that involve containerized culture methods of hatchery-produced oysters that can exclude predators. In the Gulf of Mexico, the major disease facing oysters is dermo disease, caused by the protozoan parasite *Perkinsus marinus*.

Despite biological and logistical competences for hatchery production developing as early as the first half of the 20th century there has been little interest in adopting these technologies in coastal Louisiana. Galtsoff (1964) characterized, in great detail, the life history and mode of living for *C. virginica*. Multiple patents are filed with the U.S. Patent Office detailing both larval and seed culture methods (e.g., Wells (1933), Glancy (1965)). There are likely explanations for the little interest in adopting intensive culture techniques. Fishermen consider independence and self-reliance integral qualities to their identity and that help to define their heritage of working the water. Deviation from the traditional practices of harvesting wild native oysters could be considered foreign, and thus, unnatural to their self-identity (Berrigan et al., 1991). In Louisiana, commercial harvests from public reefs have been supported by natural recruitment and management efforts by the Louisiana Department of Wildlife and Fisheries.

Despite high natural recruitment and reef management, recent reductions in natural oyster populations have been observed on public oyster grounds (LDWF, 2015).

New challenges have arisen that put in jeopardy the livelihoods of commercial oystermen in coastal Louisiana. Namely, a series of hurricanes (Hurricanes Katrina [2005], Rita [2005], Gustav [2008], Ike [2008], Isaac [2012]) over the past several years has devastated coastal communities and the natural resources their livelihoods depend on, including public and privately leased oyster beds. The Deepwater Horizon Oil Spill in 2010 has created angst and anxiety related to potential long-term, far-reaching damages on the fisheries that coastal communities depend on, including oysters. Finally, the re-engineering of freshwater inflows into the Mississippi River Deltaic Plain (e.g., Caernarvon Freshwater Diversion structure planned in the 2012 Coastal Master Plan in Louisiana) may render historic oyster production areas barren of oyster production (Wang et al., 2017).

1.1 Expanding and improving oyster culture in Louisiana

Oyster culture in Louisiana is practiced almost entirely with extensive culture methods, relying on natural and managed oyster populations for harvesting oysters that are subsequently grown to final harvest-size (≥76 mm) on unprotected substrate, resulting in a situation in which oyster farmers operate with little control over their investment (e.g., variation in recruitment and survival). Expanding and improving oyster culture in Louisiana requires introducing farmers to intensive culture techniques that can protect their investments as well as advancing oyster breeding efforts at the Louisiana Sea Grant Oyster Research Laboratory (located in Grand Isle, Louisiana 29º14’17.9”"N 90º00’11.1”W) that can add significant value to individual oysters.

1.1.1 Off-bottom oyster culture

Expanding oyster culture in LA in its current form is difficult. A moratorium on the issuance of new oyster leases in Louisiana was declared by the Louisiana Wildlife and Fisheries Commission in 2002. This moratorium reduces access to not only the fishery, but to off-bottom culturing as well because such an operation must operate on an existing oyster lease. This, however, does not exclude sub-leasing and purchasing of leased bottom from those already in custody of an oyster lease allowing some access into the fishery.

Off-bottom culture techniques have been well studied in the Gulf of Mexico (Leonhardt, 2013; Maxwell, 2007; Walton et al., 2013). While culture techniques can be continuously improved, it is important to also improve access to off-bottom culture technologies. One way to improve access to technology and oyster culture in general is through the development of an aquaculture park. Aquaculture parks are areas zoned specifically for aquaculture activities using the public water column and bottom and allowing multiple users to share infrastructure. Aquaculture parks allow new farmers to gain experience with intensive culture techniques, with reduced risk while new leases are not being issued. Maxwell (2007) provided the framework for permitting and policies for public aquaculture parks in the coastal zones of Gulf of Mexico states. In the interim, the Grand Isle Oyster Farming Zone, a twenty-five-acre aquaculture park established in the waters of Grand Isle, LA through Act 583 of the 2012 Louisiana Legislature and Act 160 of 2017, allows for immediate development of off-bottom oyster culture to test the commercial feasibility and potential of new oyster production methods in Louisiana, to be administered by the Grand Isle Port Commission in concert with the Louisiana Sea Grant College Program.

1.2 Selective breeding

Selective breeding describes the process by which the genetic composition of a population is manipulated with the goal of increasing the frequency of a desired trait or suite of traits. Selective breeding has increased production in farmed populations in a variety of aquatic species often leading to gains in growth and yield those of their wild counterparts by a factor of three or more (Bentsen and Gjerde, 1994; Knibb, 2000).

There are three main breeding strategies that have been used for the genetic improvement of livestock: 1) selection within a breeding line (hereafter referred to simply as a line): using the 'best' individuals within a line as parents for the next generation, 2) selection between lines: substituting one strain for another that has better performance in a given environment, and 3) crossbreeding: mating parents of disparate lines that produce hybrid vigor (Simm, 1998). The first two strategies employ controlled inbreeding to reduce heterozygosity and increase frequency of desired alleles, while the third seeks to exploit non-additive genetic variance. Nevertheless, to use any of the three described selection strategies, separate and distinct breeding lines are required.

Selective breeding has been used to genetically improve a variety of traits in a number of oyster species, including *C. virginica*, so applying these techniques to oysters is not novel but it has yet to be done programmatically for regional development in the Gulf of Mexico. Evidence for increased performance through selection has been documented highlighting the need for rigorous selection efforts (Casas et al., 2017). *C. virginica* has been successfully improved for both survival and growth in the Chesapeake Bay, USA (Frank-Lawale et al., 2014). In the northeastern United States, *C. virginica* has been improved for growth and survival under the pressures of juvenile oyster disease (Barber et al., 1998; Davis and Barber, 1999).

Most breeding experiments to date have focused selection pressure on adult oysters, however interest in larval selection is increasing. *Crassostrea gigas* larvae were found to be influenced by unintentional selection of broodstock that resulted in lower survival but greater success in metamorphosis (Taris et al., 2007). Much work has been conducted on adult *C. gigas* investigating the effects of selection and inbreeding including the potential for heterosis (Evans et al., 2004; Evans and Langdon, 2006a, 2006b; Hedgecock et al., 1996; Langdon et al., 2003). For the Chilean oyster (*Ostrea chilensis*), heritability estimates for increasing live whole weight ranged between 0.43 ± 0.18 and 0.69 ± 0.11, indicating great potential for a selection response (Toro et al., 1996).

1.2.1 Polyploidy

For oysters, triploidy (having three chromosome sets) was first developed in *C. virginica* (Stanley et al., 1981) and later in the Pacific oyster (*C. gigas*) for commercial use on the North American West Coast (Allen et al., 1989). Development and use of triploid oysters was driven by a reduction in marketability in the Pacific Northwest of gravid and spent animals during months of reproductive activity (Allen and Downing, 1991; Chew, 2000). Oysters undergo significant gonadal development during the spring, summer, and fall months. Triploids were developed to avoid either gravid or spent conditions and provide a summer oyster that could be marketed (Nell, 2001).

There are two principal methods currently being used for the production of triploid *C. virginica*. The first is by chemically inducing the triploid condition by treating fertilized eggs collected from diploid oysters with a chemical solution that impairs the completion of meiosis. The second makes use of another ploidy level, tetraploid (having four chromosome sets). Crossing a tetraploid oyster with a diploid oyster results in triploid offspring, also known as

natural triploids. Throughout this dissertation, the terms diploid(s), triploid(s), and tetraploid(s) refer to diploid, triploid, and tetraploid oysters (*C. virginica*), respectively, unless otherwise defined.

Chemical induction of polyploidy in oysters is achieved by treating embryos shortly after fertilization with either cytochalasin-B (CB) or 6-dimethylaminopurine (6-DMAP), though there are a variety of other treatments that provide similar results (Allen Jr and Bushek, 1992; Desrosiers et al., 1993; Guo et al., 1996; Piferrer et al., 2009). Embryos of bivalve mollusks are arrested at the metaphase of meiosis I, providing the opportunity to manipulate the release of polar bodies in either meiosis I or meiosis II; both of which will produce triploid progeny, although meiosis II treatments are more common (Figure 1.1) (Guo et al., 1992; Piferrer et al., 2009). Chemical induction of triploidy, by any means, is difficult because of the variable nature of the treatments. That is to say, there are a variety of considerations that must be made to ensure a successful treatment: temperature of egg development, dosage, time of initiation, duration of treatment, and possibly salinity variations (Desrosiers et al., 1993). These difficulties, compounded with toxicity of chemical inductions and the typical 40–80% triploid induction rate (Allen and Bushek, 1992; Allen and Downing, 1986), led to the development of a safer and more consistent method for inducing triploidy in oysters using tetraploids (Guo et al., 1996).

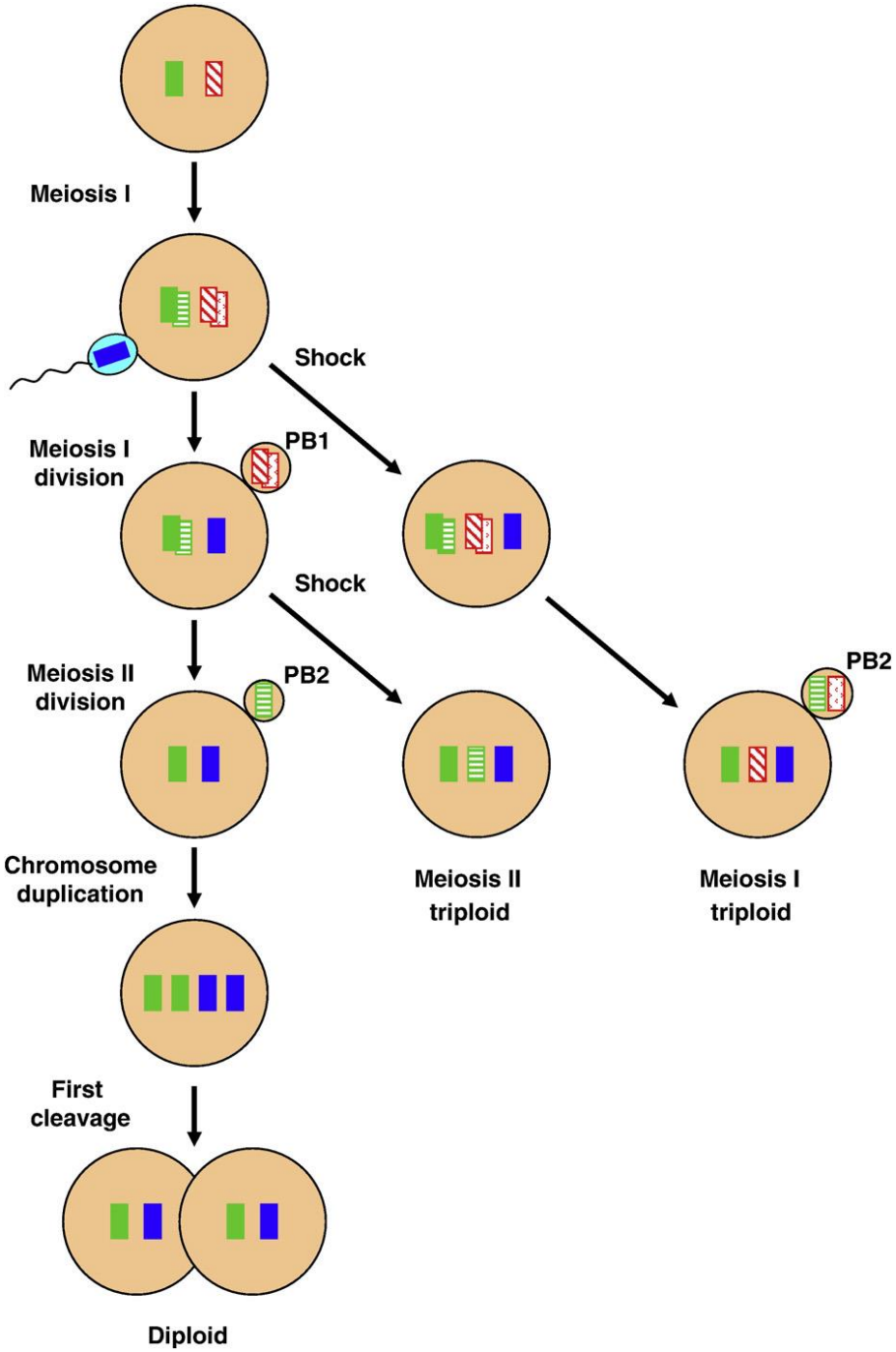


Figure 1.1. Ploidy manipulation in shellfish. Eggs are released at metaphase of meiosis I. Fertilisation resumes meiosis. Physical or chemical shock applied during meiosis I or meiosis II can suppress cell division, producing triploids by retention of the first (PB1) or second (PB2) polar body. For simplicity, in this hypothetical species 2n=2. Thus, each bar inside the cell represents one chromosome and overlapping bars indicate the sister chromatids after DNA replication during meiosis I. From Piferrer et al. (2009).

Tetraploids are obtained by treating eggs obtained from a triploid female and fertilized with sperm from a diploid male, with a similar chemical treatment as described above (Eudeline et al., 2000a, 2000b; Guo and Allen, 1994b). Triploid *C. gigas* larvae were the first triploid oysters to be obtained by mating tetraploids with diploids. While using tetraploid parents to produce triploid progeny was not a new concept, it was not previously possible with oysters because of a lack of tetraploids (Guo et al., 1996). The primary advantage of mating tetraploids and diploids is that the success rate (the percentage of larvae that are triploid) is generally 100%, compared to 40–80% for chemically induced triploids (Allen and Bushek, 1992; Allen and Downing, 1986; Guo et al., 1996). Another advantage is higher growth rates than those observed for chemically-induced triploids (Wang et al., 2005). The authors showed that while the magnitude of growth and survival rates varied by environment, they were consistently superior to both chemically-induced triploids and diploids (Wang et al., 2006).

The spread of commercial triploid oyster production to other areas was enabled by the development of tetraploids. Breeding programs in Europe and Australia began using the technique of mating tetraploids with diploids for triploid oyster production around 1999–2000 for *C. gigas*, *Saccostrea glomerata*, and *Ostrea edulis* (Nell, 2002). Tetraploid oysters made producing triploids convenient and efficient as tetraploid-diploid cross produced 100% triploid offspring. With the rapid expansion of tetraploid broodstock in these areas, triploids now compromise about half of hatchery-reared oysters worldwide. The majority of the research conducted on triploid *C. virginica* has taken place in the Chesapeake Bay, USA. Research with triploid *C. virginica* began with the proposed introduction of the non-native *Crassostrea ariakensis* (Allen et al., 2005). These studies compared triploid *C. ariakensis* and *C. virginica* for use in an aquaculture setting (Calvo et al., 2001; Grabowski et al., 2004; Kingsley-Smith et

al., 2009; Paynter et al., 2008). As a result of this research, oyster growers experienced firsthand the effects of triploidy that make the triploid oyster advantageous to grow. These effects, when positive, are characterized by faster growth and increased survival compared to diploid oysters grown in similar conditions to market-size. In Louisiana, where natural oyster production is high, triploids gain much of their potential from greater meat yields during summer spawning months when diploid yield decreases due to spawning efforts (Supan, 2000).

1.2.2 Spat-on-shell

One of the ways selectively bred and triploid oysters may be adopted with greater ease is by combining these hatchery-produced oysters with existing culture techniques of planting juveniles on-bottom. The investment cost of obtaining selectively bred and triploid oysters makes mitigating risks that accompany culture practices paramount. Off-bottom culture methods serve this purpose, but one method that may bridge the gap between extensive and intensive oyster culture is called 'spat-on-shell'. Spat-on-shell is the product of allowing pediveliger larvae to settle onto whole oyster shells; when done remotely by growers the process is termed 'remote setting'.

The general process of remote setting from spawn to spat can be divided into two phases: a hatchery phase and a setting phase. The setting phase is typically carried out by the grower in a location separate from the hatchery and is a simple process. Mesh bags or cages of clean setting substrate called 'cultch' (usually whole oyster shell) are placed into aerated tanks of ambient salt water for 24–48 hours, which allows bacteria to colonize the cultch. These bacteria provide larvae with chemical cues that attract larvae to the substrate for settlement (Weiner et al., 1989). After the soaking period of 24–48 hours, tank water is exchanged to provide fresh food to newly settled spat. Pediveliger larvae are then evenly distributed across the surface of the tank in an

attempt to avoid larvae setting in a single location in the tank. After 48 hours, unfiltered seawater is then pumped through the tanks to provide food so the new spat can grow while operators prepare transportation to a planting location. Water heaters may be used in colder climates to facilitate larval setting as well as tank covers to reduce light penetration through the water, as pediveliger oyster larvae are negatively phototactic (i.e., swim away from light sources during settlement). Several manuals and technical papers have been written on remote setting (Congrove et al., 2008; Jones and Jones, 1983; Supan, 1991) though the most recent was targeted for regional application in the Chesapeake Bay. Environmental conditions vary by region and, as a result, it is valuable for these instructive manuals to be targeted locally for easier adoption and application of the technology.

Chapter 2
A Regional Breeding Plan for Diploid and Tetraploid Eastern Oysters (*Crassostrea virginica*)

2.1 Introduction

The primary role of an oyster breeding program is to provide users a steady, reliable, and continuously improving product to culture. Currently, the Louisiana Sea Grant Oyster Research Laboratory (LSGORL) is fulfilling two of the three aspects of an oyster breeding program for the Gulf of Mexico region: steady and reliable source of improved oysters. Triploid oysters are LSGORL's principal product developed for industry use. Continued improvement of triploid oysters will come via similar selective breeding efforts that have proven successful for diploid oysters, namely through additive gains.

Additive gains have been documented in triploid oysters in two species when the diploid parents were selectively bred: the Sydney rock oyster (*S. glomerata*) and the Eastern oyster (*C. virginica*) (Callam et al., 2016; Hand et al., 2004). Additive genetic effects describe the portion of the phenotypic value of an individual for a given trait, from which dominance, environmental, and interaction deviations are subtracted. Additive genetic variance is what is typically targeted for exploitation in breeding programs. Hand et al. (2004) hypothesized that if observed gains in body weight were additive, triploids made from their selected lines would be 30% heavier than the selected diploids and 60% heavier than control diploids (based on historical data). The authors reported gains above the expected 30% and 60% gain over selected and control diploids and suggested that combining triploidy to selection may provide a previously unknown advantage that may account for the additional phenotypic gain. Callam et al. (2016) also observed gains in performance that could not be explained simply by additive genetic effects, though the source was not identified.

Triploid oysters are best produced by mating a tetraploid male with a diploid female (4n x 2n). Evidence has been presented that improving the diploid parent in this cross can improve triploid performance but, to date, no study has yet investigated how the tetraploid parent influences triploid oyster performance at any life stage. The primary reason for this is the lack of unique tetraploid populations with which proper breeding trials may be performed. If improving the diploid parent of a triploid oyster can improve triploid performance, then it follows that improvements in the tetraploid ought to translate in its triploid offspring as well.

2.2 Breeding goals

For commercial oysters in the Gulf of Mexico, important economic traits for production include growth, dermo resistance, and low salinity tolerance. Growth rate is generally recorded as time to market size (~73 mm). For commercial oyster aquaculture, survival to harvest is paramount.

In the Gulf of Mexico, survival may be linked to resistance to dermo disease and/or low tolerance to low salinity exposure. Concurrent progress in growth and survival under dermo disease pressure have been shown in *C. virginica*, but any genetic correlation has not been previously estimated (Frank-Lawale et al., 2014). Oysters selected for fast growth have been also been shown to be resistant to other disease (juvenile oyster disease) (Barber et al., 1998).

Low salinity stress for oysters is highly dependent on temperature (Heilmayer et al., 2008). In the Gulf of Mexico region, frequent freshet events occur in the spring due to spring floods and heavy rains and often coincide with increasing temperatures. The stress from low salinity may be exacerbated by the higher metabolic demand oysters experience with temperature increase. Pierce et al. (1992) showed that oysters from different populations (based on geography) exhibited different physiological responses to changes in salinity suggesting low

salinity tolerance may be, in part, genetically driven. Dégremont et al. (2012) and Callam et al. (2016) showed that line selection in the Chesapeake Bay may be tailored to develop environment specific lines of diploids and triploids.

2.2.1 Diploids

Genotype-by-environment interactions (GxE) occur when different genotypes do not respond in the same way to different environments. Differences in the environments (i.e., grow-out sites) may have a greater effect on some genotypes than others. This interaction can result in a change in the ranking of genotypes when measured under different environmental conditions (Falconer, and Mackay, 1996). Thus, selection decisions made under one set of conditions may correlate poorly with genetic gains when progeny are reared in disparate environments (Kvingedal et al., 2008). GxE has been observed in several oyster species: silver-lip pearly oyster (*Pinctada maxima*), Pacific oyster (*C. gigas*), and *C. virginica* (Dégremont et al., 2012, 2005; Evans and Langdon, 2006a; Frank-Lawale et al., 2014; Kvingedal et al., 2008; Leonhardt et al., 2017; Mallet and Haley, 1983; Swan et al., 2007). These studies have shown that both growth and survival traits are subject to GxE. Prior to undertaking highly targeted selection efforts, it is crucial that a breeding program incorporate the implications of GxE so that achieved gains are not undermined. That is to say, the best genotype for one environment may not be the best for another, thus breeding oysters in one environment for use in any environment that does not have the same conditions may not be a sound long-term breeding strategy.

LSGORL currently produces a single diploid breeding line: OBOY. This line was developed from wild oysters originating in Oyster Bayou, LA and subsequently selected for dermo resistance by Dr. Jerome La Peyre's program at Louisiana State University. Leonhardt (2013) compared diploid OBOY against three wild diploid populations across varying

environments in Louisiana and found evidence of GxE. Because of this evidence of GxE, a single diploid breeding line may not be appropriate for all potential grow-out sites and thus result in poor performance for growers in some areas.

2.2.2 Tetraploids

There are two main objectives of a tetraploid breeding program: 1) the creation and maintenance of a production tetraploid population, meaning a population whose primary function is creating commercial triploids and 2) improve tetraploids so that value is added to their triploid progeny. For the first, simply spawning tetraploids to increase broodstock population numbers to ensure a standing population size sufficient to meet commercial triploid demands will suffice. For the second, there are two principle methods of improvement that will be taken. To start improving tetraploids, tetraploids will be created from diploids that exhibit traits of interest (such as dermo resistance and low salinity tolerance). Another method of improvement involves the creation of a surplus of production tetraploids from which to focus for specific traits using mass selection. Currently (2018), efforts are being taken to identify desirable traits for tetraploid selection.

It is important to emphasize the establishment of tetraploid breeding lines is not as straightforward as it is for a diploid population in that there are no wild-type tetraploids to serve as a founder population. Making a tetraploid population is a multi-generational process: diploid to chemically-induced triploid to chemically-induced tetraploid to natural tetraploid. Care must be taken at each step to ensure the population size is great enough to avoid any deleterious effects of inbreeding while completing the chemical induction to the next ploidy state. Due to the toxicity of the chemical treatments, inducing triploids from diploids and tetraploids from triploids have highly variable success rates (40–80% triploid induction rate (Allen and Bushek,

1992; Allen and Downing, 1986) and 7–90% tetraploid induction rate (Guo and Allen, 1994b). Creating tetraploids from triploids is an especially difficult task due to the severely retarded reproductive efforts characteristic of triploid oysters. Fecundity of triploids and viability of gametes are estimated to be extremely variable, ranging from no eggs to several millions of eggs (Guo and Allen, 1994a; Que et al., 1997).

Triploids, that will in turn be used to create new tetraploid breeding lines, will be created alongside new diploid lines as they are developed. Once new tetraploid lines are created, testing can begin by evaluating field performance (growth, survival, dermo disease infection intensity) of tetraploid populations in different environments beginning as early as the induced tetraploid generation, evaluating GxE in tetraploids, examining important hatchery-based traits (e.g., gamete quality and fecundity), and evaluating triploids produced from new tetraploid lines in advance of full implementation in commercial production. At each phase of tetraploid creation (triploid induction and tetraploid induction), efforts will be made to ensure that the effective population size is as large as possible, though this will likely be difficult given the low success rate of each ploidy state induction. Significant inbreeding and reduction in effective population sizes over only few generations has been estimated in tetraploid oysters and reinforces the importance of proper breeding schemes (Miller et al., 2014).

2.3 Selection methods

Breeding programs across the world have shown that oysters can be improved by individual, line, and family selection for a variety of traits. In the proposed breeding plan, mass selection is proposed as a method of selecting within distinct populations because of research and testing capacity reasons. The breeding program should also be carried out to limit the accumulation of inbreeding across generations. This will be done by achieving large population

size in each successive generation and limiting the number of progeny between pair matings by keeping families separate during the larval stage and combining as seed to reconstitute the line.

The goal in creating these breeding lines is to reach an effective breeding number (N_e) of at least 50 for each generation, determined by the equation:

$$N_e = \frac{4N_m * N_f}{N_m + N_f} \quad \text{(Equation 2.1)}$$

where N_m is the number of males and N_f is the number of females (Table 1.1). This will aid in controlling the rate of inbreeding and potential deleterious effects of inbreeding that may manifest in the future.

2.4 Base populations

2.4.1 Diploids

Because of the evidence of GxE in oyster populations in Louisiana, the locally adapted populations used by Leonhardt (2013) (Sister Lake, Breton Sound, Calcasieu Lake) will serve as founder populations for new diploid breeding lines. Populations were chosen from these three locations because they are all major seed sources for the Louisiana oyster seed fishery. Sister Lake (29.234171°N 90.917221°W) experiences salinity ranging from 5–10 from the Spring through the Fall and 10 – 20 during the winter months. Breton Sound (29.5910°N 89.6425°W) experiences salinity below 15 for most of the year. The salinity in Calcasieu Lake (29.0003°N 90.2323°W) typically ranges between 15 and 25. Another diploid line, 'OBOY', housed at the LSGORL was developed for dermo disease resistance and will be included in the populations on which selection efforts will be applied. The OBOY line gets its name from 'Oyster Bayou' in Cameron Parish, LA but has been grown at the LSGORL at Grand Isle, LA for several generations.

Table 1.1. Effective population size (Equation 1.1) examples.

No. males	No. female	Effective popn. Size
25	25	50
35	20	50
45	17	50
55	16	50
65	16	50
100	14	50

2.4.2 Tetraploids

LSGORL, at the writing of this breeding plan, has two tetraploid populations: GNLs and VBOYs (Figure 2.1). The 4n-GNL line is derived from a 4n x 2n cross to make 3MVBOYs (the 'M' standing for mated). This cross consisted of a 'generic' tetraploid line from the Virginia Institute of Marine Science (4MGEN) and the diploid OBOY line from LSGORL. 3MVBOYs were then crossed with diploid OBOYs to make 4CVBOYs (the 'C' standing for chemically induced). These tetraploids would contain about half of their genetic material from the VIMS line and half from the OBOY line. The 4n-VBOY line has been propagated for several generations at LSGORL. The second tetraploid line is a hybrid, or 'general use' (abbreviated 'GNL'), line produced using 4MVBOY broodstock crosses with VIMS tetraploids (4MGEN). The current generation is an F_4.

2.5 Mating system

For both diploids and tetraploids, broodstock conditioning will occur naturally given the early spawning season in Louisiana and the frequent natural reconditioning that occurs over short periods of time (Supan and Wilson, 2001). Tetraploids must be verified as tetraploid prior to mating using flow cytometry (Allen, 1983). Oysters will be induced to spawn in isolated 3L tanks by heat shocks, stimulation via denatured sperm, or a combination of both. Randomized pair matings will be performed for each breeding line in an attempt to maximize effective breeding number (Equation 2.1). Larvae will be reared individually by family until settlement to keep the variation in progeny between pair matings as low as possible as the larval phase typically has the greatest mortality. Two weeks post-settlement, oysters are large enough to be combined proportionally to constitute a breeding line.

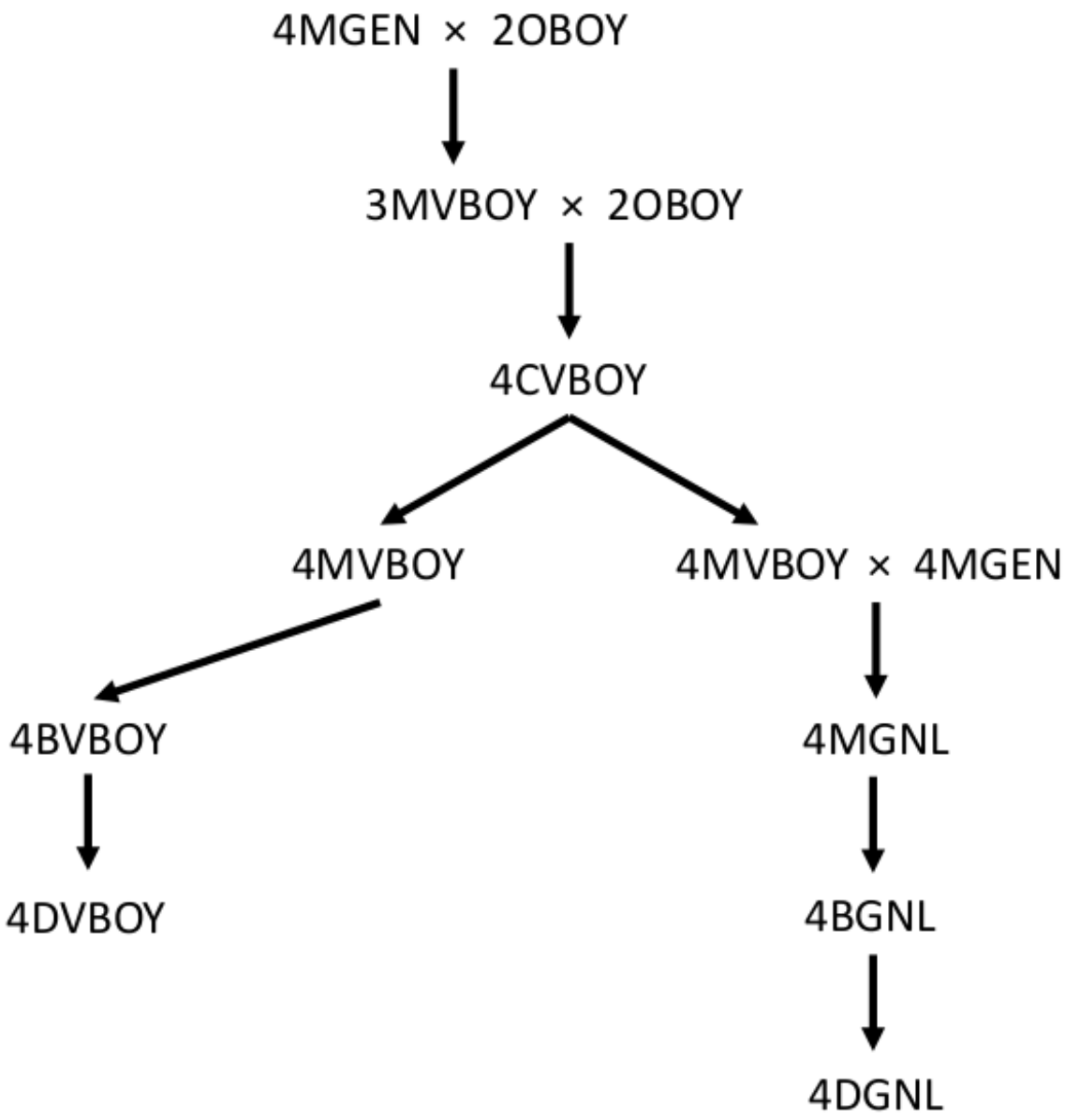


Figure 2.1. Lineage of tetraploid breeding populations. Naming convention is as follows: Number designating ploidy, generation label (C – chemically induced, M – mated, then following B, D, E, F, …, Z), line name. The diploid 'OBOY' line does not have a generational indicator.

2.6 Selection of broodstock

Diploid and tetraploid oyster breeding can be considered separate breeding programs that converge at mating to produce triploid oysters. Because of this, each of these breeding programs can operate independently, i.e. have separate selection criteria, as long as their convergence at triploid production is synergistic.

At the time of selection, based on current field testing capacity, there will be an estimated 2,250 oysters per breeding population. To ensure adequate effective breeding number (Equation 2.1) for the next generation, the 400 *best* (see 2.6.1 and 2.6.2 for what is *best*) oysters will be selected. 400 oysters are adequate to achieve desired male-female ratios and ensure that there are enough broodstock that meet selection criteria for dissemination of genetic material (see Section 2.7).

2.6.1 Diploids

Salinity is one of the main factors influencing *C. virginica* physiology. *C. virginica* have been shown to have a broad tolerance of salinity variations and changes which may be gradual or abrupt and episodic or isolated. The optimum salinity range is generally considered to be 14 to 28 (Galtsoff, 1964). Oysters have been shown to occur naturally and under artificial conditions in salinities ranging from 0 to 42.5 (Galtsoff, 1964; Ingle and Dawson, 1949; Loosanoff and Davis, 1963). However, salinity tolerance is not absolute and deleterious effects of low salinity exposure can be exacerbated by factors like duration of exposure and temperature: namely mortality, growth reduction, and reproductive effort disruptions (Butler, 1952; Chanley, 1957; Ingle and Dawson, 1949; La Peyre et al., 2013; Rybovich et al., 2016; Southworth et al., 2017). In the northern Gulf of Mexico, especially in areas near the Mississippi River, frequent

freshwater runoff is common and typically coincides with periods of high temperature (Butler, 1952; Gunter, 1950).

C. virginica is poikilosmotic, meaning it lacks the ability to osmotically regulate its extracellular fluids. The consequence of this is that cells must conduct volume regulation in the face of osmotic changes. For *C. virginica*, this is done in part with free amino acids (FAAs). Total FAAs increase proportionally with increases in salinity but, interestingly, individual FAAs do not always respond proportionally with salinity changes (Lynch and Wood, 1966). This trend has been observed in muscle tissue, mantle tissue, and isolated gill tissue (Lynch and Wood, 1966).

Genetic differences in tolerance to low salinity among oyster populations have been inferred from numerous studies. Newkirk et al. (1977) showed that there were genetic differences in salinity tolerance as well as some evidence for GxE. More recently, Callam et al. (2016) documented genetic differences in salinity tolerance and GxE as well, but also showed that these differences can manifest in triploid progeny when diploids are used in a 4n x 2n cross.

2.6.2 Tetraploids

General combining ability (GCA) describes the average performance of a line in a hybrid combinations (Sprague and Tatum, 1942). The practical use of tetraploids is in hybrid combinations with diploid breeding lines to produce triploid offspring. To date, there has not been any research into the relationship between tetraploid parent and triploid offspring performance. The current use of tetraploids to produce triploid offspring involves, generally, using tetraploids from a small number of populations in crosses using a large number of diploid populations. Typically, tetraploid sperm is delivered to hatcheries for use with in-house diploid broodstocks. Because these broodstocks vary by hatchery, it is not practical to test the

performance of triploid progeny from all possible tetraploid-diploid combinations. For this reason, general combining ability will be used as a metric to gauge the usefulness of extant tetraploid breeding populations.

Specific combining ability (SCA) describes cases in which offspring from certain combinations perform better or worse than expected, when considering the average performance of each parental line (Sprague and Tatum, 1942). Given the LSORL breeding program's development of diploid breeding lines, evaluating specific combinations of tetraploids with these diploids is needed to ensure triploid performance is desirable, but also to identify any potential combinations of tetraploids and diploids that perform above expectations.

GCA and SCA will be estimated using Griffing's diallel method 4 (Griffing, 1956). Method 4 describes a diallel cross where parents are not selfed (i.e., self-fertilization) and reciprocal crosses (i.e., parents used as both male and female) are not included. Reciprocal crosses are excluded because only male tetraploids are used in practice in the 4n x 2n cross to produce triploids. The model for combining ability is:

$$x_{ij} = \mu + g_i + g_j + s_{ij} + \frac{1}{bc}\sum_k \sum_l e_{ijkl} \begin{cases} i, j = 1, \ldots, p, \\ k = 1, \ldots, b, \\ l = 1, \ldots, c, \end{cases} \quad (2.2)$$

where μ is the population mean, g_i and g_j are the GCA effects of parents i and j, s_{ij} is the SCA, and e_{ijkl} is the error term.

2.7 Dissemination of improved broodstock to hatcheries

Delivering improved broodstock as quickly as possible to users is critical to maximizing the benefits realized from the breeding program. To this end, only improved oysters that have been properly vetted will be made available. The current model of dissemination of improved

broodstock at LSGORL is when available, for diploids, dissemination as larvae or seed oysters and, for tetraploids, as quantities of sperm.

Going forward, efforts will be taken to train hatchery operators in proper tetraploid broodstock handling procedures. This will allow hatchery operators to condition tetraploid broodstock for spawning under localized conditions. Localized conditioning offers two main benefits: 1) osmotic acclimation to conditions under which fertilization will occur and 2) reduce strain on the LSGORL breeding program from handling and maintaining ripe broodstock.

In addition to distribution of tetraploid broodstocks, cryopreservation of sperm collected from tetraploid broodstock under varying conditions will be performed in collaboration with the Louisiana State University's Aquatic Germplasm and Genetic Resources Center. Cryopreservation of sperm offers a safety net for catastrophic loss as well as convenience of viable sperm when oysters may not be conditioned for spawning.

2.8 Control to estimate genetic gain

To establish a control group against which genetic gains may be estimated, when breeders are selected, 100 breeders of average size will be randomly selected with the goal of obtaining 25 females and 25 males. This will occur for both diploids and tetraploids. Creating control groups in this way is of limited value. Inferences about performance relative to oysters obtained from outside the breeding program will be tested, providing information on relative performance within the breeding program from generation to generation, by using wildtype broodstock.

2.9 Potential problems and contingency plans

For a contingency plan, a significant number (25%) of the tetraploids produced from each line will be sent to the Auburn University Shellfish Laboratory, on the Dauphin Island Sea Lab

campus located on Dauphin Island, Alabama. This location will serve to safeguard the advances made in tetraploid production to avoid catastrophic loss. Sperm from every group of breeders selected to propagate breeding lines will also be cryopreserved to act as an archive of germplasm and as backup genetic material for any future efforts to recreate specific generations or reintroduce lost genetic variation.

Chapter 3
Analysis of Gonadal Development by Diploid, Triploid, and Tetraploid Eastern Oyster (*Crassostrea virginica*) Broodstock in Louisiana

3.1 Introduction

Production of large and frequent larval broods is of great importance for the successful operation of commercial oyster hatcheries. Understanding gonadal development of potential broodstocks is critical for such efforts because broodstock condition is related to consistent larval production (Supan and Wilson, 2001), larval survival (Lannan, 1980a), and egg quality and viability (Utting and Millican, 1997). For *C. virginica* (Gmelin, 1791), diploid-triploid and diploid-tetraploid reproductive assessments have been made (Guo and Allen, 1994a; Normand et al., 2008, 2009), but never before has the reproductive effort of all three ploidy conditions been assessed concurrently nor in subtropical northern Gulf of Mexico estuaries.

A multiple-ploidy reproductive assessment will provide information on the relative reproductive activity of animals essential to any oyster-breeding program that uses polyploid oysters. This includes the addition of new genetic material into polyploid breeding lines as triploid oysters are created from diploid oysters (Stanley et al., 1981) and tetraploid oysters in turn from triploid oysters (Eudeline et al., 2000b, 2000a; Guo and Allen, 1994b). Documented gametogenic activity will inform critical breeding efforts, specifically the temporal dynamics of reproductive stage and sex ratios.

Allen and Downing (1986) observed, in *C. gigas* (Thunberg, 1793), retarded gametogenesis in triploids relative to diploids and, interestingly, in sexually dimorphic patterns with females maturing less than males. These authors also report evidence of some spawning activity in triploids, but given the retarded stages of gametogenesis they hypothesized that the gametes released were likely not viable following reports of Lannan (1980a, 1980b) that the

larvae obtained from poorly conditioned diploid females had reduced viability compared to well-conditioned diploid females. Guo and Allen (1994a) reported triploid female *C. gigas* fecundity to be 2% of diploid female fecundity. Diploid and triploid *C. gigas* in France were observed to have similar sex ratios and percentage of sexually differentiated individuals within each population (Normand et al., 2008). Normand et al. (2009) examined reproductive effort of 5-month old diploid and triploid (both chemically induced and mated (4n × 2n cross) *C. gigas* and found the effect of triploidy was reduced reproductive output relative to a diploid control. This study provided critical information on the differences in gametogenesis between induced and natural triploids. Interestingly, there were no differences in total reproductive effort between the two triploid types, but, within natural triploids, females had lower reproductive output than males following observations by Allen and Downing (1986) in chemically induced triploids.

For natural triploids (those obtained from a 4n × 2n cross), recent studies have suggested that triploid oysters follow one of two gametogenic pathways. Jouaux et al. (2010) described, for *C. gigas*, an α pathway corresponding with the production of a significant number of gametes and a β pathway in which only a few gametes are produced and gonadal proliferation appears to be locked. α triploids appeared to undergo gametogenesis following the development pattern observed in diploids, though at reduced relative effort, but showed no signs of spawning. β triploids appeared to be locked in prophase of mitosis in germinal cells.

Tetraploids, unlike triploids, do not show evidence of retarded gametogenesis relative to diploid oysters. One-year old tetraploid *C. gigas* were reported to have similar sex ratios as diploid oysters grown under the same conditions (tetraploid – 46.7% female and 50.0% male; diploid – 43.1% female and 55.4% male) (Guo and Allen Jr, 1997). Fecundity of diploid and tetraploid females were also estimated in this study and found not to be significantly different.

Diploid females produced an average of 5.3 x 10^6 eggs (n = 11), ranging from 0.9 to 10.7 million. Tetraploid females produced an average of 3.5 x 10^6 eggs (n = 11), ranging from 1.3 x 10^6 to 7.7 x 10^6 eggs. Temporal dynamics of gametogenesis in oysters have not been studied. The literature detailing tetraploid reproductive activity focuses on viability of triploid offspring when tetraploids are used in the 4n × 2n cross.

The objective of this study was to determine the relative gonadal development efforts through time of diploid, triploid, and tetraploid oysters to better understand the effects of ploidy on reproductive efforts and for better practical broodstock management within a breeding program that exploits polyploid oysters (H_0: No differences in gonadal development by ploidy; H_A: Gonadal development was different between ploidy states).

3.2 Methods

3.2.1 Oysters and ploidy analysis

Diploid, triploid, and tetraploid oysters were spawned in June 2014 at the Louisiana Sea Grant Oyster Research Laboratory, Grand Isle, Louisiana LA (29˚14’17”N 90˚00’11”W). Diploids and tetraploids were spawned from the primary hatchery broodstocks and triploids from a spawn destined for commercial use. The resulting oyster seed were reared in a land-based nursery prior to being maintained in an adjustable long-line system adjacent to the Louisiana Sea Grant Oyster Research Laboratory.

Prior to spawning and each sampling point, ploidy was determined for each individual by flow cytometry (Allen, 1983).

3.2.2 Histology

Fifteen oysters per ploidy were haphazardly selected monthly from appropriate long-line mesh bags for histological examination and shell height (mm) measurement for one year. Five

millimeter cross-sections were removed from each individual sampled with a scalpel beginning just anterior of the adductor muscle ending just posterior to the palp-gill junction. Cross-sections were then preserved in Davidson's Fixative for 48 hours. After 48 hours, Davidsons's Fixative was removed and replaced with a 70% ethanol solution and stored until processing. For processing samples, tissues were embedded in paraffin wax, sectioned at 5 μm, and stained with hematoxylin then counterstained with eosin (Kim et al., 2006).

3.2.3 Reproductive assessment

Slides of each month's fifteen sampled oysters per ploidy were first examined to determine sex and then reproductive stage. Reproductive stages were assigned to one of five stages: early development (ED), later development (LD), spawning (S), advanced spawning regressing (ASR), and recycling (R) originally described by Kennedy and Krantz (1982) and modified by Supan and Wilson (2001). Briefly, early development for females is described by a single layer of germinal cells beginning to project into the lumina of the follicles and for males the beginning of stratification of the germinal epithelium. Later development, for both sexes, is accompanied by large and distended follicles, with the gonad occupying a majority of the space between the digestive gland and mantle. Females in later development have large numbers of oocytes that are free in the lumina, while males have large quantities of spermatozoa in the central follicular area. Spawning females are characterized with ova discharge from follicles that disrupt the regular oocyte arrangement. Spawning males are characterized with sperm that have been discharged, which disrupts the organization of the lamellar cellular arrangements. During ASR, in both sexes, the follicles contract. Oocytes may remain free in the lumen in females and typically no spermatozoa remain for males. Recycling includes the presence of early and later development stages as well as the presence of atresia. For triploid oysters, α and β gametogenic

patterns were assigned following Jouaux et al. (2010), where α corresponds to animals producing significant numbers of gametes and β corresponds to animals producing few gametes and exhibiting evidence of arrested or disrupted gametogenesis.

Sex ratios and differences in reproductive stages were analyzed from the monthly fifteen oysters per ploidy with chi-square contingency tables, using ploidy as row variable and sex as columns for sex ratios and reproductive stage as columns for reproductive stage analysis. Chi-square analyses were run in R using the base package 'stats' function 'chisq.test()' (R Core Team, 2017).

Gonad-to-body ratio (GBR) was estimated from the fifteen monthly samples per ploidy using image analysis following Quintana et al. (2011) measuring the total body area. Slides were scanned at a resolution of 800 dots per inch (dpi) and imported into the GNU Image Manipulation Program (GIMP) version 2.8.10. The images were magnified 12 times. To determine GBR, the body was first outlined using a digital drawing tablet. The gonad was then traced following the inner and outer margins. The image manipulation software automatically determined the areas selected by counting the number of pixels within each tracing. GBR was estimated with the following equation:

$$\frac{Total\ body\ area\ (pixels)}{Total\ gonad\ area\ (pixels)} \qquad \text{(Equation 3.1)}$$

The GBRs were analyzed using an analysis of variance model to identify differences in GBR at different reproductive stage by ploidy within each month using the R base package 'stats' function 'aov()' (R Core Team, 2017).

3.3. Results

The monthly mean shell height (± 95% CI) of oysters (distance between the umbo and the ventral valve margin) increased over time, as expected. At the start of sampling, mean

monthly shell lengths were 68.1 ± 2.1 mm for diploids, 87.5 ± 2.5 mm for triploids, and 74.4 ± 3.2 mm for tetraploids. At the culmination of the study, shell lengths had increased to 108.6 ± 2.7 mm for diploids, 136.4 ± 2.9 mm for triploids, and 107.6 ± 3.5 mm for tetraploids.

Water temperatures varied by month and followed expected seasonal trends of increasing from a minimum of 9.4 ºC in February to a maximum 33.4 ºC in July and then decreasing the fall (Figure 3.1). Salinity varied throughout the study period. In the spring of 2016, salinity dropped below 10 from April to July due to heavy rain and fresh water input from Mississippi River flooding events, with only three weeks during that period having salinity above 15. Beginning in late June, salinity began to rise, peaking at 32 in October before decreasing into the winter.

3.3.1 Sex ratio

Based on analysis by Pearson's chi-square test for independence, sex ratios were different among ploidy groups in all months except February 2015, September 2015, and January 2016 (P <0.001). The proportion of diploid females increased to mid-summer, then males dominated for most of the late-summer and fall (Figure 3.2). The proportion of triploid females increased to a peak in July and decreased through the rest of the year. All but one observed hermaphrodites were triploid. The majority (80%) of α triploids were male with 18% female and 2% hermaphrodites. Thirty-six percent of β triploids were male, 58% female, and 6% hermaphrodites. From April to July, tetraploid sex ratios were approximately 0.5. For tetraploids, females were the majority in August and declining back to near 0.5 in October. Tetraploid males were in greater abundance than females from February—March and November—January. The only sexually undifferentiated oysters were observed in the triploid group in November and December.

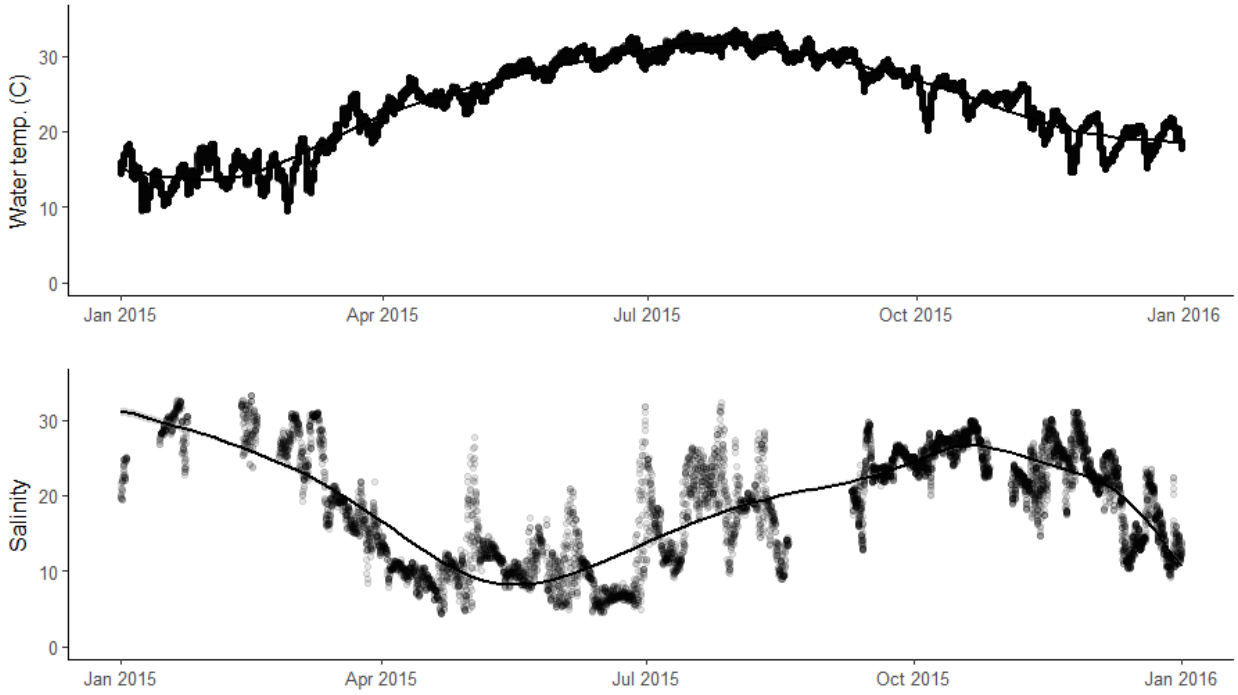


Figure 3.1. Daily average water temperature (°C) and salinity at the testing location across the testing period.

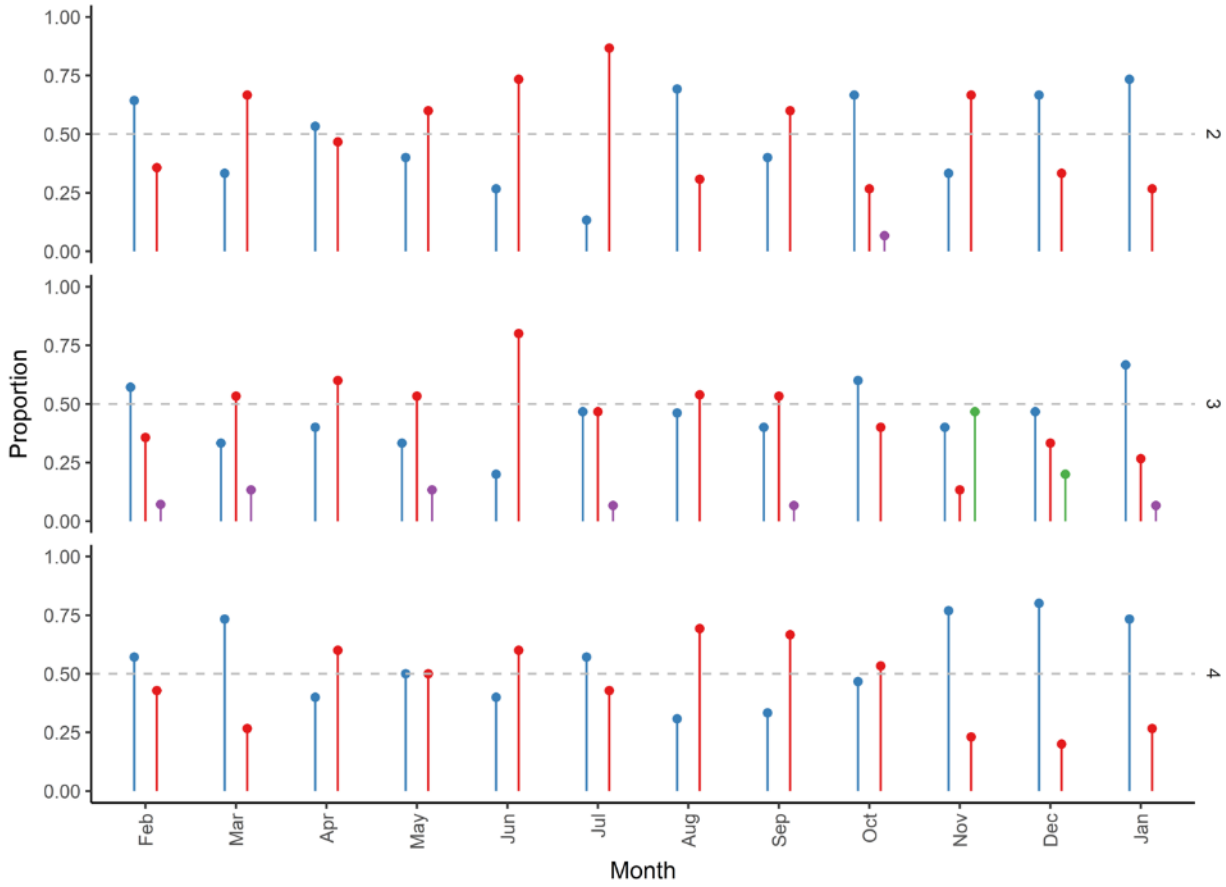


Figure 3.2. Monthly proportions of males (blue), females (red), hermaphrodites (purple), and undifferentiated (green) for diploid (2), triploid (3), and tetraploid (4) oysters. Gray dashed line indicates a proportion of 0.50.

3.3.2 Reproductive stage

Gonadal development was different for diploids, triploids, and tetraploids as seen in reproductive stages at each time point (all $P < 0.001$). Stage appeared to progress logically from early development through to spawning, then ASR and finally recycling was observed for all ploidy states (Figure 3.3). This general trend was observed in all three ploidy states.

Similar proportions of diploids were observed spawning monthly from April through August. Following this regular monthly spawning activity, a significant proportion of the sampled population spawned in September and October. This is in contrast to the tetraploids, where the proportion of oysters in the S stage began increasing in May, peaking in July and August, and then culminating with only 7% in the S stage in October. 73% of diploids were still in the S stage in October.

Triploids in the ED stage were observed at every sampling point, including summer months when diploid and tetraploids had developed beyond ED and no ED were observed for diploids or tetraploids. ED triploids were observed from May—September in proportions below 0.25. Few triploids were observed in LD, S, or ASR stages throughout the year, but from May—September a significant proportion of triploids were in the R stage. From gross examination, triploids had not appeared to have spawned. Of the sampled triploids, 75.2% showed clear signs of disturbed gonadal development and were categorized as β-triploids following (Jouaux et al., 2010). For early stages, β-triploids were mostly designated by the presence of a disruption in chromosome condensing within nuclei.

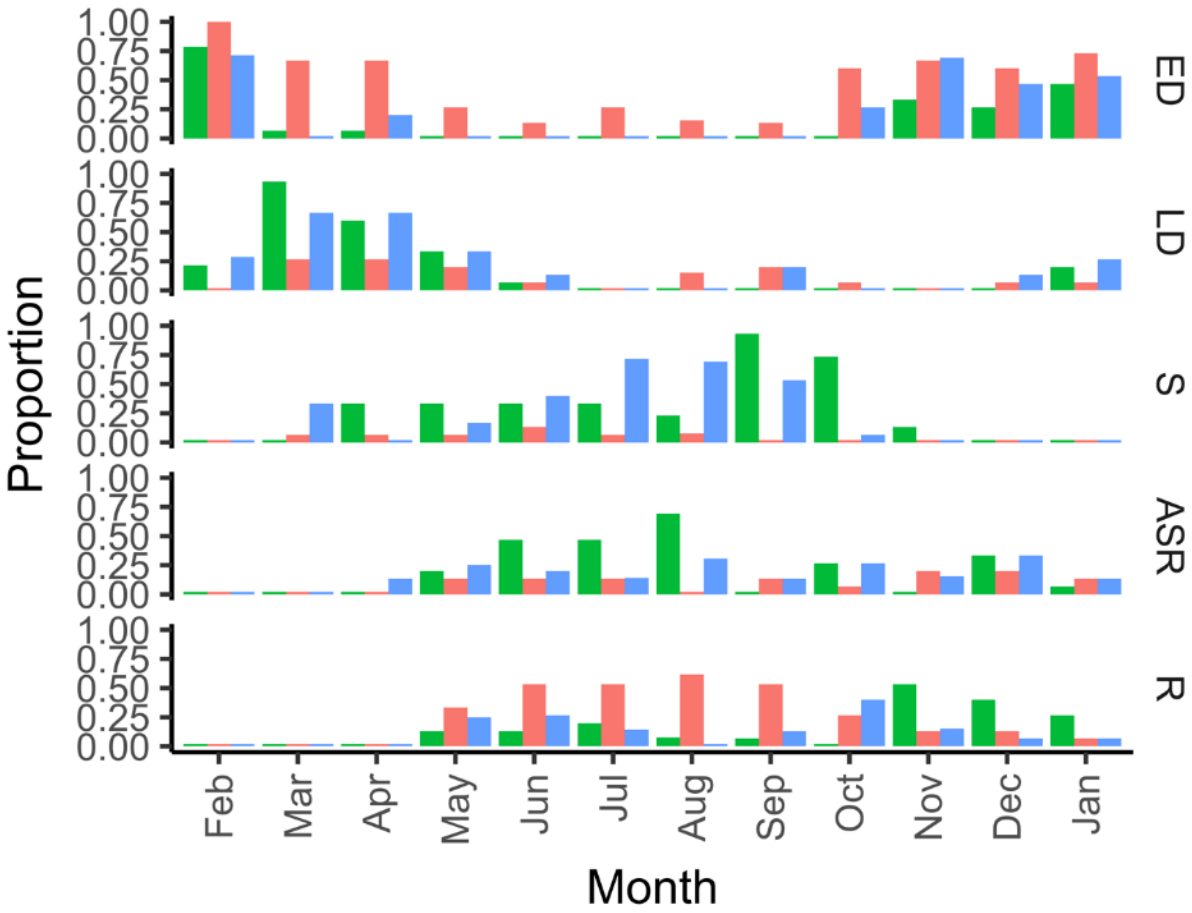


Figure 3.3. Monthly proportion of gonadal development stages for diploid (green), triploid (red), and tetraploid (blue) oysters. Early development (ED), later development (LD), spawning (S), advanced spawning and regression (ASR), and recycling (R) are shown.

For later stages, while mature oocytes and spermatocytes were observed, gonadal tubules were often reduced and irregular, gonadal tissue was dominated by oogonia and spermatozoa, and gonadal area was dominated by Leydig-cell connective tissue. The gonadal development of α-triploids resembled that of diploid oysters at similar reproductive stages.

3.3.3 Gonad-to-body ratio

Mean GBR increased with development stage for each ploidy level. Reproductive effort measured by GBR was greatest in the later development stage just prior to spawning for all oysters (Table 3.1). Triploids had the lowest GBR at each development stage except ED. Diploid reproductive effort was greater at every development stage than both triploids and tetraploids.

Gonad development increased through the spring, peaking in March-April for all oysters (Figure 3.4). Following this peak, a sudden drop in gonad area occurred, indicating a spawning event. After this initial spawning event, diploid and tetraploid oysters began to develop gonads further, while triploids continued to reduce total gonadal area throughout the remainder of the year. Diploid and tetraploids had a second, but reduced, gonadal area peak in August (diploids) and September (tetraploids), after which gonadal area decreased significantly. Compared to the first gonadal area peak, the second gonadal area peak was reduced by 24% for diploids and 39% for tetraploids. All oysters, regardless of ploidy, began to redevelop gonad in December-January.

Of triploids sampled, 24.8% were α-triploids with proliferating gonia. The α-triploids, when separated from their β-counterparts, follow the gonadal development observed for diploid and tetraploid oysters: a gonadal area peak in March followed by gonadal redevelopment and another peak in August (Figure 3.5).

Table 3.1. Mean gonad-to-body ratio (with 95% confidence interval) for diploids (2n), triploids (3n), and tetraploids (4n) by developmental stage. Comparison letters indicate significant differences by pairwise comparisons.

		Ratio		
Developmental Stage	**Ploidy**	**Mean**	**95%CI**	**Comparison**
Early Development	2n	0.17	0.02	A
	3n	0.16	0.03	A
	4n	0.09	0.01	B
Later Development	2n	0.42	0.03	C
	3n	0.25	0.03	D
	4n	0.31	0.04	D, E
Spawning	2n	0.32	0.03	E
	3n	0.25	0.03	D
	4n	0.26	0.03	D, E
Advanced Spawning & Regression	2n	0.25	0.03	D
	3n	0.11	0.01	B
	4n	0.15	0.02	A
Recycling	2n	0.15	0.03	A
	3n	0.11	0.01	B
	4n	0.13	0.02	A

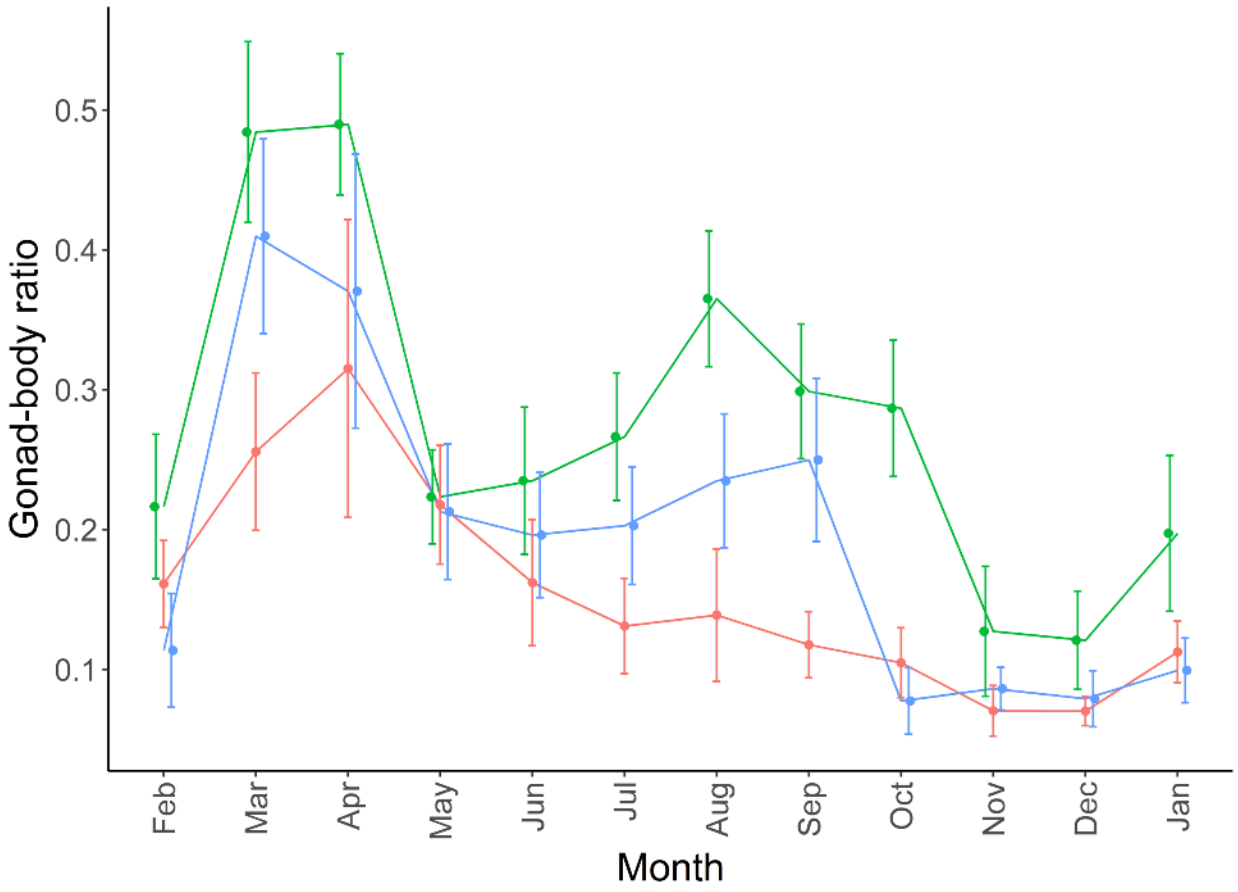


Figure 3.4. Monthly mean (± 95% CI) gonad-to-body ratio of diploid (green), triploid (red), and tetraploid (blue) oysters. Points and accompanying error bars are shifted for easier viewing.

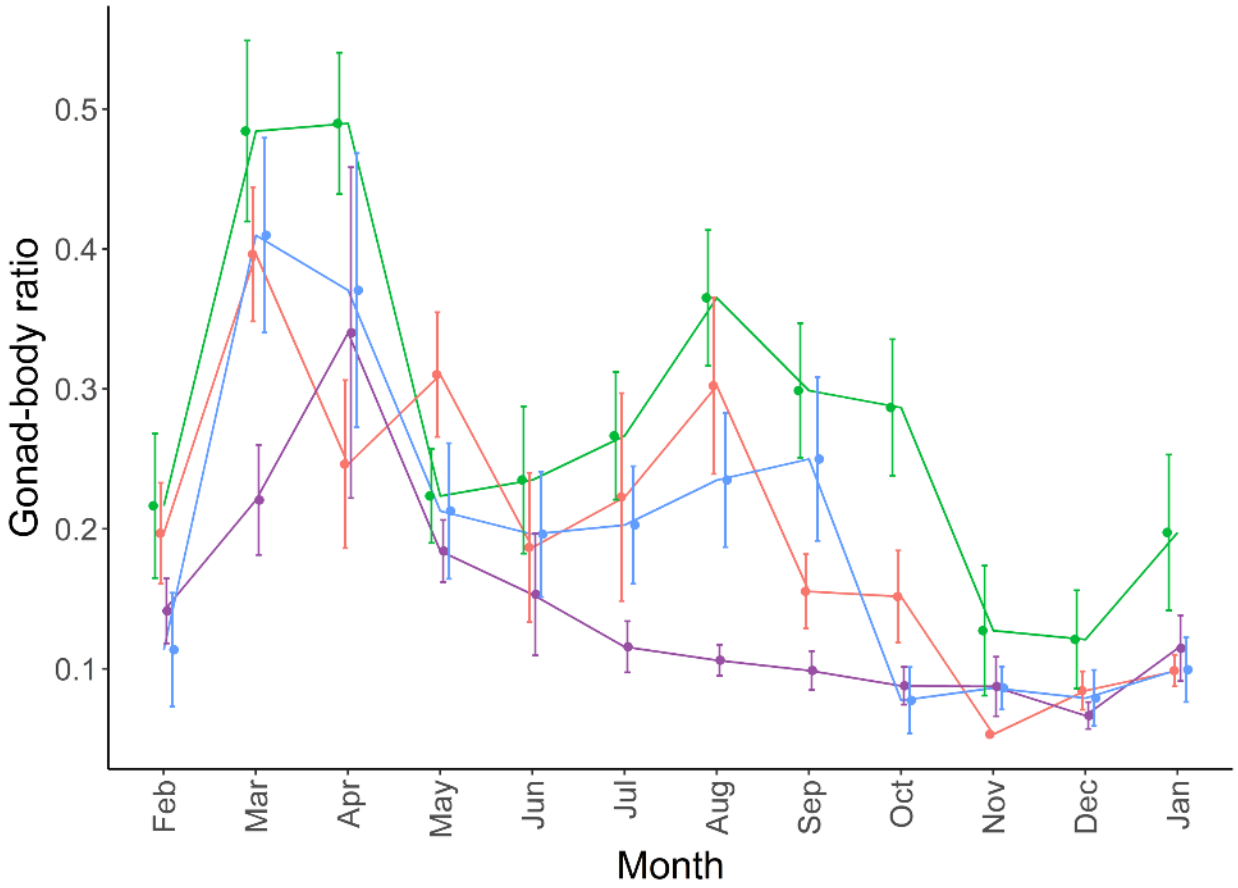


Figure 3.5. Monthly mean (± 95% CI) gonad-to-body ratio of diploid (green), α-triploid (orange), β-triploid (purple), and tetraploid (blue) oysters. Points and accompanying error bars are shifted for easier viewing.

3.4 Discussion

Sex ratio fluctuated across the sample period for all ploidy states. The ratio across the study was roughly equal males and females, but females dominated during the spawning months for all ploidy states. Sex ratio for *C. virginica* is known to vary with time, location, nutritive stress, and environmental stress (such as disease) (Bahr and Hillman, 1967; Cox and Mann, 1992; Galtsoff, 1964; Tranter, 1958). Triploids that showed significant gonadal development (i.e. α triploids) were overwhelmingly male (80%). It is unclear why observed α triploids skewed male (which occurred during peak spawning months) while the majority of β triploids, diploids, and tetraploids were female during the same time period.

Gonadal development in diploids appeared normal throughout the year (Supan and Wilson, 2001). The majority of triploid *C. virginica* sampled in this study (76%) exhibited abnormal and retarded gametogenesis (classified as β triploids). However, 24% of triploid oysters examined exhibited normal gametogenesis and were thus classified as α triploids, though none appeared to have spawned from gross examination. This is similar to the 25% of triploid *C. gigas* examined by Jouaux et al. (2010) classified as α triploids. Allen and Downing (1990) observed evidence of spawning in six of 11 chemically-induced triploid *C. gigas* sampled and 'most' of the females sampled during the same time period as *C. gigas* diploids. Since these animals spawned, they would be classified as α triploids as well, though there were observed in a greater proportion than seen in this study. Barber and Mann (1991) examined chemically-induced triploid *C. virginica* and found no evidence of spawning during typical spawning summer months. They did not rate developmental stage, but reported that triploids "produced only a few isolated oocytes or ova … and spermatocytes" suggesting no α triploids observed. Gonadal development in tetraploids proceeded similarly to diploids throughout the year.

Two periods of peak gonadal development were observed in the Spring and Fall following historic trends for the northern Gulf of Mexico (Gauthier and Soniat, 1989; Supan and Wilson, 2001). Diploids and tetraploids both had GBR peaks at those points. Tetraploids were in spawning condition roughly one month later than diploids, as evidenced by reproductive stage. It is possible that the observed delay could be due to an increased metabolic cost of producing gametes with extra genomic material. Another explanation could be due to the geographic origin of a portion of the founding population of the tetraploids studied. Tetraploids used in this study contain genetic material from mid-Atlantic oysters. Genetic variation has been shown to influence spawning time and duration for *C. virginica* (Barber et al., 1991). Further evidence of this source of variation can be seen when considering when the greatest proportion of oysters in the spawning stage were observed for α triploids and tetraploids relative to diploids (June-July vs. May-June, respectively). Mated triploids obtain two of their three chromosome sets from their tetraploid parent and if timing of gonadal development is in part genetically controlled, the triploids may follow a similar pattern as their tetraploid parent.

Reproductive effort as measured by GBR for β triploids was less than that of diploids and tetraploids at every development stage. α triploids had large variation in GBR, possibly due to the low number of animals observed, but generally had similar GBR to tetraploids (which was consistently lower than for diploids). Triploid *C. virginica* have been shown to have decreased gonadal development relative to diploids and this study confirms observations of decreased gonadal development (Barber and Mann, 1991).

An understanding of the differences in gonadal development of diploid, triploid, and tetraploid broodstock populations is of practical concern for hatchery operations. Diploids and tetraploids are required for triploid production, and triploids are required for introducing new

genetic material into and creating new tetraploid populations (Guo et al., 1996; Guo and Allen, 1994b). It appears that tetraploids and α triploids may require additional time for gonadal development relative to diploid broodstock under similar conditions. Artificial conditioning regimes or holding systems can be implemented for efficient handling of broodstocks to maximize broodstock availability and spawning success. An example of this is see through promoting gonadal development earlier than would be expected or through preventing oysters from spawning by reducing water temperatures.

Chapter 4
Variation in survival and growth rates among triploid eastern oyster (*Crassostrea virginica*) larvae obtained from different tetraploid lines

4.1 Introduction

Investigations into the influence of the diploid parent in a tetraploid x diploid cross have been few, but these have shown that improvements in the diploid parent can be transferred to their triploid offspring. Hand et al. (2004) compared diploid and chemically induced triploid Sydney rock oysters (*S. glomerata*) from a third-generation breeding line and an unselected control group. Triploids were chemically induced, so all three sets of chromosomes originated from the selectively bred population. The authors hypothesized that if observed gains in whole body weight were simply additive, the triploids made from selected lines ought to be 30% heavier than the selected diploids. Previous data on the whole weight gains of selected diploids over the control provided predictive information on possible gains. The authors predicted expected additive gains from triploids would be 60%, but in fact, whole body weight of the chemically induced triploid progeny was, on average, 74% greater than the control diploids – 14% greater than predicted gains.

Callam et al. (2016) examined the effect of the diploid female on triploids in several environments for *C. virginica*. They showed that, except under low salinity conditions, the diploid contribution to triploid growth performance was significant. Dégremont et al. (2014) showed that, in *C. gigas*, OsHV-1 resistance in the diploid parent contributed to increased resistance in triploid offspring. These studies all suggest that improvements in the diploid parent can be transferred to triploids. Following these revelations, it is then necessary to begin investigating the role of tetraploid parent in important traits for their triploid offspring.

A variety of studies have been conducted investigating aquaculture-relevant larval traits in oysters. Among these traits are growth and development rates to determine genetic control over how long it takes to complete the larval phase of the oyster life cycle. Losee (1978) estimated that heritability for larval growth rate ranged from 0.40 to 0.55. Larvae that metamorphosed early during the setting period for a larval brood were larger as spat at 29 weeks than larvae that metamorphosed late during the setting period (Losee, 1979). For *C. gigas*, larval development rate (i.e., time to metamorphose) was negatively correlated with metamorphose success (Ernande et al., 2003). Survival is another important trait that contributes to hatchery success. A key factor in survival is salinity, and larval survival in different salinities has been shown to be genetically controlled (Eierman and Hare, 2013).

The goal of this study was to determine differences in hatchery traits between the triploid offspring of the two current tetraploid broodstocks at the Louisiana Sea Grant Oyster Research Laboratory. Important hatchery traits of triploid oyster larvae were measured by comparing performance of triploids produced from two tetraploid broodstocks with two diploid broods as a reference. These traits were hatching rate, survival to pediveliger, and growth rate.

This study follows from an objective of a tetraploid breeding program: improving tetraploids so that value is added to their triploid progeny. In this instance, the added value being investigated is in hatchery traits related to commercial production. High fertilization, hatching, survival, and growth rates as well as short pediveliger harvesting period all improve commercial triploid production efficiency. The major objective of this study was to identify potential hatchery-based traits of triploids affected by their tetraploid parents for potential exploitation within a tetraploid breeding program (H_0: No differences in larval traits by ploidy; H_A: Larval traits were different between ploidy states).

4.2 Methods

4.2.1 Experimental oysters

Parental oysters were obtained from two tetraploid lines and two diploid lines: 4nVBOY, 4nGNL, SL, and CL. '4nVBOY', an F2 line of mated tetraploids (i.e., not chemically induced but from tetraploid x tetraploid cross), originated from the diploid 'OBOY' line. The 'OBOY' line was developed from wild oysters in Oyster Bayou, LA and subsequently selected for dermo resistance by Dr. Jerome La Peyre at Louisiana State University. The 4nGNL line is an F4 line that was developed by crossing the original 4nVBOY population with a tetraploid line developed by Dr. Stan Allen at the Virginia Institute of Marine Science (4MGEN) (see Figure 2.1 for tetraploid population lineages). SL consists of wild broodstock collected from Sister Lake, Louisiana where salinity conditions typically range from 4 to 12. LC are wild broodstock originating from Lake Calcasieu, Louisiana where salinity ranges from 12 to 24.

Oysters were naturally spawned using a thermal shock (temperature increased from 28 C to 34 C to induce spawning) after conditioning in the wild at the LSGORL Research and Demonstration Farm.

4.2.2 Mating design

For each broodstock group, parental oysters were randomly chosen from the total available broodstock. Three females from SL and CL were used as dams for crosses. Three males from each broodstock group were crossed with each female as represented in Figure 4.1.

		Female parents					
		CL			SL		
Male parents		1	2	3	4	5	6
4nGNL	1	X	X	X	X	X	X
	2	X	X	X	X	X	X
	3	X	X	X	X	X	X
4nVBOY	4	X	X	X	X	X	X
	5	X	X	X	X	X	X
	6	X	X	X	X	X	X

Figure 4.1. Mating design. Crosses are designated with 'X'. Each female was crossed with three unique males. Colors indicate crosses that were combined.

4.2.3 Larval rearing

Eggs from individual females were screened through a 65 µm screen and retained on a 15 µm screen to separate debris from eggs. Eggs collected in this way from individuals were counted and then divided into nine equal portions. Once eggs were divided into nine aliquots, each aliquot was then fertilized with sperm obtained from their assigned males. Embryos were pooled by individual female within each combining group (see Figure 4.1) after fertilization. Embryos from individual females within each combining group were split into two replicate culture tanks. Embryos were stocked into lightly aerated 180 l flat bottom tanks.

Larvae were fed a cultivated micro algae mixture *ad libitum*. Larval tanks were drained and cleaned every 48 hours. During cleanings, larvae were collected on nylon screens, transferred into beakers, and counted by sampling 100 µl three times from the beakers. Survival rate was calculated as the number of larvae alive on a given day divided by the number of embryos stocked into a given tank. Growth was monitored at this time by measuring shell lengths of 25 larvae per offspring group. Sample size was determined by an *a priori* power analysis using historical larval data from the Louisiana Sea Grant Oyster Research Laboratory by using the recommended statistical power of 0.8 (Cohen, 1988) for an effect size (d) of 0.45.

When eyed larvae were observed, 15 shell length measurements of eyed-larvae were made per offspring group to establish average eyed-larval shell length. Sample size for eyed larvae measurements derive from an *a priori* power analysis to achieve a statistical power of 0.8 with an effect size (d) of 0.5. Oyster larvae develop a circular pigmented eye-spot which marks the ending of the larvae stages of the oyster life cycle (Figure 4.2).

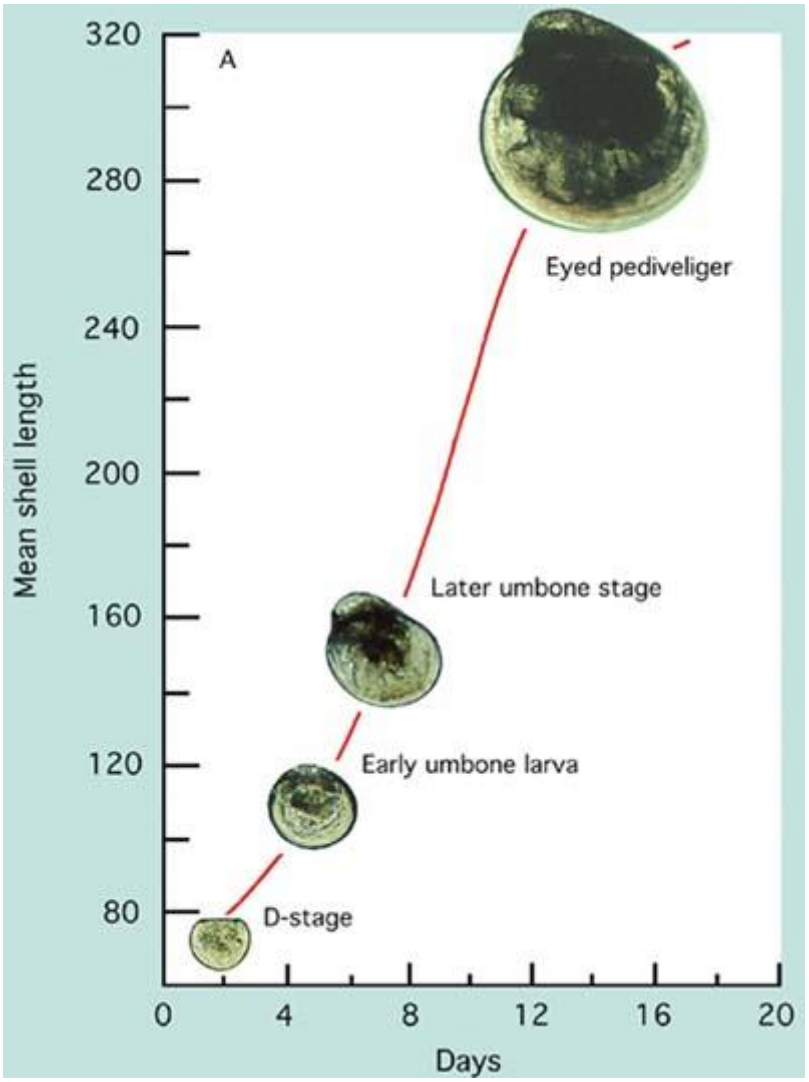


Figure 4.2. Oyster larval development stages from embryo through the terminal larval stage of pediveliger. From Helm et al. (2004).

Eyed larvae were harvested (i.e., collected from a larval brood) every 24 hours on a screen size determined by the smallest eyed larval size. Once harvested, eyed larvae were transferred to shallow mesh-bottom trays filled with oyster shell chips (microcultch) between 250–500 µm in size.

4.2.4 Ploidy analysis

Ploidy analysis was performed at various stages of this study using flow cytometry (Allen, 1983). Each parental oyster was individually verified as the expected ploidy prior to spawning. Each offspring group was analyzed at Day 2 post-fertilization and again post-settlement.

4.2.5 Statistical analysis

Hatching rates and larval survival were arcsine transformed prior to analysis. No transformation was needed for growth data. All traits were then analyzed by ANOVA in R using the base package 'stats' function 'aov()' (R Core Team, 2017). All error bars shown are ± 95% confidence intervals unless otherwise stated. Post-hoc comparisons were estimated using Tukey's HSD test. A Cox proportional hazard model was used to compare survival of larvae until the first harvest of pediveligers. The Cox proportional hazard model was fit using the 'survival' package in R (Therneau, 2015). Significance was determined using an α of 0.05.

4.3 Results

4.3.1 Hatching success rate

Hatching rate is a term common in bivalve hatcheries and is defined as survival at Day-2 post-fertilization (i.e. at the initial straight-hinge veliger larvae, or D-stage). There were significant sire, dam, and line effects on hatching rate ($P<0.001$). Hatching rate for triploids

from the 4nVBOY tetraploid was greater, regardless of the diploid parent, than for triploids from 4nGNL (mean hatching rate 0.48 and 0.28, respectively, Figure 4.2).

For triploids produced with 4nVBOY, there was no difference in hatching rate between diploid dams. For triploids made from 4nGNL, hatching rate differed between those produced from the diploid CL and SL (0.18 and 0.38, respectively). Considering triploid hatching rate on the basis on dams, CL-triploids had greater hatching rate than SL-triploids (0.53 and 0.31, respectively). By line, 4nVBOYxCL had the greatest hatching rate (0.49) followed by 4nVBOYxSL (0.47), then 4nGNLxSL (0.38), and 4nGNLxCL (0.18).

4.3.2 Survival to harvest

Survival was analyzed every 48-hours from embryo through the first harvest of pediveliger larvae from each larval brood (Figure 4.3). Only the tetraploid parent significantly affected the probability of survival of fertilized eggs to the first harvest of pediveligers ($P<0.025$). 4nVBOYxSL had the greatest probability of survival at first pediveliger harvest (0.066±0.027), followed by 4nGNLxSL (0.024±0.018), 4nGNLxCL (0.015±0.031), and 4nVBOYxCL (0.006±0.001). There were also differences in the time to the first harvest. The brood with the earliest pediveliger harvest was 4nVBOYxSL (Day 6 post-fertilization), then 4nVBOYxCL (Day 8 post-fertilization), then 4nGNLxSL (Day 10 post-fertilization), and 4nGNLxCL (Day 12 post-fertilization).

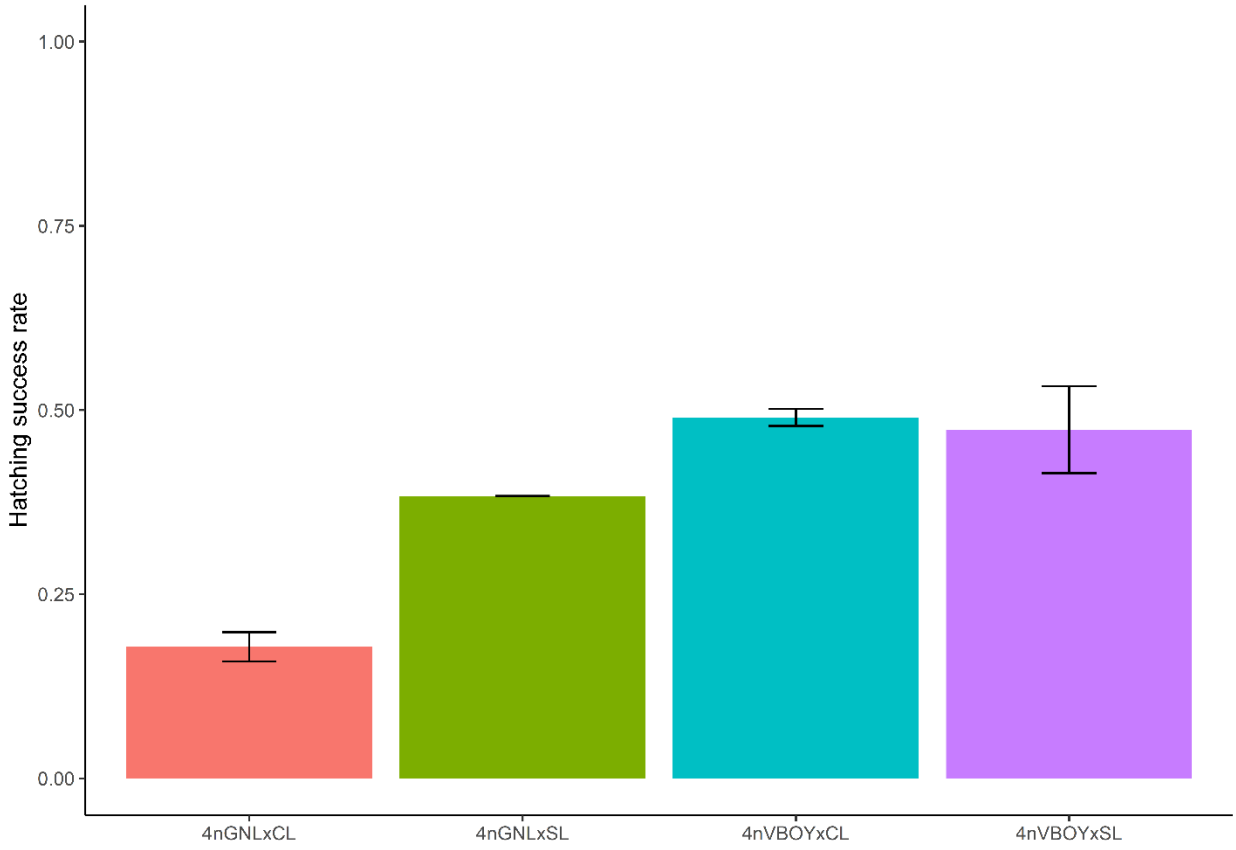


Figure 4.2. Average hatching success rate (±95% CI) of the four triploid broods.

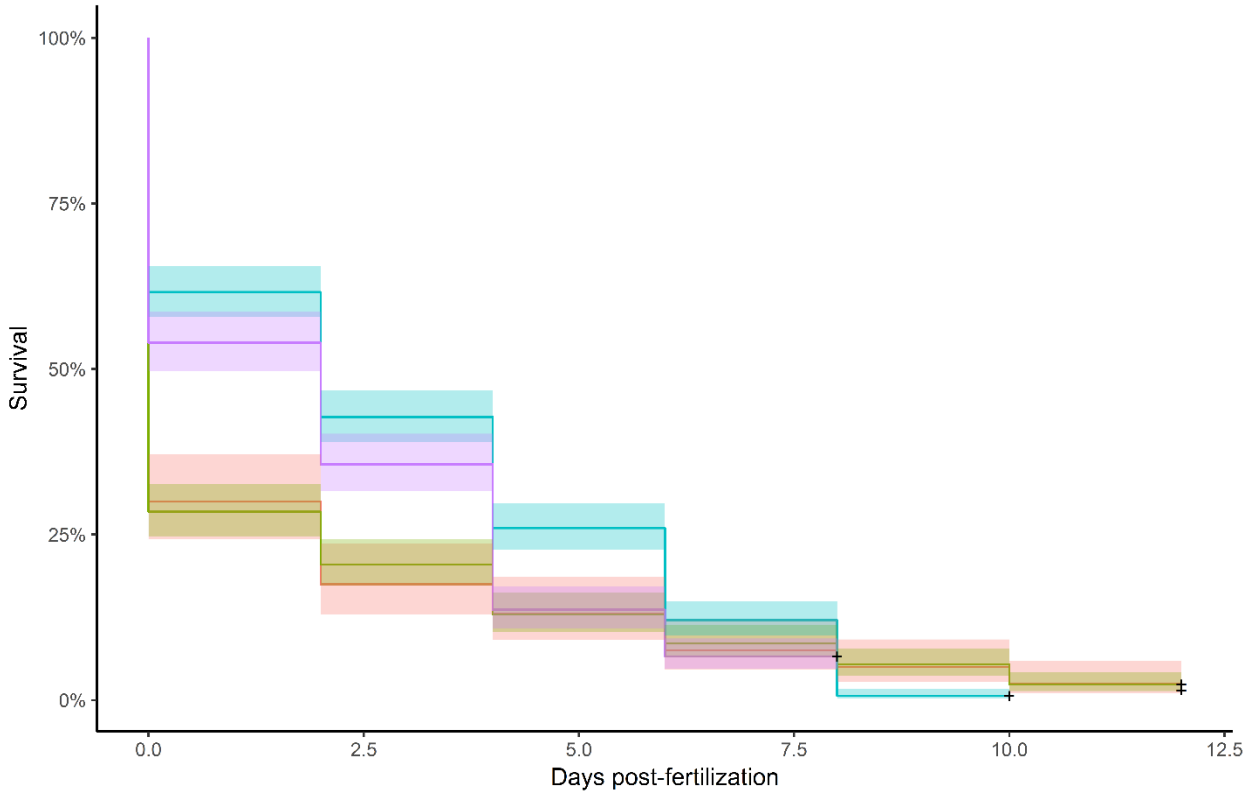


Figure 4.3. Survival proportions (±95% CI) of the four triploid broods as estimated by a Cox proportional hazard model. Each brood is represented by the following colors: 4nGNLxCL, 4nGNLxSL, 4nVBOYxCL, and 4nVBOYxSL.

4.3.3 Shell length

Shell lengths increased throughout larval rearing as expected until the first harvest of pediveligers (Figure 4.4). There were no significant differences in shell lengths of larvae prior to the first harvest of pediveligers when considering either the tetraploid parent (P=0.54), the diploid parent (P=0.29), or specific tetraploid-diploid combinations (P=0.14). Shell lengths of non-pediveliger larvae at the first harvest were 143.0±40µm for 4nVBOYxCL, 173.5±62.7µm for 4nVBOYxSL, 259.6±74µm for 4nGNLxCL, and 228.0±163.4µm for 4nGNLYxSL.

The choice of tetraploid parent had no significant effect on shell lengths of pediveliger larvae (Figure 4.5). Diploid parent and specific combinations of tetraploids and diploids did not significantly affect shell length of pediveliger larvae. Shell lengths, generally, increased to approximately 320µm where the shell lengths then remained until the end of the larval-rearing period. Pediveliger shell lengths were within the range typically observed for triploid oyster larvae in this hatchery (unpublished data).

4.3.4 Harvest proportion

Harvest proportion is defined as the proportion of the total pediveliger larvae harvested from a brood at on a given day (Figure 4.6). The period of time over which pediveliger larvae were being harvested (i.e., harvest period) was shorter for triploids with the 4nGNL parent than the 4nVBOY parent (3 days and 5 and 10 days, respectively). All broods had an initial peak of the proportion of harvested pediveligers followed by a smaller peak and then a sharp decline in harvest proportion until all pediveligers were either removed manually or were dead. Triploids from the 4nVBOY tetraploid had an initial peak on the third harvest day, while those from the 4nGNL tetraploid peaked on the second harvest day.

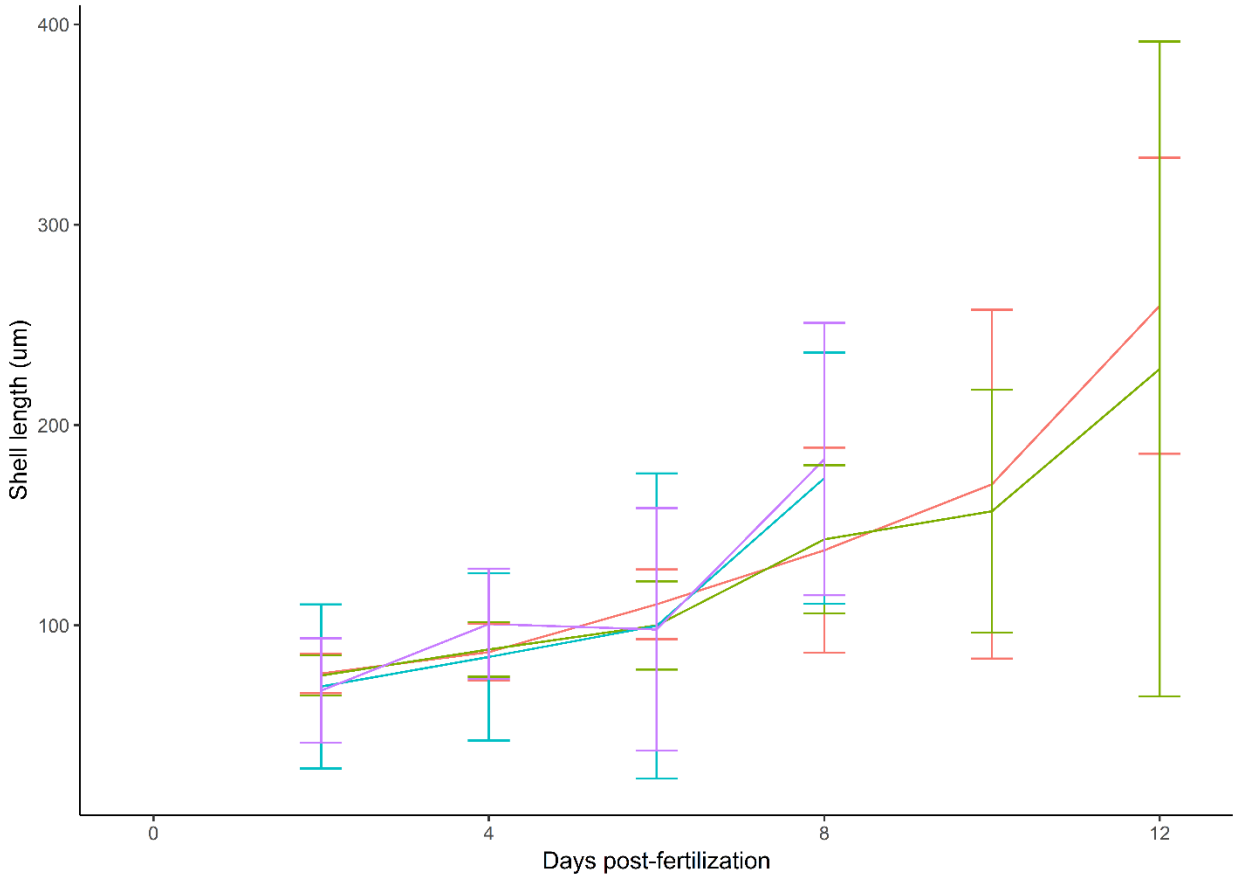


Figure 4.4. Shell length (±95% CI) of the four triploid broods by days post-fertilization until the first harvest of pediveliger larvae. Each brood is represented by the following colors: 4nGNLxCL, 4nGNLxSL, 4nVBOYxCL, and 4nVBOYxSL.

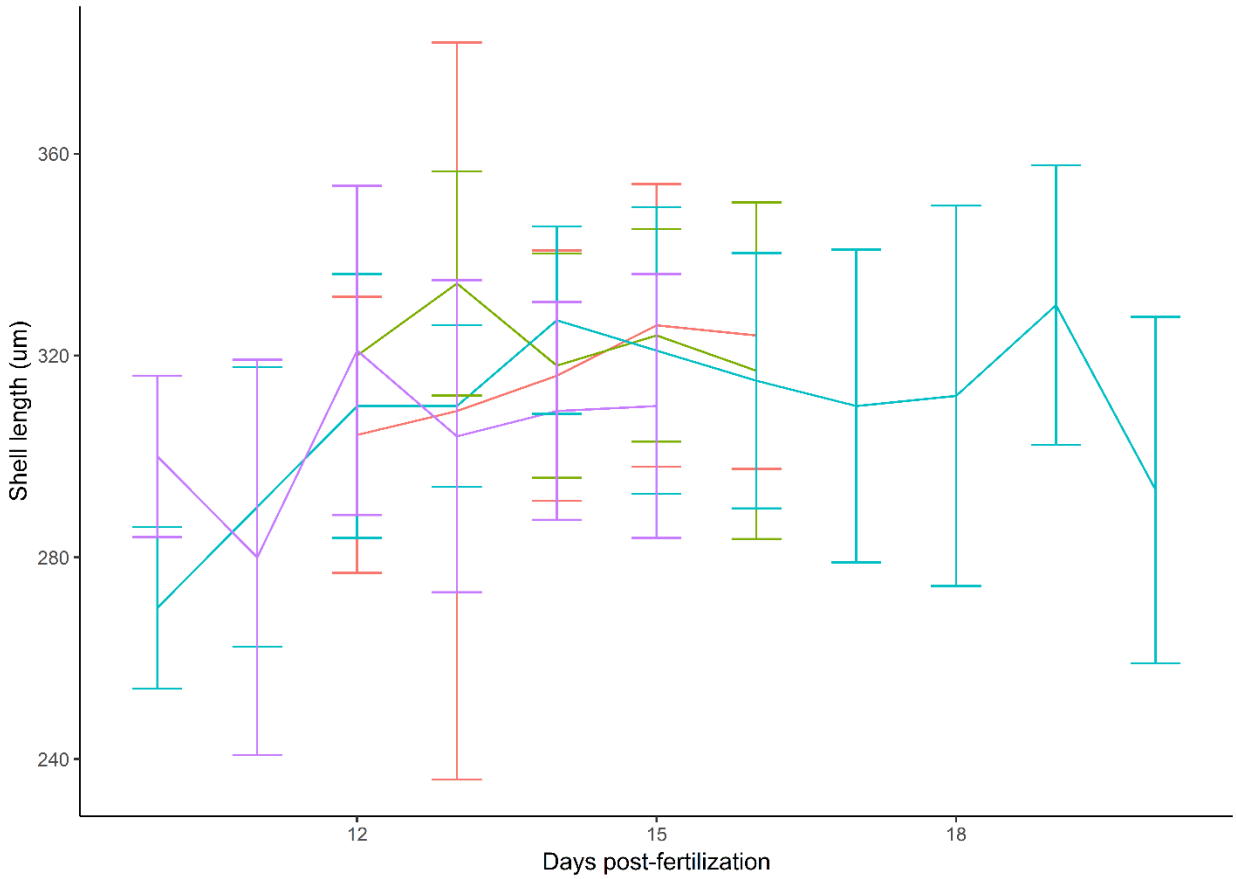


Figure 4.5. Pediveliger shell length (±95% CI) of the four triploid broods by days post-fertilization until the first harvest of pediveliger larvae. Each brood is represented by the following colors: 4nGNLxCL, 4nGNLxSL, 4nVBOYxCL, and 4nVBOYxSL.

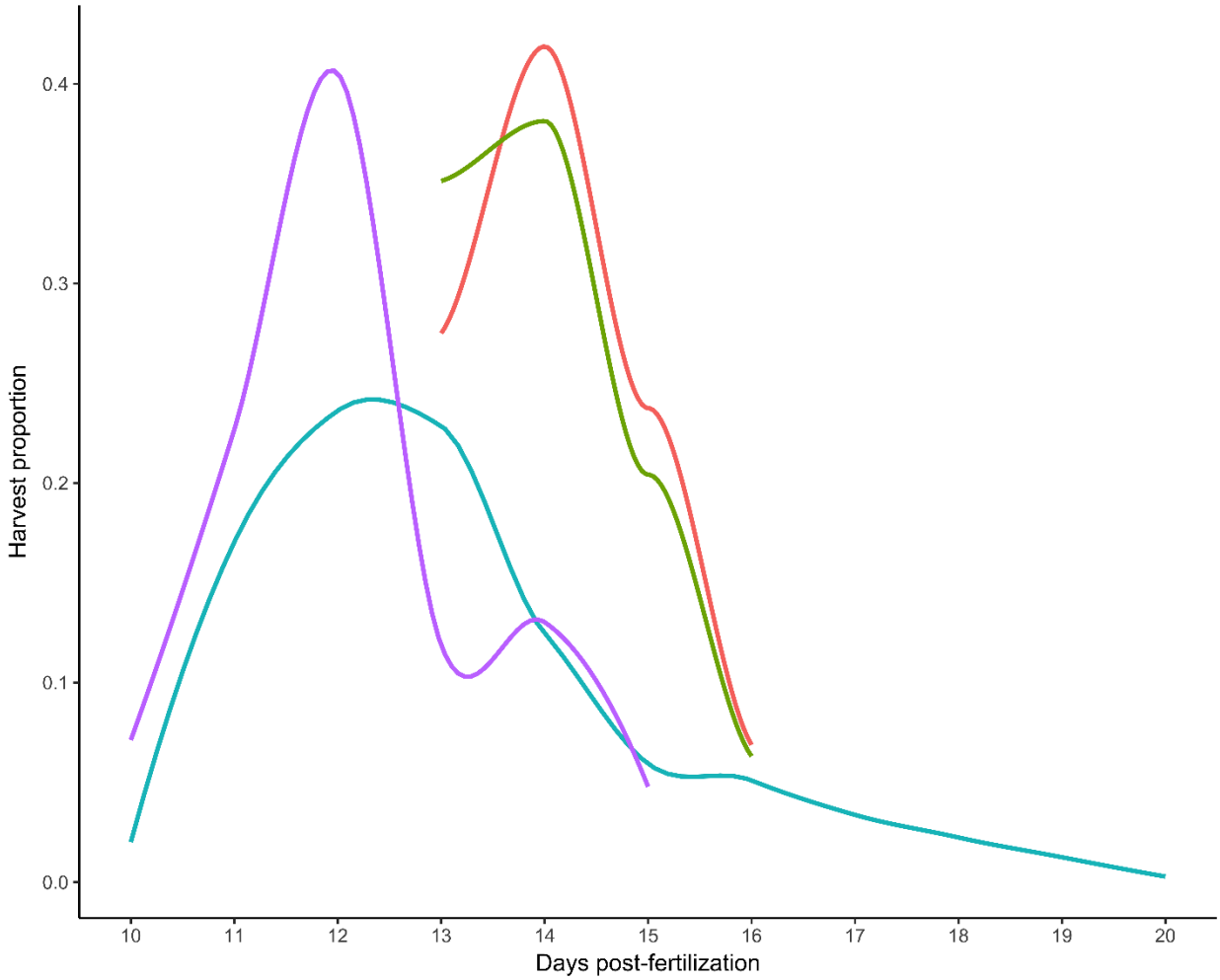


Figure 4.6. Harvest proportion of the four triploid broods by days post-fertilization. Harvest proportion is the ratio of the pediveliger larvae harvested on a given day to the total pediveliger larvae harvested for a given brood. Each brood is represented by the following colors: 4nGNLxCL, 4nGNLxSL, 4nVBOYxCL, and 4nVBOYxSL.

4.4 Discussion

Hatching rate, or survival to the initial straight-hinge veliger stage known as D-stage, is an important trait for efficient oyster hatchery operation. Successful hatching and optimized hatching rates mean that a hatchery will maximize production while minimizing wasted resources (time and money). Environmental conditions such as ocean acidification, salinity, and temperature have been shown to influence hatching rates (Barros et al., 2013; Eierman and Hare, 2013; Galtsoff, 1964). For endogenous factors, Boudry et al., (2002) suggested that, for *C. gigas* at least, three groups of factors explained variations in reproductive success (i.e. hatching rate) among parents: gametic, zygotic, and genetic. Gametic quality has been shown to directly influence reproductive success in *C. virginica* through measures of oocyte lipid content (Gallager et al., 1986; Gallager and Mann, 1986) and variations in sperm motility. The zygotic factor is described by specific sperm-egg interactions, which have been shown to effect reproductive success in matings of wild and cultured *C. virginica* (Gaffney et al., 1993). Boudry et al., (2002) suggested that much of the mortality observed in early larvae are attributable to high 'genetic load' of deleterious genotypes.

In this study, gametic quality (both oocyte and sperm) were not examined, but inferences about specific parental combinations and genetic contributions to hatching rate can be drawn. Maternal and paternal effects on hatching rate of triploid oysters were observed. While maternal effects on adult traits (e.g., growth) have been documented for *C. virginica*, paternal effects on important traits for culturing oysters have not been studied in detail (Callam et al., 2016). Specific combinations of male tetraploids and female diploids also significantly influenced hatching rate. There was no single combination with the greatest hatching rate, but one combination (4nGNLxCL) had significantly lower hatching rate than other combinations (Figure

4.2). Gametic incompatibility has been shown in *C. virginica* (Gaffney et al., 1993). Identifying combinations of extant tetraploid and diploid breeding populations that may be subject to gametic incompatibility has major implications for commercial production. Here, identifying one possible combination with such incompatibility will lead to either avoiding such combinations for commercial productions or exploring introducing genetic material into either or both parental breeding lines to potentially reduce the observed incompatibility.

Beyond successfully hatching from egg, an oyster larva is only valuable to a hatchery insofar as it survives to the pediveliger stage. Differences in survival were observed due to the tetraploid male parent. Triploid broods from the 4nVBOY tetraploids had a greater probability of survival during the first week of culture, then dropped to the same proportion as triploids produced with the 4nGNL tetraploid males. Survival rates at first pediveliger harvest were equivalent. Larval survival has some genetic control, so these differences in early stage survivals suggests there may be variability on which selection pressure may be applied to increase survival of triploid *C. virginica* larvae (Eierman and Hare, 2013). Increased survival is a direct increase in hatchery efficiency and, thus, a worthwhile endeavor to further investigate.

Increased growth of larvae is of value for an oyster hatchery when it translates to earlier pediveliger development. While there were no observed differences in growth between triploid broods when considering either male or female parent or specific combinations of each, larvae did develop to the pediveliger stage at different rates based on the tetraploid parent. Larvae with 4nVBOY tetraploid male parents developed, on average, to the pediveliger stage four days earlier than those with a 4nGNL tetraploid male parent. Developing to the pediveliger stage earlier will allow hatcheries to have greater brood turnover and more efficiently utilize tank space leading to greater production.

Pediveliger harvest proportion over time provides valuable insight on a larval brood's production quality. The less time a hatchery needs to spend rearing a brood to harvest the required amount of pediveligers the greater production that hatchery is capable of. In addition to increased production, it has been established that larvae in a brood that reach the pediveliger stage earliest also have greater growth as spat and adults (Losee, 1979, 1978). The harvest of pediveligers for each larval brood had two peaks of harvest activity and were skewed right toward longer brood culture times. Selective breeding may prove useful to select for larvae that fall within the first peak of pediveliger harvest. These larvae would all reach harvest over a shorter period and also exhibit greater growth at subsequent life stages.

The observed differences attributable to the tetraploid parent suggest potential routes of selective breeding to add value to triploid offspring and make the hatchery production of triploid oyster larvae more efficient. Among these differences were hatching rate, survival, time to pediveliger development, and duration of pediveliger harvest. Improvements in these traits would increase hatchery production capacity and efficiency.

Chapter 5
Using Remote Setting to Produce Seed Oysters in Louisiana and the Gulf Coastal Region

This chapter is the culmination of work to modernize and add up-to-date information to a remote setting manual originally produced by Louisiana Sea Grant (Supan, 1991). Below is the updated body text of that manual. This chapter will also be published by the Louisiana Sea Grant College Program to aid current and future culturists in establishing and improving remote setting operations in the Gulf Coastal Region.

5.1 Introduction

Oyster production in Louisiana has ranged from a low of 4.8 million pounds in 1966 to over 12 million pounds in 2016. Approximately 80 percent of Louisiana's oyster harvest has traditionally been taken from private leases. Although production since the early 1980s has been well above the long-range average, much of this increase can be traced to increasing effort. Issuance of oyster dredging licenses by the Louisiana Department of Wildlife and Fisheries has remained well over 1,000 per year since 1985. Although privately leased acreage has risen to approximately 400,000 acres, total production from private grounds has shown very little growth, with declining production per acre.

For over 100 years, the Louisiana oyster industry has relied on the state's public oyster reefs for a supply of seed oysters. There are many reasons for the decline in the productivity of these reefs, including saltwater intrusion with its resulting marsh loss, natural oyster predation and disease, and increased harvesting pressure. Lack of consistent seed supply for oyster farming has been a significant detriment to the economic development of the state's oyster industry.

During the 1970s, the Pacific Northwest also experienced a similar problem with seed supply. However, research and development by university and industry members in the region

has given the individual oyster farmer the ability to produce seed oysters using ready-to-set or *eyed* larvae (as known as pediveligers) from oyster hatcheries.

Oyster larvae are microscopic, free-swimming oysters that develop from eggs and sperm spawned by adults. Oyster larvae have a velum (similar to a tiny tongue with hair-like structures that beat back and forth), which propels them through the water and gathers food. The larvae begin to grow their shells within the first 24 hours. During a 10- to 15-day period, the larvae swim and feed on microalgae (microscopic, free-swimming plants). As they grow, the shell changes from a straight hinge (similar to a clam) to a typical adult oyster shape. Near the end of their swimming period, the larvae develop an eye-spot (seen as a black dot) on their shells and grow a foot that allows them to crawl. During the last 48 hours, they search for a place to set, or cement, themselves by crawling on whatever surface they find suitable. After setting, they then change from a swimming existence to a sedentary one; their velum and foot are absorbed and other internal organs change.

In the wild, larvae swim up and down in the water and are moved by tides and currents, but in the hatchery, they are raised in large tanks using filtered seawater. Commercial hatcheries commonly use several tanks that can each hold well over 8,000 gallons of seawater for high-volume larval production.

Larvae are small and are measured in micrometers (commonly called microns, of which there are approximately 25,000 to an inch). Larvae begin as fertilized eggs measuring 40-50 microns in diameter and grow to 285-350 microns when they finally set. At that size, the eyed-larvae can be seen, without magnification, swimming inside a white bucket, looking like fine grains of brown-black sand.

Remote setting methods allow the grower to utilize a hatchery without the expense, special abilities, or location needed to operate one. In the Pacific Northwest and East coast, for example, large commercial, high-volume oyster hatcheries now produce billions of larvae for sale to growers.

Remote setting of the Pacific oyster (*C. gigas*) was first investigated in 1972 through laboratory studies by D. S. Lund, at Oregon State University (OSU) and commercially by W. W. Budge. Larval handling and remote setting was further refined by west coast oyster growers and B. A. Henderson, also of OSU. Prior to these efforts, oyster hatcheries were not economically feasible because of cultch (the material on which oyster larvae will set) handling and grow-out problems. Remote setting techniques helped hatcheries become viable by allowing a division of labor between the hatchery operator and the oyster farmer, and enabled the hatchery to concentrate on high-volume larval production. Successful commercial remote setting methods have been well documented from the Pacific Northwest and East coast.

The general process of remote setting is as follows:

> Hatchery-raised larvae are wrapped in moist nylon cloth or coffee filter and paper toweling then shipped to the grower in an insulated container at 41°F (5°C). Upon arrival, the larvae are suspended in a covered tank of filtered seawater at about 86°F (30°C), containing washed and aged cultch. Oyster shells are generally used as cultch, held in plastic-mesh bags (shellbags), wire mesh or stainless steel cages. The larvae are introduced at a density of 100 per shell, anticipating a 20 to 50 percent successful attachment to the cultch during the setting period of two to three days. Setting activity and spat condition are verified by microscopic examination prior to planting on leased water bottom. When and

where to plant are very important decisions, since the resulting spat can be lost to crab and oyster drill predation (Figure 5.1).

Since larvae can close their shells like adult oysters, they can be stored in the refrigerator. Researchers found that they have a shelf life of up to seven days, so long as they are kept moist and at the proper temperature, but long storage times are not recommended. Larvae should be delivered from the hatchery overnight and set as soon as possible.

The remainder of this manual will discuss important points that need to be considered before larvae are set.

5.2 Choosing a remote setting site and grow-out areas

Water quality is the most important factor in choosing a site. Consistently good water quality can mean the difference between having regular successes or failures.

5.2.1 Setting site

A common sense approach to determining water quality is by looking at the activities in the watershed or drainage area of the bay or bayou under consideration. Herbicides and pesticides are lethal to larvae at strong doses. Check to see if local road maintenance efforts include the use of herbicides to control ditch weeds and if mosquito fogging occurs close by. Runoff from boat yards can cause problems for setting operations, especially from antifouling paints. Bilge water, which usually contains oil, fuel, and hydraulic fluids, can cause problems if pumped into setting tanks.

Very high tides over the marsh can result in brown-colored dead water, caused by decaying marsh grass. Failed larval sets are likely when such water is used.

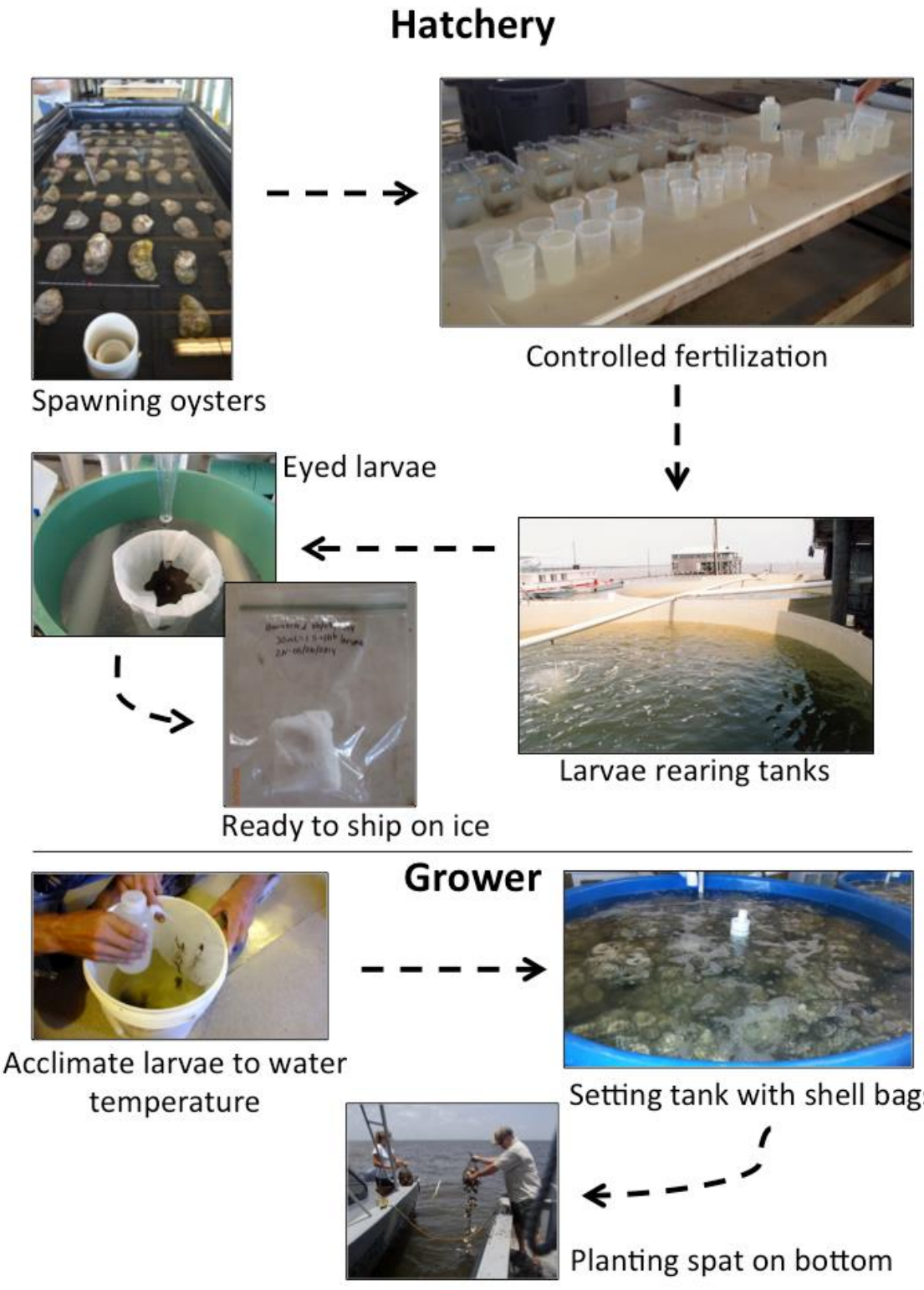


Figure 5.1. Oyster production from start to finish, using hatchery-raised larvae and remote setting.

Check the salinity (salt content) of the water you are considering. This can be done with an inexpensive hydrometer (Section 5.11) or, for a slightly greater investment, a refractometer (Figure 5.2). Larvae do not set well in water that is too fresh. The salinity should be above 10 parts per thousand.

Other factors to consider include:

- access to reliable electric power;
- ease of getting to the site for providing surveillance, monitoring, maintenance, and delivery of larvae from the hatchery;
- ability to transport cultch to the site and seeded cultch to the grow-out area;
- ease in pumping water from below the surface to avoid surface pollutants and rainwater and off the bottom to ensure that the water does not contain sediment.

5.2.2 Grow-out sites

Grow-out areas should be chosen the same way a lease for bedding oysters would be chosen. Oysters grow faster in areas with swift-moving currents because more food is available. This is important if placing stacked containers or shellbags of spat onto the lease. When placed in slack water, the growth of spat located in the inner areas of containers or shellbags will be less than that of spat in the outside areas. Stunted seed oysters do not grow quickly when bedded.

- Bottom type is also important if the cultch is to be placed on the bottom. A bottom firm enough to walk on without sinking is the minimum requirement for placing pallets of containers or shellbags. If containers or shellbags are going to be thrown directly on the bottom, a hard reef is necessary to prevent burial.

Figure 5.2. Tools with which salinity (salt content) may be estimated: hydrometer (top) and refractometer (bottom).

Other factors to consider include:

- location close to the setting site, to avoid drying out the freshly set spat and to reduce transportation costs,
- use of the site during rough weather,
- water deep enough to keep the spat submerged,
- water quality,
- theft.

5.3 Remote setting system construction and preparation

The system needed for setting larvae includes a tank, air-lines, a blower, a cover, and a seawater pump and filter.

5.3.1 Tank design, construction, and preparation

Setting larvae in a tank containing cultch and seawater provides the oyster grower complete control over seed production, as opposed to releasing the eyed-larvae into the wild over a reef or planting the cultch and expecting a wild set. The tank can be simple and of any shape. The cultch and water should not be more than four feet deep to allow complete penetration by the larvae.

The tank volume depends on the amount of seed you want to produce from each batch. Larger tanks are more efficient and economical for high volume production per unit of effort and construction costs. Larger tanks require a great deal of structural support because increased water weight creates greater stress. Smaller tanks can be made much more simply and cheaply.

A tank 10 x 8 x 4 feet holds 300 shellbags, which are approximately three feet long and hold about 1 cubic foot or 215 oyster shells. About 1.3 million spat can be produced from each set with a tank this size, if approximately 100 larvae per shell are added, and 20 percent of the

larvae set, an acceptable setting success rate. Figures 5.3, 5.4 and 5.5 illustrate examples of tank designs.

Most setting tank interiors are light in color. Keep in mind that larvae like to set on darkened surfaces.

The tank should have at least a two-inch drain. Draining and cleaning are easier if the tank drains from the bottom.

After construction, the tank should be thoroughly soaked to leach out toxic chemicals from the construction materials. This is especially necessary for fiberglass, gelcoat, painted material, and cement. Completely fill the tank with water and soak for at least two days. After draining, repeat the process twice more. Rinse the tank with water between soakings. Exposing the tank to direct sunlight during leaching will also help. Fiberglass, gelcoat, and paints should leave no odor in an empty tank once leached. Cement tanks are well leached when the pH is neutral (close to 7).

A steam cleaner can be used to quickly leach fiberglass. The nozzle is fixed toward the center of a covered tank and the tank is steamed for six hours. This method should avoid burning a hole in the fiberglass and make the surface very hot (Jones and Jones, 1988). Afterwards, the tank should be rinsed until there is no remaining fiberglass smell.

A tank cover is necessary to keep rain out and to darken the inside of the tank during setting. Since eyed-larvae swim away from light, a cover helps in obtaining an evenly distributed set on the cultch by darkening the water column. In Louisiana, a tightly fitted tarp works well.

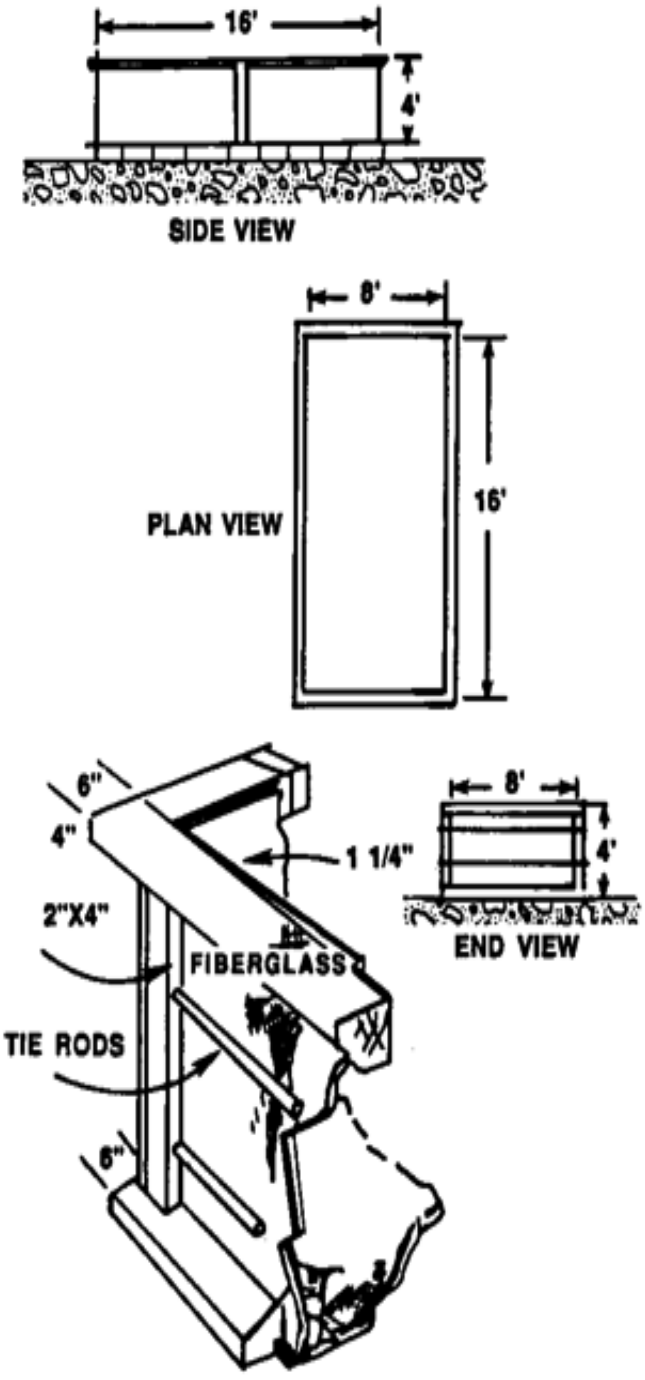


Figure 5.3. Plywood and fiberglass tank design.

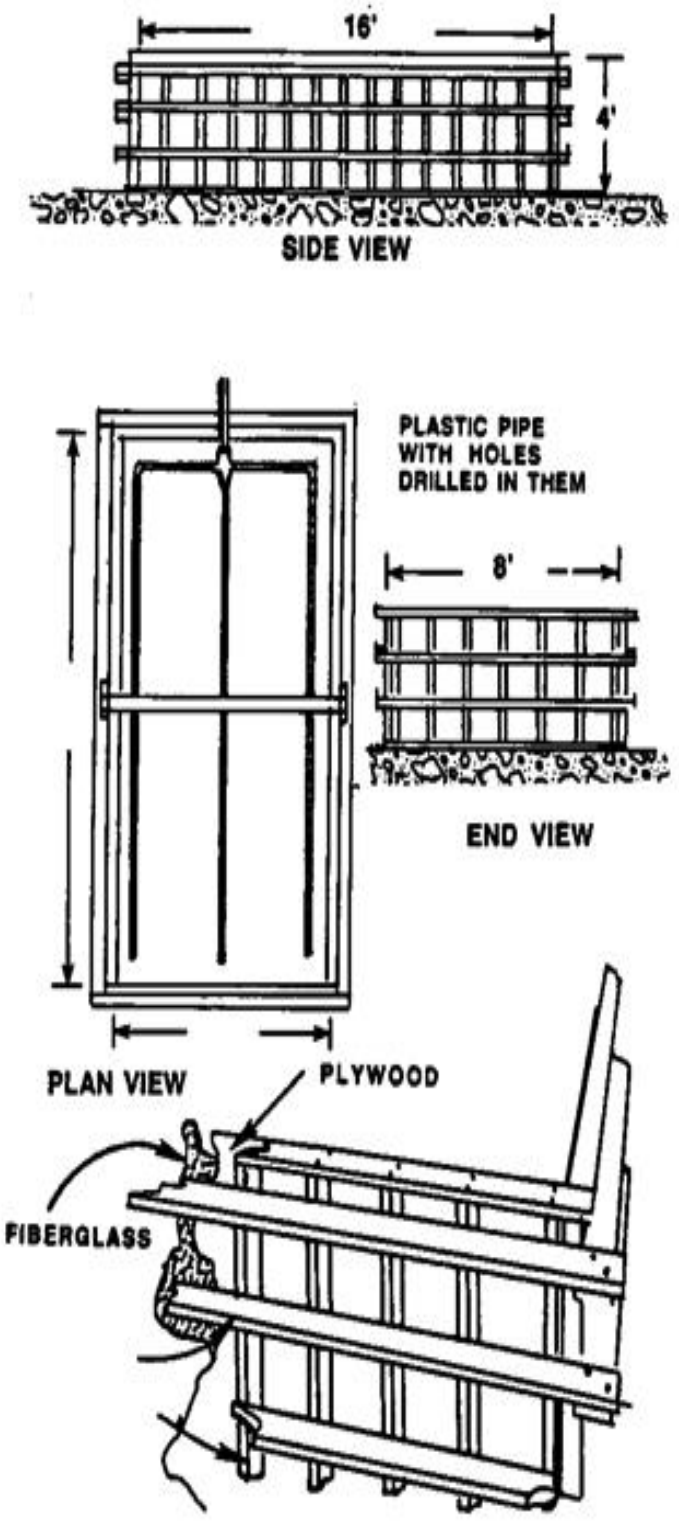


Figure 5.4. A setting tank design. Note that 2 x 4s and 2 x 6s are used instead of tie rods, as in Figure 5.3 (from Jones and Jones, 1988).

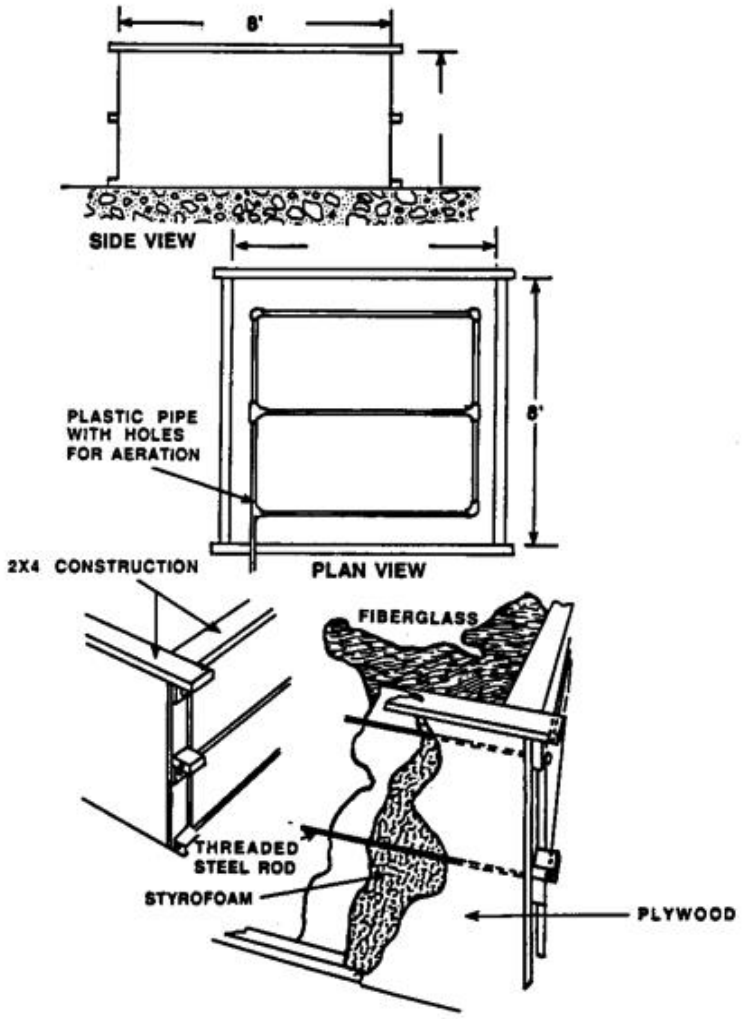


Figure 5.5. An insulated tank design. Insulation is particularly important if the seawater is heated, to keep heating costs low (from Jones and Jones, 1988).

5.3.2 Aeration

Air is bubbled through the tank during setting to help keep the larvae evenly distributed. Plastic pipes with small holes (1/32 inch to 1/8 inch) drilled about every foot, laid evenly across the tank's bottom, work well. The air-lines should be designed to allow easy removal and cleaning (see Figure 5.4 and 5.5).

Air can be supplied by any means so long as it is oil-free. Compressors and pumps can be used, but blowers work best (i.e., high volume, low pressure). Aquaculture or aquarium specific blowers can be purchased through the internet (Figure 5.6). Connect the blower's outlet to the tank's air pipe system. A valve may be placed between the blower and tank to control the airflow and bleed off excess air. It is also necessary to locate any air source higher than the water level in the tank, so that the pump will not fill with water when it is turned off or when the power goes off.

5.3.3 Seawater pumping and filtration

Remote setting systems do not have critical pumping requirements. Ideally, a pump should be chosen to match the total dynamic head of any system, which includes the type and length of the plumbing, number and type of fittings, and the height to which the water is being pumped. The pump size depends on how fast you want to fill the tank because once the tank is filled, the pump can be turned off. Radial (centrifugal) pumps with plastic, cast iron, or brass impellers are commonly used. Swimming pool pumps with plastic impellers and intake baskets to catch debris work well with seawater. Locate the pump near the water to eliminate impeller cavitation. Keep silt from fouling the cultch inside the tank by locating the pump's intake off the bottom and away from boat wheel wash in shallow water.

Figure 5.6. An aquaculture specific air-blower.

Seawater should be filtered before it enters the tank to remove most of the larger microscopic plankton that eat or compete with the larvae. Filter bags (rated for 50 microns) are effective enough for remote setting, easy to use, and economical (Figure 5.7). They can be purchased from most aquaculture supply companies. Commercial swimming pool sand filters work well also, but are more expensive and difficult to maintain (e.g., interior fouling).

5.4 Cultch preparation and handling

The majority of the labor required for seed oyster production using remote setting techniques involves cultch preparation and handling. Cultch must be washed free of grit, chips, and dirt; the tanks must be loaded and unloaded and the cultch bedded on the lease.

5.4.1 Cultch preparation

Clean oyster shells (cultch) are important for obtaining a good set on a wild reef, and remote setting is no different. Depending on the cultch type used, preparation may include the following:

5.4.1.1 Aging

Shucked shells should be aged at least six months to one year before using in a remote setting tank. Meat remaining on fresh-shucked shells will quickly sour the water in the setting tank, which will kill larvae and spat. Weathering the shells (i.e., piling them up outside) will help remove the remaining meat and fouling organisms (e.g., barnacles, mussels, sea grasses, and jelly-like animals).

5.4.1.2 Grading

Grading shells helps obtain a uniform size by removing smaller shells and fragments. Motorized tumblers and shakers can be used, which can also remove much of the powder and grit from the air-dried shells.

Figure 5.7. Filter bag that fits over the setting tank source of seawater.

5.4.1.3 Washing

Washing is very important, because dirt and grit will keep the larvae from setting on the cultch. Washing can be as simple as rinsing the cultch off with water from a hose or it can be part of a mechanized process that grades, washes, and containerizes the cultch.

5.4.1.4 Leaching

If plastic, rubber, fiberglass, cement or other artificial cultch materials are used, leaching is important for removing toxic residues that may remain from manufacturing. Soaking the material in water is a common practice, but fouling may be a problem if it is soaked in seawater. A leaching period of four to eight months is recommended for plastic clutches (Roland and Broadley, 1990), however, the water climate in the Gulf region will quicken the process. Cement or cement-coated cultch must be soaked to neutralize the pH (i.e., a pH of 7), because cement mix is very alkaline.

5.4.2 Cultch handling

Oyster shell is the cultch most used for remote setting and is usually handled in shellbags averaging approximately 40 pounds each. The bag material (a roll of 13-inch lie-flat heavy-duty shellbag material or tubing with 9/16-inch mesh) is available from most aquaculture supply companies (Figure 5.8). The material is cut at the desired length (typically three to six feet), knotted at one end, slipped over a three-foot length of eight-inch PVC pipe (thin-walled) and filled with shells. Some operators have designed bag-filling tables with built-in lengths of PVC pipe over which the bags may be fitted. Cultch is then loaded onto the table and pushed into the pipes holding the shellbags (Figure 5.9). The pipe is then removed and the shellbag is tied shut. Generally, shellbags are stacked on wooden shipping pallets for forklift handling.

Figure 5.8. Roll of shellbag material (top) and shellbags cut, filled, and ready for handling (bottom).

Figure 5.9. Shell bags being filled at a table designed to aid in filling. Bags are slipped over the PVC pipe affixed to the table; cultch is then loaded onto the top of the table and pushed into the openings filling the bags.

Front-end loaders can be used to dump shells into a machine that grades and washes shells (Figure 5.10) and then sends them into a hopper. Laborers cut and knot the shellbag material, slip the bag onto the loading pipe(s) at the bottom of the hopper, remove the bagged shells, knot the bag again, and stack it on a pallet.

Smaller operators simply load shellbags using a hand-held hopper next to a shell pile, then wash with a fire hose (Figure 5.11). Sometimes a table-loader may be used.

Wire mesh cages or stainless steel cages may also be used to hold shell in setting tanks allowing for other mechanized handling techniques of cultch material and resulting spat (Figure 5.12). Sunlight will break down plastic over long periods of time, so bagging fresh-shucked shells to be washed a year later may result in brittle shellbags that break or tear easily when handled.

5.4.3 Tank loading and unloading

Shellbags or other containers can be loaded into and out of the tanks by hand, but shellbags stacked on pallets are also used in setting tanks. Conveyors, front-end loaders, and various hoists are used for loading and unloading tanks.

5.4.4 Other cultch types

Clam shells, limestone and crushed concrete will tightly pack within the 13-inch lie-flat bagging or other containers and may not allow larvae to penetrate for a good, even set. Past research has shown that such cultch should not be contained more than 9 inches thick to allow the aerated water to move the larvae through the cultch mass.

Figure 5.10. Oyster size graders that can be used to grade and wash seed simultaneously. Seed oysters or cultch materials are loaded into the grader as the drum rotates and water is sprayed into the rotating drum washing shells and allowing oysters/shells to fall through appropriate sized holes in the drum for grading.

Figure 5.11. Debris being washed off of oyster shells using a seawater fire hose.

Figure 5.12. Cylindrical vinyl-coated wire mesh cage (top) with shock cord closures for drop-out bottom. Rectangular vinyl-coated cultch cage (bottom).

Crushed shell, sold in feed stores as chicken scratch, can be used to produce single oysters and small clusters. The cultch can be spread onto the bottom of shallow setting tanks (like soft crab shedding tanks). After setting, the cultch must be handled gently to reduce crushing the freshly set spat, preferably with a water hose.

Artificial cultch, such as *French tubes* and cement-coated materials require special handling as well. Some of these materials are used in other regions with good success. For information on handling these cultch types, consult the west coast setting publication mentioned in the first part of this manual.

5.5 Cultchless setting system construction and preparation

Cultchless oyster setting is one alternative to setting oyster larvae on cultch (whole oyster shells) and produces single-seed oysters rather than clumps of oysters typical of setting on whole oyster shells. Single-seed oysters are mainly produced for use in containerized culture practices (e.g., cages or bags). Single oysters lack much of the protection from predation that forming clumps or reefs provide. Containerization is mainly for protection from predators and to improve growth rates and quality control of hatchery-produced oysters. Using containers also allows growers to manipulate growing conditions to maximize yield.

5.5.1 Setting material

Though often called cultchless setting, oyster larvae are set on what is called microcultch. Microcultch is made of very small pieces of cultch material (usually sand particle size of crushed oyster shell but can be made from any material typically used as cultch). Crushed oyster shell can be purchased from farm supply stores or aquaculture supply companies, usually in 50 pound bags. To make mostly single seed, the microcultch should be graded to approximately 250 microns in size.

There are two ways of using microcultch: 1) simply spreading the microcultch on the bottom of the tank and 2) holding the microcultch in setting containers. Spreading microcultch on the bottom of the setting tank is simple and easy to do, however care must be taken when collecting freshly set spat from the tank because these spat are extremely fragile and can be crushed when sweeping up the microcultch material after a set. In such cases, water should be used to collect the spatted cultch. Holding microcultch in containers allows for more careful handling but requires either constructing or purchasing setting containers.

5.5.2 Tank design, construction, and preparation

Cultchless spat are often set in shallow tanks or troughs with a large reservoir to provide enough water exchange to the spat. Spat are held within the tank in setting containers with mesh bottoms. These setting containers are made of untreated wood (typically cedar or cypress) or PVC that has been well leached of toxins.

The operating principal of a cultchless setting system is that setting containers (trays or cylinders) act as downwellers (meaning water flows down through the containers) delivering food and water to freshly set spat. Water flows into each tray from the reservoir delivering food and water and is then returned to the reservoir through a standpipe at the end of the trough that can be fitted to maintain desired water depth (Figures 5.13 and 5.14).

Figure 5.13. Setting tank. A blower is connected to a manifold that runs along the length of the tank. Flexible rubber tubing connects the manifold to the airlifts attached to setting trays. Water is recirculated from a reservoir by a sump and discharged through a hose positioned at the end opposite the stand-pipe.

Figure 5.14. A 35 gallon trash can acts as a reservoir for a setting tank. It is placed under the standpipe drain. Water is then pumped from the reservoir back to the setting tank by a submerged pump and hose.

The tank size depends on the amount of seed you want to produce from each batch. Unlike tanks for setting larvae on cultch, the size of the tanks for cultchless setting depends on how many and what size setting trays/cylinders are being used. Tank depth depends on the setting container design. Microcultch and larvae set in these setting containers do not need to be in more than a couple inches of seawater. If the containers are too deep the larvae may set on the containers rather than the microcultch. A tank 8' x 4' x 1' can hold 5 setting trays (32" x 16" x 6"), in which approximately 5 million larvae can be set.

Tank construction and materials follow the same guidelines set out in Section 5.3. Briefly, tanks can be made of fiberglass, concrete, polyethylene, etc. and should be thoroughly soaked to leach out toxic chemicals from construction materials. The tank should have a drain that is at least two-inches diameter. Draining and cleaning are easier if the tank drains from the bottom.

5.5.3 Setting containers

Setting containers in a cultchless spat production system require a mesh bottom to retain the microcultch and larvae while simultaneously allowing water to pass through. A setting container can be as simple as a square or rectangle of wood with mesh tacked to the bottom or more complex by gluing mesh to cut cylinders of PVC (Figure 5.15).

The mesh used to retain microcultch and larvae is a critical component of the system. Eyed-larvae are graded to retain on mesh screens measured at 212 microns or greater. This means that the mesh screen used to retain microcultch and setting larvae should be slightly smaller than this to ensure no larvae are washed through the screen and into the reservoir tank (any larvae that do so are essentially wasted). Mesh screens rated as 150 microns are generally used in the construction of setting containers.

Figure 5.15. Wooden (cypress) setting tray with mesh bottom (measuring 32” x 16” x 6”).

The size of the setting container depends on how many larvae are going to be set at a time. A container that is too large for the amount of larvae setting may result in wasting too much microcultch and one that is too small will result in larvae setting on each other (forming clumps) rather than as singles. A simple formula can aid in determining the setting container size based on setting no more than 1500 larvae per square inch of setting container. This ensures larvae have enough room to set as singles without wasting space (Section 5.14).

5.5.4 Aeration

Aeration demands are dependent on the method of water delivery to spat (i.e., downwelled to setting containers or microcultch spread along the bottom of the tank). If microcultch is simply spread along the bottom of the tank, air can be bubbled through the tank via a similar setup as described in Section 5.3. If water is delivered to the spat through downwellers, then aeration occurs through the method of water delivery.

Water can be delivered to spat by either being dripped into the setting containers or through airlifts. Airlifts can also aerate water as it is delivered to the setting containers. Simple airlifts can be built using PVC pipe. A hole at the elbow piece is drilled large enough to insert the flexible hose. An air-stone, small enough to fit inside the PVC, is attached to the hose and pulled up into the pipe (Figure 5.16). Air-stones must be completely inside the pipe or water will not be lifted into the setting container. Airlifts are then connected to a manifold attached to a blower.

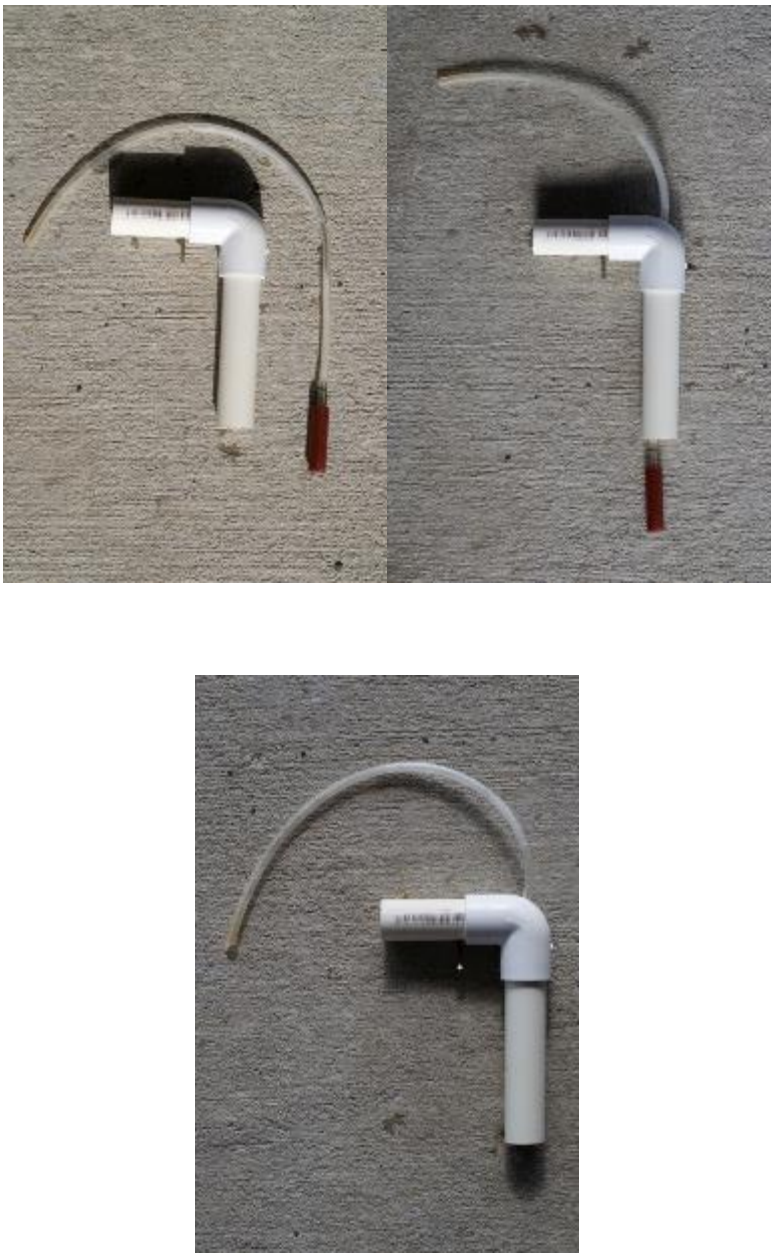


Figure 5.16. An airlift. 'L' shaped airlift with flexible air-hose and air-stone (top left). The air-hose is inserted into the airlift through a hole in the elbow and the air-stone attached to the hose (top right). The air-stone has to be pulled up into the airlift for water to be lifted (bottom).

Water can also be delivered to setting containers through another method. Water from the reservoir tank can be pumped through a manifold running across the top-center of the tank with flexible rubber hosing inserted along the length of pipe which then directs the water into setting containers. It is important to have the hosing suspended above the water so the water can aerate as it drips into the setting container.

5.5.5 Filtration

Cultchless setting systems operate under the same filtration requirements as cultch-based remote setting systems (see Section 5.3). The pump size depends on how fast you want to fill the tank because once the tank is filled, the pump can be turned off. Radial (centrifugal) pumps and swimming pool pumps work well with seawater. Locate the pump near the water to eliminate impeller cavitation. Keep silt from fouling the tank by locating the pump intake off the bottom and away from boat wheel wash in shallow water. When using setting containers with mesh bottoms, filtration becomes ever more important because the mesh can foul rapidly.

5.6 Ordering, evaluating, and handling larvae

It is important to know the amount of larvae needed for each set. Knowing how to tell good larvae from poor larvae (evaluation) and how to handle the larvae are also skills that must be developed.

5.6.1 Ordering larvae

To know how much larvae is needed, a basic recipe can be used. Each grower can improve or change his recipe after evaluating seed production results to decide whether to use more or less larvae during later sets. Oyster hatcheries can customize an order by making individual quantities of larvae within an order, so the order does not have to be split up for each

setting tank. Assuming good larval quality and that everything is done correctly, the following calculations are a good start.

5.6.1.1 Setting Tank

Average setting success is 20 to 30 percent. Adding 100 larvae per oyster shell to the tank may produce on the average approximately 20 to 30 spat per shell. Therefore, a tank that is 10 x 8 x 4 cubic feet containing 300 shellbags averaging 215 shells per bag should receive approximately 6.5 million larvae, resulting in approximately 1.3 to 1.9 million spat.

5.6.1.2 Setting Container

Setting success within setting containers is roughly equivalent to setting on whole oyster shells (20 to 30 percent). Adding 1500 larvae per square inch of setting container area may produce approximately 200 to 300 spat. Therefore, a setting container that is 30 x 18 inches should receive approximately 1 million larvae, resulting in approximately 200,000 to 300,000 spat.

5.6.1.3 Grow-out

Of the twenty spat per shell bedded, three market oysters may be achieved, or a 15 percent survival rate. Most Louisiana oyster farmers have years of experience in bedding seed oysters, so individual results may be better. In general, 3 percent of the larvae added to the setting tank should reach market-size. Grow-out of cultchless seed involves containers (e.g., cages or bags) and is not covered in this manual.

5.6.2 Evaluating larvae

Larvae are usually shipped in a styrofoam cooler containing the ball(s) of larvae wrapped in nylon cloth or coffee filter and moist paper toweling with frozen gel packs. The cooler

contents should be 40º-50ºF (4º-10ºC) and the gel pack still partially frozen. There should be no *fishy* odor.

The moist toweling should be removed and the individually wrapped larvae balls allowed to reach room temperature prior to placing in a clean bucket containing setting tank water. This will allow the larvae to acclimate or get used to the setting tank water conditions, especially salinity. A 15-30 minute acclimation period is recommended to reduce stress on the larvae and ensure that the larvae swim when added to the setting tank (Roland and Broadley, 1990). After the larvae begin to move in the bucket, do not wait long, or the larvae will begin setting on the bucket.

The wrapped balls of moist larvae can be weighed to keep track of the accuracy of each order. Weigh the wrapping again after the larvae have been removed and subtract the cloth weight from the previous total. A scale sold at local hunting supply stores for weighing gunpowder is suitable.

Buying a microscope to look at larvae and freshly set spat is a good investment (see Section 5.12). During microscopic examination, good-quality larvae can be determined by size, eye-spot development, and movement.

5.6.2.1 Size

Individual larvae should be approximately 285-350 microns from hinge (umbo) to bill (see Figure 5.17).

5.6.2.2 Eye-spot

This should be darkly colored and 15-17 microns in diameter (see Figure 5.17).

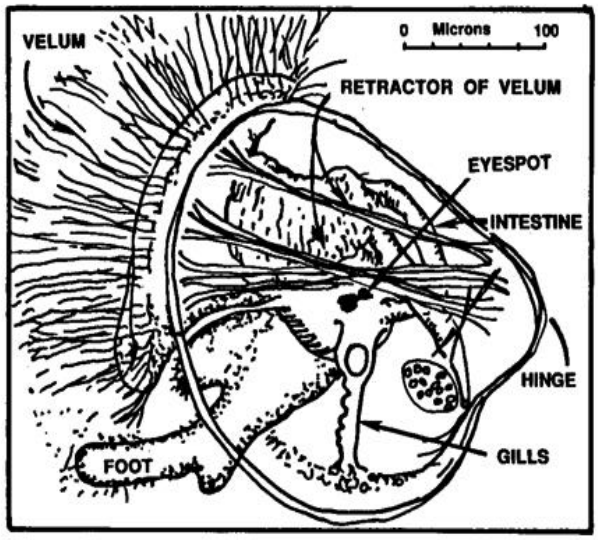


Figure 5.17. An oyster larva (from Jones and Jones, 1988).

5.6.2.3 Movement

Most larvae should be actively swimming with an extended velum (swimming organ). Swimming may not occur, however, after extended refrigeration storage (>3 days). Crawling with the extended food should be noticeable. Give the larvae time to acclimate to the temperature and salinity of the water if no movement is noticed immediately. A cracked shell and a fouled velum are signs of poor handling and raising practices. Some larvae may swim together in strands of mucus, and if this is the case, add more water to the container.

5.6.3 Handling larvae

It is important that once the larvae arrive from the hatchery they are either refrigerated or used immediately. Heated or dried larvae result in failed sets. *The larvae are fragile*. Do not squeeze or drop the larval ball, and handle it gently. Though the larvae look like sand, they must not be treated as such.

5.7 Setting methods

The actual setting of the larvae is relatively easy and very similar for setting on cultch or microcultch, but certain methods should be followed.

5.7.1 Cultch and microcultch soaking

A 24-hour soaking period is recommended before the larvae are added for both cultch and microcultch. It has been said that soaking the cultch in the tank before adding the larvae *gets the land smell off*. Research has found that it may put a *sea smell* on the cultch. During soaking, a film that attracts larvae is formed by naturally occurring bacteria, originally called LST because they were found growing in a larval setting tank. The *scent*, or chemical cues, that the bacteria expel stimulate the larvae to search, by crawling with their foot, and cement themselves to the filmed surface (Weiner et al., 1989).

Microcultch needs to be rinsed thoroughly with seawater but not be soaked because when the water depth is only several inches to cover the microcultch larvae have little choice in where they set. This is different from setting on whole oyster shells because in that case larvae have many more surfaces to set on.

5.7.2 Water change

It is recommended that the water be changed after the soaking period so that fresh food can be provided before the larvae are added. However, successful sets have been obtained without doing so.

5.7.3 Aeration

Constant aeration is generally used, giving the water a light rolling, but not boiling action. In British Columbia, a fairly high aeration (a good roll but not boiling) for a half hour after the larvae were added produced a more even set than constant aeration. Constant aeration may have produced water circulation patterns that caused the larvae to collect in certain areas. There was no difference in the distribution of spat in the upper, middle and lower portions of the setting tank with the shorter aeration period (Roland and Broadley, 1990).

5.7.4 Larval distribution

Even sets start with evenly adding the larvae to the tank or setting containers. The simplest way is to gently mix the larvae in a plastic bucket of tank water and pour them evenly into the tank or setting containers.

Some growers ladle the larvae into the tank or use a plastic sprinkling can. Others use a spoon on a pole. Make sure the aerator is on when adding the larvae.

5.7.5 Water heating

A setting tank water temperature of 77º to 86ºF (25º to 30ºC) is recommended. Successful remote setting has been conducted in Louisiana with local seawater temperatures as high as 91ºF (33ºC).

If setting is going to be attempted during the early spring and fall in the Gulf region, tanks should be insulated and heaters should be used to maintain the water temperature near 86ºF. During October 1990, a cold front passed through southeastern Louisiana, resulting in a setting tank water temperature drop from 82º to 65ºF (28º to 18ºC) within 36 hours. The remote setting attempts during and after that temperature drop resulted in failed sets. Those sets could have been saved had an electric immersion heater been available. An insulated tank would have helped as well.

Keep in mind that many metals are toxic to larvae. Incoloy 800 or titanium stainless steel is recommended for metal heating elements in direct contact with the setting tank water. Twenty-five amp quartz-sheathed electric immersion heaters are used in the Northwest (Figure 5.18).

5.7.6 Tank cover

Covering the tank will help obtain an even set, since “eyed” larvae tend to swim away from light. A tarp works well. Heated tanks should have insulated lids.

5.7.7 Setting time

Allow 48 to 72 hours for the larvae to set after they have been added.

Feeding the larvae during setting is not necessary. After the setting time (i.e., after the set has been verified using a microscope), raw seawater should be pumped into the tank in a “flow-through” manner to provide fresh food for the new spat.

Figure 5.18. Small electric titanium immersion heater available from online retailers. Larger heaters would be used for larger tanks.

The more water flow the better. Leaving the freshly set spat in the tank without enough food or in a dry condition for too long will cause high mortality.

When setting spat on microcultch (creating cultchless single seed oysters), food needs to be added to the reservoir tank after the 48-hour setting time. Simple algal paste that can be purchased from aquaculture supply companies can be used (Figure 5.19) or, the spatted cultch can be moved to an appropriate, nearshore, nursery system receiving wild food from local seawater (not covered in this manual).

5.7.8 Salinity

Salinity in the setting tank should be above 10 parts per thousand (often abbreviated as ppt), according to previous remote setting attempts in Louisiana. Setting efforts at lower salinities have proved unsuccessful.

If the local seawater at the remote setting site becomes too fresh at times, artificial sea salts, available from aquarium shops and aquaculture supply companies, can be added to increase the salinity and improve remote setting success. How much to add can be determined with a hydrometer (see Section 5.11).

5.7.9 Maintenance between sets

After the tank has been drained and unloaded, the tank interior (and setting containers if used) should be cleaned to remove any remaining grit and freshly set spat from the tank walls and bottom. Rubbing the interior with a nylon abrasive pad works well, and these are commercially available with plastic swivel-heads and long handles or poles to save your back. A stiff floor broom should work also. After rinsing the tank, check to see if the spat have been removed by wiping some of the surface with your bare hand.

Figure 5.19. Bottles of algal concentrate available from online mariculture retailers.

Freshly set spat look and feel like coarse sandpaper. Failure to scrub the tank between sets will result in more troublesome spat removal later on.

The filter bags are made of polypropylene-felt material and need to be cleaned soon after use. Turn them inside out and hose them out thoroughly and allow them to air-dry. They can also be cleaned in an old washing machine without detergent or bleach.

5.8 Evaluating the set and keeping records

It is very important to check the set, especially before the tank is drained and the cultch is removed from the tank. Much can be learned from failed and successful sets with good evaluation and record keeping.

5.8.1 Checking for swimming larvae

Evaluating the set begins by counting swimming larvae and spat. A one-cup sample of water should be taken from the setting tank at intervals of 1, 24, and 48 hours after the larvae have been added. Pour the sample into a clear glass container and hold it up to or above a light and you should be able to see and count any larvae in the sample. You may also use a microscope or magnifying glass (at least 10X magnification). Use the one-cup measure each time and take all three samples from the same tank location so that accurate comparisons can be made between samples and sets.

Obviously, there should be fewer swimming larvae over time. A fast decrease in the number of swimming larvae found in the samples means that the larvae were ready to set and the cultch, tank and water conditions were right. If five or more larvae are still found swimming in the one-cup sample after 48 hours, the larvae were not ready to set, the cultch was not suitable or the tank conditions were not right and a longer setting time is needed. When the water gets too

cold (e.g., at night in the late spring or early fall in an uninsulated tank), live larvae may stop swimming, though the internal organs can be seen moving under a microscope (Section 5.12).

Checking for larvae for single speed production is slightly different because the larvae are held in significantly smaller volumes of water within the setting container. Instead of using a one-cup sample, larvae can be checked by carefully collecting water using an eye dropper from inside the setting container. Checks should be done at 1, 24, and 48 hours after larvae have been added, and as with setting on cultch, fewer swimming larvae should be visible as time progresses.

5.8.2 Checking for spat set

Counting spat is very important and simple, once you get an eye for seeing freshly set spat (Figure 5.20, 5.21). Air-drying the cultch sample before counting makes it easier to see the tiny spat. When a larva successfully completes metamorphosis (changes) to become a spat, a new edge of shell will have grown onto the cultch from the bill of the larval shell, with the hinge pointing slightly upward. A hand-held tally counter helps to keep track of counts.

A new shell should be hung or placed in the tank when the larvae are added and replaced with another clean, new shell after 24 hours. This will give you a spat count for the first and second 24-hour periods of the setting time. These can be hung overboard for later reference. There should be a decreasing number of spat on these shells over time. The new shells should also be placed in the same section of the tank each time for accurate comparison.

As the setting tank is being unloaded, check some of the cultch from different sections of the tank to determine the evenness of the set. If shells are being used, count at least six shells from each section of the tank, such as the middle, side and corner. Be sure to take shells from the top, middle, and bottom layers of the cultch.

Figure 5.20. Newly set spat on an oyster shell. Several spat are circled for easier identification.

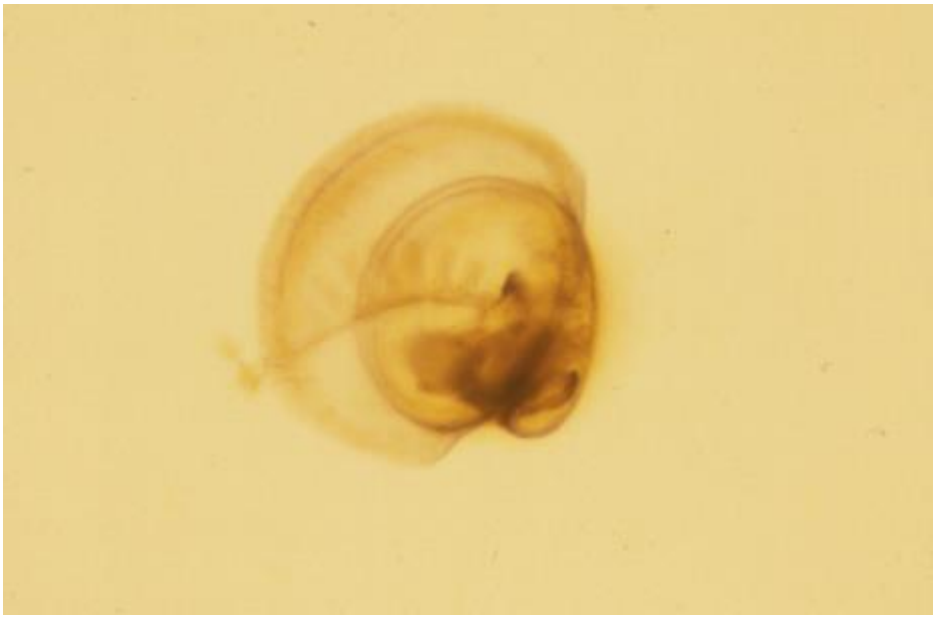


Figure 5.21. A 2-day old spat at 100X magnification. Note the newly formed shell growth from the larval shell: a sign of successful metamorphosis.

Accurate data collection and record keeping can help determine how good and poor seed production occurs. Thorough counting and record keeping will make future setting easier and provide better results. Setting results can vary for many reasons (i.e. different individuals, water sources, setting techniques, nursery and grow-out locations, seasons, larval sources, cultch types, etc.). Section 5.13 contains data sheets for remote setting. Copy them and use them regularly.

5.9 Evaluating seed growth and survival, and determining when to plant

Spat growth of up to one inch (25 millimeters) within 30 days was common in Louisiana during 1987-90 shellbag-nursery trials in salinities ranging from 11 to 18 ppt. Research conducted in Maryland, however, showed that planting freshly set spat-on-shell obtains similar results to planting larger seed.

While the cultch is being emptied, it is important to evaluate some of the shells from different shellbags or containers to obtain an accurate average of seed production. Average number of spat per shell, the number dead and alive (percent survival) and average size are valuable information for evaluating the effectiveness of remote setting efforts and its cost. Such information will help individual oyster farmers improve their techniques and allow them to customize production, depending on the conditions encountered.

Determining when to empty cultch containers for bedding onto the lease bottom is up to the individual oyster farmer. During summer nursery trials in Louisiana, spat began to grow through the shellbag within 40 days. This should be avoided, because high mortality will result from damaging the thin-shelled seed during bedding.

Damaged seed is more likely to attract crabs and other predators to the bedding site. Crab predation can be quite heavy when bedding is done during the summer months; however, the larger the seed size, the fewer predation problems. Crab predation can be reduced by

harvesting crabs from the bedding area using baited crab traps. Black drum prefer single oysters and have difficulty feeding on clusters of oysters. Spat smaller than 1/4 inch are especially vulnerable to mud crabs.

Flatworms (i.e. the oyster leach, *Styloccus* spp.) (Figure 5.22) are a minor predator of spat on oyster reefs, since crabs and fish may prey upon them when they crawl from one spat to another. However, during Louisiana shellbag-nursery trials, flatworms caused up to 33 percent spat mortality. The flatworms appear to be protected by the mesh covering as well. Overnight air drying and concentrated saltwater dips are effective in treating the cultch to reduce flatworm predation. The simplest treatment is bedding seed at the proper time of year or season, which is important if flatworms become a problem. Bedding the seed before too many losses occur exposes the flatworms to their natural predators. Keep in mind, however, that early seed bedding to reduce flatworm predation must be weighed against possible crab predation later.

5.9.1 Planting seed

Since most oyster farmers have generations of experience with bedding oysters, little needs to be mentioned here. The characteristics of seed produced by remote setting are different from those of seed dredged from oyster reefs. Seed produced by remote setting usually are not as cupped and are more thinly shelled. The advantage of this technology is the large quantity of seed produced. Fewer boat loads of such seed may be needed. Seed survival obviously depends on whether the seed is mishandled before bedding, the bottom type, salinity of the bedding area, and predators. Smaller seed obviously needs a longer grow-out period.

Figure 5.22. An oyster leach or flatworm in a glass ash tray. Note the size relative to the quarter-sized coin.

5.10 The cost of remote setting

The total cost of seed production using remote setting depends on the individual oyster farmer; the kind of equipment and labor available; the farmer's experience; and bedding ground conditions. Since the costs are likely to be variable, the farmer should take great care in his own estimates. A more detailed cost accounting follows as a guide (Tables 5.1 and 5.2).

Estimates for Table 5.1 are based on standard setting tank designs found in this manual. The tank interior is made of fiberglass for waterproofing and for providing a strong surface against wear from repeated loading and unloading of shells. The fiberglass surface is painted with epoxy paint to eliminate a reaction of the water with the styrene in the resin. Using wood glue and good carpentry, three or four coats (two to three gallons) of epoxy paint may be good enough as a tank interior coating.

Two filter bags are used at a time during tank filling (Figure 5.7). Since the bags may be worn by repeated cleaning, two extra are included in the cost.

Estimates for Table 5.2 are calculated from remote setting experiences in Louisiana. The labor costs include bagging the shells, loading, and unloading the tank, and planting the spat. Boat fuel is not included. A 1,500-foot roll of bag material costs about $75 (delivered). Generally, a 1 cubic-foot bag of seed averaging 20 spat per shell cost about $6. Note that Table 5.2 included a depreciation charge for the setting system. The grower should realize that this is not an out-of-pocket expense but is an allowable deduction for income tax purposes.

Table 5.1. Materials and equipment for an 8’ x 8’ x 4’ setting tank for a single tank remote setting system*

6 sheets of 3/4” exterior-grade plywood	$120.00
6-8’ 2x4s (treated)	$24.00
16-8’ 2x6s (treated)	$104.00
1 gal. preservative (for tank exterior)	$13.00
fiberglass materials:	$244.00
16 yds of 1.5 oz. mat @ 5.00/yd.	($80.00)
6 gal. resin & hardener	($150.00)
1 gal. acetone	($14.00)
1 gal. white epoxy paint (for tank interior)	$30.00
1/2” PVC pipe & fittings for air lines	$11.00
4-50 micron-rated filter bags & 2 support discs	$57.00
1-1/2” PVC plumbing for water pump & bag support discs	**$5.00
1-13’ 8” thin-walled PVC pipe (for making shellbags)	***$38.00
tank cover (plastic tarp)	$15.00
hardware	$30.00
air pump	***$290.00
water pump	***$135.00
Optional:	
microscope (1-100x & 1-30x pocket scopes)	$120.00
hand tally counter	$12.00
Total	**$1,248.00**

*Heaters and tank insulation not included
**Plumbing costs depend on how far the tank and/or pump is from the water source
***Can be used with additional tanks

Table 5.2. Approximate costs for setting 240 shellbags per set

Depreciation of setting system with options (Table 5.1) *	
Electricity for air and water pumps	$5.00
Eyed-larvae at $300/million x 6 million	$1800.00
Larval shipping	$48.00
Shellbags: $0.95/bag x 240	$229.00
9.5 cubic yds. Of oyster shell @ $16.50/yd.	(**$157.00)
6'mesh material/bag @ $0.05/ft x 240	(**$75.00)
Labor:	***$540.00
3-men each earning $60/day ($7.25/hr) for 3 days	_____
Total	**$2,802.00**
Cost/bag averaging 250 shells/bag and 20 spat/shell	$6.17

*The entire investment was treated as 5-yr property under IRS Modified Accelerated Cost Recovery System (MACRS). Depreciation charges vary year to year under this system. The depreciation charge in the table reflects the setting system in its third year. The yearly depreciation is $219.00.
**Shucked shell prices range from $14-$19.00 (delivered).
***1,500' roll.
****Labor includes making shellbags, loading and unloading tank, and placing bags of spat in nursery area. Include an extra work day ($150) for removal from the nursery area to grow-out area. Management and security are not charged here.

A 20 percent setting success rate is guaranteed by most commercial hatcheries. Each set from a tank is bedded on an acre. Other growers may do their planting differently. A 3 percent return of market-size oysters from the amount of larvae added to the tank may be attainable. Higher setting rates and returns may be attainable with good setting, nursery, and bedding techniques, water quality, evaluation, and record keeping.

5.11 How to measure salinity with a hydrometer

Whether you farm oysters, make soft crabs, or own a saltwater aquarium, knowing the salinity of seawater (the amount of salt present in the water) can be valuable information. This can be obtained inexpensively with a hydrometer.

A hydrometer is a precisely weighted, sealed glass tube that measures the density of a liquid by how high or how low it floats. A numbered scale is located inside the stem to obtain a reading of the density, also known as the specific gravity. The density changes with temperature and with differences in the amount of salt in the water (Table 5.3).

Hydrometers are made specifically for various liquids, such as syrups, alcohol, and battery acid, so make sure that the one you use for measuring salinity is specific for seawater or brine. Hydrometers for seawater can also be purchased with thermometers built inside. They can be purchased at aquarium shops and aquaculture supply companies.

To help in reading the hydrometer, try using it in a bucket of water. You could also use a short length of three-inch PVC pipe, with a cap on one end, filled with sea water.

Table 5.3. Converting density to salinity.

Density		**Salinity**		
(sp. gravity)	59°F	70°F	80°F	90°F
1.000	0	2	4	6
1.002	4	5	7	9
1.004	6	8	9	11
1.006	9	10	12	14
1.008	11	13	15	17
1.010	14	15	17	20
1.012	17	18	20	22
1.014	19	21	23	25
1.016	22	24	25	28
1.018	25	26	28	30
1.020	27	29	31	33
1.022	30	32	33	36
1.024	33	34	36	
1.026	35	36		
1.028	38			

Most hydrometers are built to be used at a temperature of 59ºF (15ºC), so when the seawater temperature is different, the observed reading on the scale must be converted to the actual temperature.

5.12 Buying and using a microscope

Buying a microscope to look at larvae and freshly set spat is a good idea and investment. A poor larval shipment can be determined beforehand, reducing the possibility of a failed set and the resulting wasted labor. Cultch should also be checked to make sure you have a good set before unloading a tank.

Microscopes generally use a combination of lenses to magnify. Scopes usually have a 10 power (10X) eyepiece and different lenses (objectives) below the eyepiece that can be switched for different magnification. Some cheaper models have interchangeable eyepieces and one lens below them. Whichever is used, keep in mind that it is the combination of the two that determines the magnification or power (X). For instance, a 10X eyepiece with a 10X objective will magnify 100 times (100X). With a 3X objective, it magnifies 30X. A knob is turned for focusing.

Buying a microscope to look at larvae and freshly set spat need not be too expensive, but like everything else, you get what you pay for. The more you spend, the better the field of view (i.e., the circle you view may be the size of a jar lid as opposed to a dime). There may be two eyepieces as well. Such options make it easier on your eyes, especially if you are going to look in the scope for long periods of time.

The least expensive are pocket scopes with one or two eyepieces (biscopes) having 100X or 30X magnification (sold as separate scopes). These have a battery-operated light and a vinyl carrying case ($20-$50). Scopes used for students in high school and college have more than

one lens and are more expensive ($250-$800). These are sold as compound scopes with magnification in the high range (100X-400X) and stereo (two eyepieces or oculars) dissecting scopes for close handwork with low to midrange magnification (10X-40X). Used scopes are available. If you are going to invest that much money, buy or build a carrying case as well.

To check larvae closely (i.e., to see internal organs moving to verify that nonmoving larvae are dead or alive), 100X is needed. You will be able to see the extended foot as well, meaning that the larvae are ready to set.

If you want to measure the larvae, a scale (micrometer) is needed. The scale is marked from 0 to 100 and is made from clear plastic or glass. With expensive scopes, the scale is placed in the removable eyepiece, so when you are looking in the scope, the 0 to 100 scale is in view over what you are measuring.

Less expensive scopes have eyepieces fixed in place, so if you want to measure larvae, you can place the scale down in the sample that you are observing or lay it on top of the sample as a cover slip. Inexpensive, hand-held pocket scopes can be used to measure larvae by placing the clear scale (sold as a cover slip) onto the bottom of a clear glass ashtray with a little seawater and a few larvae. The larvae can then be evaluated for velum and foot movement and measured as they swim over the scale. If they are moving too fast, some fresh water or a capful of alcohol (gin or vodka) will stop their swimming. Too much liquid in the ashtray will make measuring troublesome. If you need to push the larvae around while looking at them to get them situated over the scale, make a probe with a straight pin inserted diagonally through an eraser on a pencil.

Freshly set spat can be easily seen at 30X. A 10X magnification will allow faster shell examination by covering more area while looking for spat, then changing to 30X to get a closer

look. Use the probe to push grit aside. Larvae that have successfully set will have grown a new edge of shell from the bill onto the cultch with the hinge pointing slightly upward.

5.13 Setting checklist

PREPARATION

Date and Time or Comment

_____ Larvae ordered from hatchery and transport arranged
_____ Setting tank preparation completed (cleaned and washed)
_____ Heating and aeration systems tested
_____ Cultch preparation completed (aged and cleaned)
_____ Tank loaded and filled with water for conditioning
_____ Test cultch hung in tank
Amount and type of cultch in each set tank
_____ Tank A _____ Tank B
_____ Tank C _____ Tank D
_____ Conditioning water dumped and filled with heated water

LARVAE INSPECITON

_____ Larvae arrival time and date
_____ Temperature of larvae in ice chest
_____ Color and smell
_____ Moisture of bundle of larvae
_____ Average size of larvae
_____ Food activity
_____ Loose velums

LARVAE INSPECTION (in bucket)

_____ Number of larvae per ml in bucket
_____ Total number of larvae (number per ml x volume)
_____ Mucus production (stringing)
_____ Behavior (drop to bottom, stick to bucket, swimming, etc.)

SETTING

_____ Time and date larvae put in tank
Number of larvae in tanks
_____ Tank A _____ Tank B
_____ Tank C _____ Tank D
_____ Bay water visibility
_____ Salinity and temperature

_____ Aeration and frequency and duration
_____ Temperature at start of set
_____ Number of larvae swimming after 1 hour in 1 cup

DAY 2

_____ Temperature in morning
_____ Number on test cultch
_____ New test cultch added
_____ Number of larvae swimming in 1 cup
_____ Food added? (amount)
_____ Water change? (amount)
_____ Temperature at end of day

DAY 3

_____ Temperature in morning
_____ Number on test cultch
_____ New test cultch added
_____ Number of larvae swimming in 1 cup
_____ Food added? (amount)
_____ Water change? (amount)

POST-SET AND PLANTING

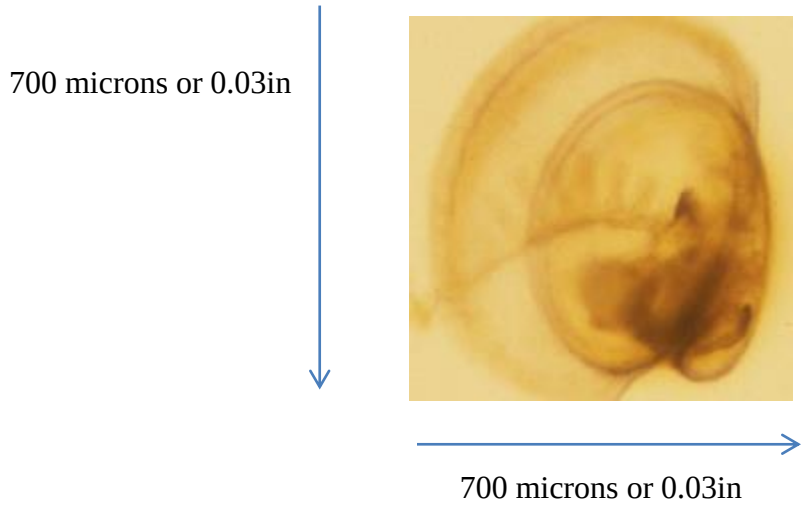
_____ Date and time of removal from tank
_____ Air temperature and weather conditions
_____ Length of time out of water
_____ Tide foot level or depth
_____ Location of cultch for nursery
_____ Tide foot level or depth

Table 5.4. Spat counts per cultch piece

	TANK A	TANK B	TANK C	TANK D
AT REMOVAL	_____	_____	_____	_____
	_____	_____	_____	_____
	_____	_____	_____	_____
AFTER 2 WEEKS	_____	_____	_____	_____
	_____	_____	_____	_____
	_____	_____	_____	_____
AFTER 1 MONTH	_____	_____	_____	_____
	_____	_____	_____	_____
	_____	_____	_____	_____

5.14 How to determine setting container larval density

- Larvae are between 300-350 microns at setting
- Spat are removed from setting containers when they catch on a 500 micron screen → Length of spat will be around 700 microns or 0.03 inches (The spat basically double in size)
- The size at which the spat catch on the 500 micron screen needs to be determined, so when we stock larvae they have enough space to grow



- Changing the oyster above into a square, the area is 0.0049cm^2
- To calculate a stocking density, find the area of the desired setting container in inches and divide by the area of a freshly-struck spat (0.0009in^2)
    - This is approximately the number of larvae that should be set in the container
    - Being conservative with stocking densities is recommended to reduce the number of larvae that set on each other
    - If spat are transferred quickly to a nursery, setting containers can be stocked from 1 to 1.5 times the number determined by setting area

First calculate the number larvae per container based on area:

Area of downwellers (in square inches) / 0.0009in^2 = _____

For less conservative density, multiply the estimated larvae per container by 1.5:

(Max larvae per container) _____ x 1.5 = _____

Chapter 6
An enterprise budget for floating cage oyster (*Crassostrea virginica*) culture in the Gulf of Mexico region

6.1 Introduction

Oyster production has a long history along the Gulf of Mexico coast. Wild oysters were harvested by hand and beginning in the 18th century fishermen were using tongs to harvest larger quantities more efficiently (MacKenzie, 1997). In Louisiana, oyster production began in the early 1800s along the Mississippi Delta. Shortly after commercial oyster harvest operations were established, immigrant fishing communities (most notably from Yugoslavia) began to apply and adapt traditional culture methods of manipulating wild oyster populations to produce a higher quality product (Wirth and Minton, 2004). Dredging (pulling a metal frame with a net) oysters began around the turn of the 20th century and dredges were raised and lowered by hand until around 1913. Even greater harvesting efficiency was then achieved through mechanization (Vujnovich, 1974).

These manipulative methods involved the transfer of small *seed* oysters from natural reefs to private waterbottom leases in more productive waters, thus transforming oyster harvesters to oyster culturists that utilize both public and private oyster grounds. The practice of seed transplanting has remained remarkably unchanged for nearly 200 years in the Gulf of Mexico. Seed planting allows culturists to manipulate growing conditions and ultimately the final flavor of oysters destined for market as well as to adjust harvesting based on market demands (Supan, 2002; Wallace, 2001; Wirth and Minton, 2004).

Leasing waterbottom for oyster cultivation began in Louisiana around the 1850s in Jefferson, St. Bernard, and Plaquemines Parishes. Leases were originally acquired through parish officials, but the management of waterbottom leasing has since been taken over at the

state level. Leases were acquired as a way to officially identify and protect a culturists location and crop. At this writing, there are over 8,000 private oyster leases in Louisiana with the vast majority of landings reported from private oyster leases (LDWF, 2015). The proportion of landings that come from seed transplanting is not known, however.

The economics of transplanting harvested wild seed from public oyster grounds to private oyster leases have been studied, though there has been limited and sporadic information prior to the 1990s. Melancon (1990) developed a conceptual model that describes seed transplanting and eventual harvesting and the environmental influences on the overall oyster lease productivity (Figure 6.1). The seed transplanting fishery in Louisiana was described by Melancon and Condrey (1992) as labor efforts to locate, harvest, and transplant seed and then harvest once adults reach market size. This model also included environmental effects on production and other associated operating costs. Melancon and Condrey (1992) described production and concluded that labor was the greatest cost, both variable and fixed, composing 43% of the total production cost. These authors determined the breakeven point (in 1992) was achieved when 14% of bedded oysters were recovered by harvesting as market-sized adults. Dugas (1977) has suggested that a recovery to plant ratio of 1.2:1 is common for Louisiana bedding leases. However, because any ratio above 1:1 suggests wild recruitment, reassessment of this expected recovery ratio may be warranted even though population sizes on public seed beds are at levels comparable to those seen in the early 1980s (LDWF, 2015).

An alternative to transplanting wild seed oysters developed during the 1970s on the US west coast. This alternative approach consists of allowing hatchery-produced oyster larvae to settle onto cleaned oyster shell to create what is known as spat-on-shell, which is accomplished remotely (i.e. away from the hatchery) and called remote setting (Lund, 1971).

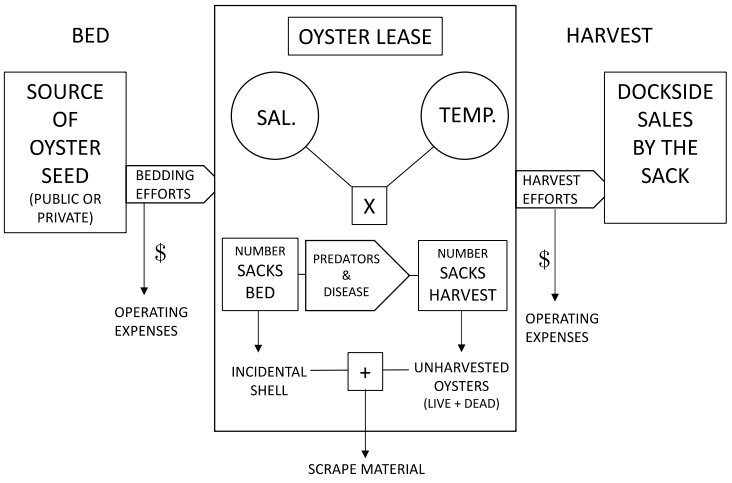


Figure 6.1. Conceptual model of a Louisiana oyster seed fishery (reproduced from Melancon, 1990).

Larvae are reared in hatcheries until ready to settle and metamorphose and are delivered to setting facilities to initiate the remote setting process. On site, larvae are introduced to tanks of cleaned and containerized cultch material and seawater and allowed to settle. Oyster shells are typically used as cultch. Remote setting produces spat-on-shell (SOS), which may be several spat attached to a single oyster shell. SOS are transported to planting sites as early as two-days post settlement, as long as newly set spat are covered from sunlight and kept cool and wet. SOS are planted on private waterbottom leases and subsequently grown and harvested similarly to traditional wild seed plantings. The detailed process of remote setting and its application has been well documented in the Pacific Northwest (Jones and Jones, 1983), the Mid-Atlantic (Bohn, 1989), and the Gulf Coast (Supan, 1991). Congrove (2008) estimated a SOS operation, with a capacity of setting larvae on 300 bushels of cultch per set, had a cost per bushel of approximately 20 USD, with an expected profit per bushel of harvested oysters of approximately 12.70 USD. This estimate was based on a yield (ratio of bushels of harvested oysters to bushels of planted cultch) of 2:1 using selectively-bred diploid oysters. The profitability of SOS operations is mainly sensitive to labor costs and the ex-vessel price of market-sized oysters (Supan et al., 1994). In the Gulf of Mexico, costs were estimated to be between roughly 14 and 20 USD depending on initial infrastructure (Supan et al., 1999). Initial investment cost depends on existing infrastructure and can range from as little as 3,000 to 50,000 USD if land, vehicles, vessels, etc. need to be purchased (Congrove et al., 2008; Supan et al., 1999). Oyster growers interested in setting up SOS operations are likely to have vessels and water bottom leases, reducing the cost of setup.

The production of oysters in hatcheries was made possible by the discovery that fertilization and development of *C. virginica* larvae occur in the water column and not in the

mantle cavity like other, brooding, oyster species (Brooks, 1879). Once larvae were able to be collected from the water column, the entire larval life cycle was able to be described (Wells, 1933). A full understanding of the larval life cycle allows exploitation for hatchery production.

Rearing larvae in hatcheries, from egg fertilization to metamorphosis, takes between two and four weeks. The general process consists of collecting mature gametes from sexually mature adults, fertilizing collected eggs, then rearing larvae in tanks of filtered seawater until metamorphosis. Periodic water changes are required to maintain water conditions suitable for culture and limit bacterial contamination. Larvae are fed a diet consisting of a mixture of microalgae. Diet composition and quantity may vary with larval size. Historically, larval cultures were reared under static conditions (i.e., a standing tank of water that is aerated, drained, cleaned, and refilled periodically), but recent developments have allowed for the use of flow-through systems (i.e., water and algae are continuously supplied to the culture tanks).

There is little information in published literature examining the economics of oyster hatcheries beyond the scope of using hatchery-produced oysters for culture practices. Lipschultz and Krantz (1978) developed a model using linear programming techniques in an effort to optimize hatchery production of cultchless oysters and SOS. Based on the analysis, labor was the biggest operating cost after initial infrastructure costs. Continuous operation of a hatchery, rather than several large pulses of production, increased production costs and was driven by labor inputs. Production of SOS, in comparison to cultchless oysters, reduced production costs by as much as 45% based on the model. Most private oyster hatcheries operate as part of a larger vertically-integrated business.

Intensive oyster culture employs mechanization, containerization, and the use of hatchery- produced oysters. For intensive culture, the type of gear used has been shown to

impact aspects of oyster growth and health, such as survival, shape, condition index, time to market-size, and even disease abundances (Mallet et al., 2013; Walton et al., 2013). Containerized gear types are generally of three varieties: containers on the water bottom, suspended in the water column, or just below the surface. In the Gulf of Mexico region, the predominant intensive methods suspend containers just below the water surface either with floating bags, cages, or an adjustable long-line system (ALS).

Adjustable long-line systems consist of oyster bags suspended by a cable that is in turn supported by pilings with a series of clips at various depths allowing the cable depth to be adjusted in the water, hence the name adjustable long-line system. If a grow-out location has sufficient tidal range, bags can be set at an intertidal height allowing for diurnal fouling control. If the intertidal zone is too shallow, manually raising the cable for aerial drying of the bags for fouling control may be required based on local fouling conditions. Oysters are generally stocked at a density of between 75 and 100 oysters per bag and remain in the bags for 10-18 months until they reach market size.

Maxwell and Supan (2010) performed an in-depth economic analysis on ALS in Louisiana operating in a proposed aquaculture park. They analyzed ALS operating under several scenarios consisting of varied fouling control regimes and harvesting either the entire crop once per year or small harvests weekly. Fouling control regimes largely controlled operating costs by driving up labor costs. Sensitivity analysis showed that the optimal production method consisted of using triploid oysters and setting bag depth in the intertidal zone to allow diurnal exposure for fouling control. There was little difference in operating costs between deploying seed oysters once or multiple times and between a single harvest and multiple harvests. The sensitivity analysis also suggested that an increase in oyster survival, when starting with at least 75%

survival, was inversely proportional to the break-even price of a 100-count box of market-size oysters. That is to say, a 10% increase in oyster survival to market-size coincided with approximately a 10% reduction in the break-even price of a 100-count box of oysters.

In Louisiana, floating cages are currently the most used intensive culture method for growing hatchery-produced oysters. Floating cage systems consist of wire shelves that hold extruded, plastic-mesh oyster bags fixed on two pontoons to suspend oysters just below the surface. Multiple cages are set in series, connected by paired, lateral lines tied along a common long-line anchored at each end to the seafloor. Cages are routinely flipped upside down for aerial drying for fouling control based on local conditions. Formal economic analysis of this culture system in the Gulf of Mexico has not been performed. In the Chesapeake Bay, USA, regional economic analyses of off-bottom oyster culture have been used, though not intended directly for floating cage systems. As with other intensive culture methods, beyond the initial investment costs, labor is likely the major driver of operating costs (Parker et al., 2013; Wieland, 2007).

6.2 Physical system description

In the Gulf of Mexico region, OysterGro® floating cages are primarily used (Figure 6.2). The cages are constructed of 12-gauge vinyl-coated 4.5 inch (11.4 cm) wire mesh, with two levels that can hold either four or six extruded-plastic, rectangular oyster bags, that measure roughly 46 cm wide x 89 cm long x 8 cm high each. Each cage has a door along the long axis to access the culture bags. Cages are attached with 8-gauge vinyl-coated wire to two air tight floats measuring 104 cm in length each. Floats have screw caps on each end to allow timely sinking to avoid damage from winter ice in the higher latitudes and tropical storms in the lower latitudes of North America. Floating cages are deployed in runs of 10-15 cages between two auger anchors.

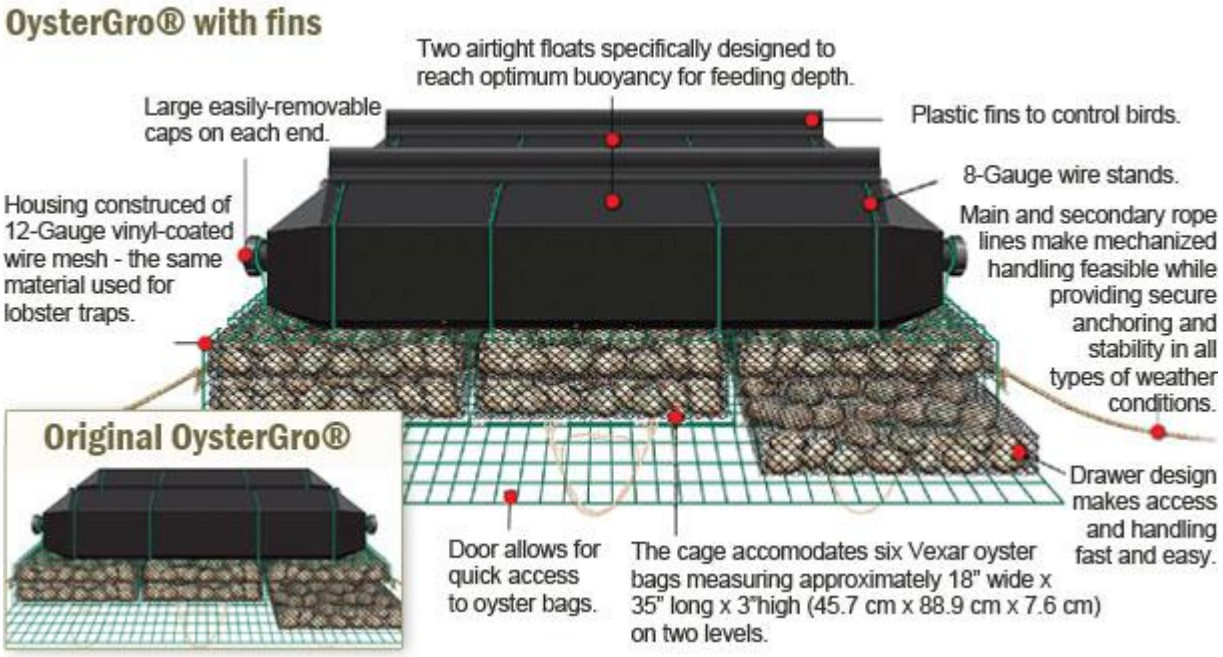


Figure 6.2. OysterGro® cage system manufacturer diagram (https://www.oystergro.com).

Each run of cages require 160 feet (48.8 m) of sinking 3/8 inch longline secured between two auger anchors (40 inch (101.6 cm) in length, 3/8 inch (0.95 cm) diameter, and 6 inch (15.2 cm) screw head). Cages are fixed to the longline by two bridles looped over rubber pucks attached to 3-6 foot (91.4-182.9 cm) lateral lines, which allows cages to be flipped for aerial drying without fouling the long-line. Runs of cages are installed in parallel with approximately 30 feet (9.1 m) between runs (Figure 6.3). Typical cage density is 100 cages per acre. The deployment site should be marked in a way that meets local regulations. Usually this is done by driving two-inch diameter PVC poles into the seafloor around the perimeter of the area that will contain the cages.

6.3 Operational plan

After installation, the operation of a floating cage oyster culture system begins with stocking with seed oysters. Seed is generally available from hatcheries for stocking into cages beginning in the spring and extending into fall. Depending on seed size, seed is deployed at approximately 225 oysters per bag (each cage holds either 4 or 6 bags). Initially, seed bags (1.5, 2, and 4 mm mesh) are used to house smaller sized seed oysters until the first sorting. Seed require weekly aerial drying to ensure that the small mesh size of seed bags do not foul. Cages are flipped so that the two pontoon floats are riding the surface of the water with oyster culture bags above the water surface. Aerial drying in the afternoon to avoid summer/fall heat is recommended, otherwise for 24 hours during the winter. Cages are left in this configuration overnight and flipped back into the feeding position in the morning so that the bags are below the pontoons just under the water surface.

Redistributing (splitting) seed will generally occur about one month after seed are initially stocked into cages. Timing of this, however, must be dictated by actual seed growth. To split seed, all seed bags are removed from cages and the seed is sorted by size.

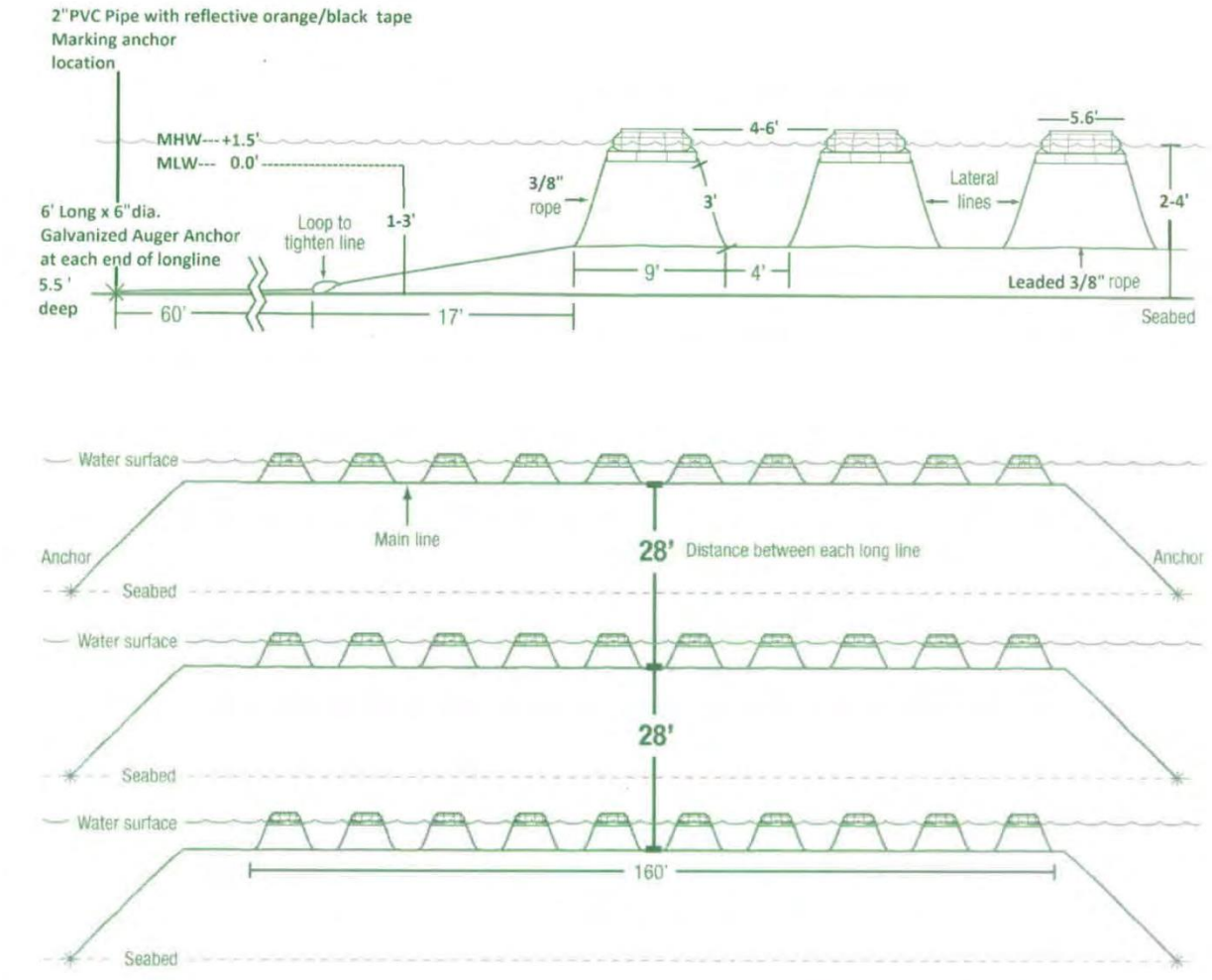


Figure 6.3. Floating cage deployment schematic depicting layout of anchors and individual cages (top) and multiple runs of cages (bottom).

Sorting can take place either on-site, using portable, stacked sorting trays of appropriate mesh sizes to separate different sizes or at remote facilities with mechanized sorting equipment (like the mechanized tumbler/sorter available for industry use at the LA Sea Grant Oyster Research Laboratory on Grand Isle, Figure 6.4). Oysters can then be restocked into larger mesh grow-out bags of appropriate mesh size for that grade of seed at the final density of approximately 225 oysters per bag and redeployed to the floating cages. Weekly aerial drying resumes until oysters have reached the desired harvest size.

Additional handling of the oysters may be required if fouling is extraordinarily bad, large size disparities within bags arise, or significant mortality is observed. Extreme fouling of gear will require simply swapping out the oyster bags with clean (pressure washed) bags while significant size variation within bags or high mortality may require additional sorting to have oysters of similar size in bags together and remove dead animals.

Harvesting oysters for sale involves removing bags from cages, counting, and packaging oysters as needed. Summer harvesting requires growers to meet state regulations for harvesting shellfish in warm months; usually requiring refrigeration within a period of time from removal from the water. After the first year, harvesting may occur more often and during the summer as needed.

6.4 Economic analysis and enterprise budget

The following budget and analyses are based on the production of 100-count boxes of oysters produced by 1 acre (0.4 hectare) of floating cages, using the typical 100 cages per acre cage density, to be sold for the half-shell market. This budget stops at the *farm gate*, and does not include marketing or transport of oysters beyond harvesting activities because the cost of these can vary widely among operators.

Figure 6.4. QuickTube Sorter manufactured by the Chesapeake Bay Oyster Company, available for use by local growers at the Louisiana Sea Grant Oyster Research Laboratory, Grand Isle, LA. Sorter consists of interchangeable rotating tubes with various hole sizes to sort seed and larger oysters. Oysters are pushed down the sloping rotating tube by water jets.

An enterprise budget is based on a simple model:

$$TNR = TR - TC \quad \text{(Equation 6.1)}$$

where *TNR* is the total net revenue (annualized), *TR* is total revenue, and *TC* is total costs. *TR* for this budget is assumed constant. Total revenue is defined as:

$$TR = P_u * U \quad \text{(Equation 6.2)}$$

where P_u is the price per unit and *U* is the number of units sold. Total cost is defined as:

$$TC = FC + VC \quad \text{(Equation 6.3)}$$

where *FC* is total annual capital (or fixed) costs and *VC* is total annual variable costs (Hansen and Mowen, 2006).

6.4.1 Capital costs

Capital costs are fixed expenditures that must be made upfront and are independent of production success. Capital costs are based on a 1 acre floating cage (specifically OysterGro®) oyster farm. Asset depreciation was estimated using straight-line depreciation over the estimated economic life of all assets (Table 6.1).

Capital costs can be critical to the success of an oyster farming operation. Floating cages (and associated installation items) are considered here as fixed capital costs. Having enough cages to maintain adequate oyster density in bags is of the utmost importance. It may be tempting for starting farmers to limit the number of cages purchased, but having adequate density will limit mortality and provide an environment for greater growth rates and is worth the initial investment (Honkoop and Bayne, 2002; Monteforte et al., 2005; Taylor et al., 1997). Other capital costs may not be necessary (e.g., boat, pressure washer, etc.), but are assumed to be part of the initial investment for this analysis (Table 6.1).

Table 6.1. Fixed costs for a 1 acre floating-cage oyster farm in the Gulf of Mexico. LDWF – Louisiana Department of Wildlife and Fisheries, AOC – alternative oyster culture, LADNR – Louisiana Department of Natural Resources, LADEQ – Louisiana Department of Environmental Quality.

Item	Unit	Number of units	Cost per unit (USD)	Total (USD)
Grow-out system (startup)				
OysterGro® 6-bag cage	Cage	100	130.00	13,000
Vexar oyster bag	Bag	600	5.00	3,000
Longline anchor	Anchor	20	25.00	500
3/8" Sinking Long Line	Feet	2,200	5.50	12,100
3/8" Floating Bridle Line	Feet	917	3.00	2,751
Rubber puck for bridle	Bridle	200	2.00	400
Floats (2 per long line run)	Float	20	15.00	300
Miscellaneous				
Vessel		1		20,000
Vessel maintenance	Per boat per year	1	500.00	0
Electric pot hauler		1		1,000
Metal flipping platform		1		500
Commercial grade pressure washer		1		1,000
Permitting, licensing, and insurance				
Commercial fishing license	Annual expense	1	55.00	55
Commercial vessel license	Annual expense	1	15.00	15
Commercial oyster harvest license	Annual expense	1	100.00	100
LDWF Alternative oyster culture	Startup cost	1		100
LDWF AOC annual permit fee	Per acre	1	2.00	0
AOC equipment and facility removal bond	Startup cost	1		1,500
LADNR Coastal use & U.S. Army Corps of Engineers	Startup cost	1		100
LADEQ Water quality certification	Startup cost	1		385
Vessel insurance	Annual expense	1		750
Commercial liability insurance (1 million USD coverage)	Annual expense	1	927.50	928
LLC registration	Annual expense	1		100
Depreciation expense				
OysterGro® 6-bag cage	Annual expense			1,857
Vexar oyster bag	Annual expense			429
Longline anchor	Annual expense			71
3/8" Sinking Long Line (ft.)	Annual expense			1,729
3/8" Floating Bridle Line (ft.)	Annual expense			393
Rubber puck for bridle	Annual expense			57
Floats (2 per long line run)	Annual expense			43
Vessel	Annual expense			2,857
Electric pot hauler	Annual expense			143
Metal flipping platform	Annual expense			71
Commercial grade pressure washer	Annual expense			143
Total fixed costs				**66,377**

Included in capital costs are application fees for permits from state and federal agencies: LDWF alternative oyster culture (AOC) permit, LDWF required AOC equipment and facility removal bond, LADNR and U.S. Army Corps of Engineers coastal use permits, and LADEQ water quality certification. Depreciation in this budget was estimated using the IRS *straight-line* method assuming no salvage value.

Grow-out system (startup) items were estimated from a local (New Orleans, LA) OysterGro® cage supplier. Vessel cost was estimated from the average cost of a 19-24 foot skiff-type vessel with motor for sale in Louisiana, Mississippi, and Alabama sourced from BoatTrader (http://www.boattrader.com, last accessed 01/01/2018). The electric pot hauler, metal flipping platform (local custom fabrication), and commercial grade pressure washer were estimated from local (southern LA) sources. Permits and licensing fees were quoted from each respective agency as of December 2017. Commercial liability insurance (1 million USD coverage policy) was quoted from the Bankers Insurance Group.

6.4.2 Variable costs

Variable costs, unlike capital (or fixed) costs, are impacted by whether or not the business is operating and the size and scale of the operation. Variable costs are also called operational costs or expenses (Table 6.2). Labor hours are based on a weekly aerial drying routine for fouling control and assuming that some harvesting activities will occur on these days and ten additional days to account for periods of high harvest rates. Total days of labor were estimates as follows: if weekly air-drying, that is 104 days of labor. If harvesting occurs on 10 days other than those 104 days designated for weekly drying, cages can be air-dried while harvesting, thus reducing the total days needed for air-drying cages from 104 to 94. Labor rates are based on the standard deckhand daily rate in Louisiana of 100 USD/day.

Table 6.2. Variable costs for a 1 acre floating-cage oyster farm in the Gulf of Mexico.

Item	Unit	Number of units	Cost per unit (USD)	Total (USD)
Seed	1,000 oysters	100	13.50	1,350
Labor				
Maintenance	Days	94	100.00	9,400
Harvesting	Days	10	100.00	1,000
Boat fuel	Gallons per year	1,040	2.50	2,600
Misc. equipment upkeep	Annual expense	1	500.00	500
Total				**14,850**

Seed price is an important input to the farm budget and may influence starting farm size. Seed in this budget is based on the average price of 2 mm seed in the Gulf of Mexico sold by the thousand following industry convention. Boat fuel is listed as a variable cost because the amount of trips and distance to farms may vary based on a number of factors (drop-off location for harvests, number of harvests, number of trips required for gear maintenance, etc.). Fuel consumption is based on an assumed 10 miles per gallon with a total traveled distance of 10 miles per working day. Miscellaneous upkeep is listed under variable costs and consists of supply replacement, minor repair work, bushel baskets, etc. These items require replacing when worn out and the amount of each supply may vary with production scale.

6.4.3 Enterprise budget

Enterprise budgets are used to estimate costs and revenues for the production of a product (in this case oyster production). This type of budget is used to gain an understanding of a production model in order to explain the relationship between costs, revenue, and ultimately profit. Enterprise budgets achieve this by assigns costs as fixed (section 6.4.1) or variable (section 6.4.2) for a specific production model. Allocating costs in this manner allows for a variety of analyses to better understand a production model and estimate important parameters such as break-even points, expected profit for given production, and how man units needed to achieve a given target profit.

Enterprise budgets, as with any model, are limited by the parameters that make up the model. Accuracy of inputs (e.g., fixed and variable costs, price per unit, labor needs, etc.) are crucial to producing a representative budget. Enterprise budgets are also limited in scope to production methods described by the budget model. In the case of a floating cage oyster farm enterprise budget, it is not applicable to other oyster culturing methods, like on-bottom spat-on-

shell grow-out operations. Seed price was estimated from the current triploid disease-resistant oyster seed cost (per 1,000 seed oysters) in the Gulf of Mexico. Fuel usage is difficult to estimate for a generic budget, especially in the Gulf of Mexico where oyster farm locations may be in remote locations. This budget estimates one-gallon of fuel per day operating. This may be drastically underestimated for locations that are in remote areas and require significant travel time to reach a farm.

An enterprise budget for a one acre floating cage oyster farm is detailed in Table 6.3. Default parameter values listed in the table were chosen as conservative estimates, but can be altered to more accurately represent operational procedures of a given farm. Costs are projected for five years. Production scale, costs, and revenues were assumed constant across years. These values may change, when considering longer-term operations. Based on the conservative parameter values used in Table 6.3, profit is achieved after year 5.

6.4.4 Volume-cost analysis

A break even analysis is an economic analysis undertaken with the goal of identifying the point at which total cost (i.e., variable and fixed costs) equal total revenues, which is the break-even point. In other words, when the total net revenue is zero (Equation 6.1). A volume-cost analysis (VCA) is simply a break-even analysis where costs (both fixed and variable), production unit volume, and production unit price are variables manipulated to show their effects on profit. Assumptions of a VCA are: 1) costs and revenues are linear and ignore scaling efficiencies, 2) costs are affected only by changes in production activity, 3) all produced units are sold, and 4) sales price, variable costs, and fixed costs are constant over the range of analysis (Hansen and Mowen, 2006).

Table 6.3. Five-year 1-acre floating oyster cage enterprise budget with default parameter values. Red values indicate negative values.

Assumptions

Item	Unit	Number of units	Year 1	Year 2	Year 3	Year 4	Year 5
Farm size	Acre	1					
Cage flipping schedule	Week	1					
Price per unit	Oyster	0.50					
Survival from seed to harvest	Percent alive		75%	75%	75%	75%	75%
Harvest							
Current year crop	Percent harvested		50%	50%	50%	50%	50%
Previous year crop	Percent harvested		0%	50%	50%	50%	50%

				Total (USD)				
Item	Unit	Number of units	Cost per unit (USD)	Year 1	Year 2	Year 3	Year 4	Year 5
1. Fixed costs								
Grow-out system (startup)								
OysterGro® 6-bag cage	Cage	100	130.00	13,000	0	0	0	0
Vexar oyster bag	Bag	600	5.00	3,000	0	0	0	0
Longline anchor	Anchor	20	25.00	500	0	0	0	0
3/8" Sinking Long Line	Feet	2,200	5.50	12,100	0	0	0	0
3/8" Floating Bridle Line	Feet	917	3.00	2,751	0	0	0	0
Rubber puck for bridle	Bridle	200	2.00	400	0	0	0	0
Floats (2 per long line run)	Float	20	15.00	300	0	0	0	0
Miscellaneous								
Vessel		1		20,000	0	0	0	0
Vessel maintenance	Per boat per year	1	500.00	500	500	500	500	500

(Table 6.3 continued)

Electric pot hauler		1		1,000	0	0	0	0
Metal flipping platform		1		500	0	0	0	0
Commercial grade pressure washer		1		1,000	0	0	0	0
Permitting, licensing, and insurance								
Commercial fishing license	Annual expense	1	55.00	55	55	55	55	55
Commercial vessel license	Annual expense	1	15.00	15	15	15	15	15
Commercial oyster harvest license	Annual expense	1	100.00	100	100	100	100	100
LDWF Alternative oyster culture	Startup cost	1		100	0	0	0	0
LDWF AOC annual permit fee	Per acre	1	2.00	2	2	2	2	2
AOC equipment and facility removal bond	Startup cost	1		1,500	0	0	0	0
LADNR Coastal use & U.S. Army Corps of Engineers	Startup cost	1		100	0	0	0	0
LADEQ Water quality certification	Startup cost	1		385	0	0	0	0
Vessel insurance	Annual expense	1		750	750	750	750	750
Commercial liability insurance (1 million USD coverage)	Annual expense	1	927.50	928	928	928	928	928
LLC registration	Annual expense	1		100	100	100	100	100
Depreciation expense								
OysterGro® 6-bag cage	Annual expense			1,857	1,857	1,857	1,857	1,857
Vexar oyster bag	Annual expense			429	429	429	429	429
Longline anchor	Annual expense			71	71	71	71	71
3/8" Sinking Long Line (ft.)	Annual expense			1,729	1,729	1,729	1,729	1,729
3/8" Floating Bridle Line (ft.)	Annual expense			393	393	393	393	393
Rubber puck for bridle	Annual expense			57	57	57	57	57
Floats (2 per long line run)	Annual expense			43	43	43	43	43

(Table 6.3 continued)

Vessel	Annual expense			2,857	2,857	2,857	2,857	2,857
Electric pot hauler	Annual expense			143	143	143	143	143
Metal flipping platform	Annual expense			71	71	71	71	71
Commercial grade pressure washer	Annual expense			143	143	143	143	143
Total fixed costs				**66,879**	**10,243**	**10,243**	**10,243**	**10,243**
2. Variable costs								
Seed	1,000 oysters	100	13.50	1,350	1,350	1,350	1,350	1,350
Labor								
Maintenance	Days	94	100.00	9,400	9,400	9,400	9,400	9,400
Harvesting	Days	10	100.00	1,000	1,000	1,000	1,000	1,000
Boat fuel	Gallons per year	1,040	2.50	2,600	2,600	2,600	2,600	2,600
Misc. equipment upkeep	Annual expense	1	500.00	500	500	500	500	500
Total variable costs				**14,850**	**14,850**	**14,850**	**14,850**	**14,850**
Total annual expenses				**81,729**	**25,093**	**25,093**	**25,093**	**25,093**
3. Revenue								
Seed	1,000 oysters	0	13.50					
Oysters	Per oyster		0.50	18,750	37,500	37,500	37,500	37,500
Total revenue				**18,750**	**37,500**	**37,500**	**37,500**	**37,500**
Total net revenue				**(62,979)**	**12,408**	**12,408**	**12,408**	**12,408**
Cumulative revenue				**(62,979)**	**(50,571)**	**(38,164)**	**(25,756)**	**(13,349)**

VCA is best shown graphically by plotting total costs and total revenues against units produced using Equation 6.2 and the following:

$$TC = FC + V_u * U \quad \text{(Equation 6.4)}$$

where *TC* is total costs, *FC* is total fixed costs, V_u is variable costs per unit, and *U* is units produced (units are equivalent to oysters). These lines are then plotted and the intersection indicates the break-even point in units produced. VCA allows manipulation of these variables to estimate the break-even point for various prices per unit and differing variable costs, which in the case of floating-cage oyster culture are dominated by handling methods. For example, flipping cages weekly vs. biweekly will alter the variable cost per unit produced.

The present VCA shows two fouling control scenarios (weekly and biweekly aerial drying) and three unit-price scenarios (0.50, 0.75, and 1.00 USD per oyster) (Figure 6.5). Unit production at a given unit price (and associated variable costs) to attain a desired target income can be estimated by the following:

$$TIS = \frac{FC+TI}{UC} \quad \text{(Equation 6.5)}$$

where *TIS* is target income sales (in units), *FC* is fixed costs, *TI* is target income (in USD), and *UC* is the unit contribution margin. Unit contribution is estimated as the difference of unit price and variable cost per unit:

$$UC = P - V_u \quad \text{(Equation 6.6)}$$

where *UC* is the unit contribution, *P* is the unit price, and V_u is the variable cost per unit. The VCA depicted in Figure 6.5 shows estimated target income sales (in units) for a target income of 50,000 USD.

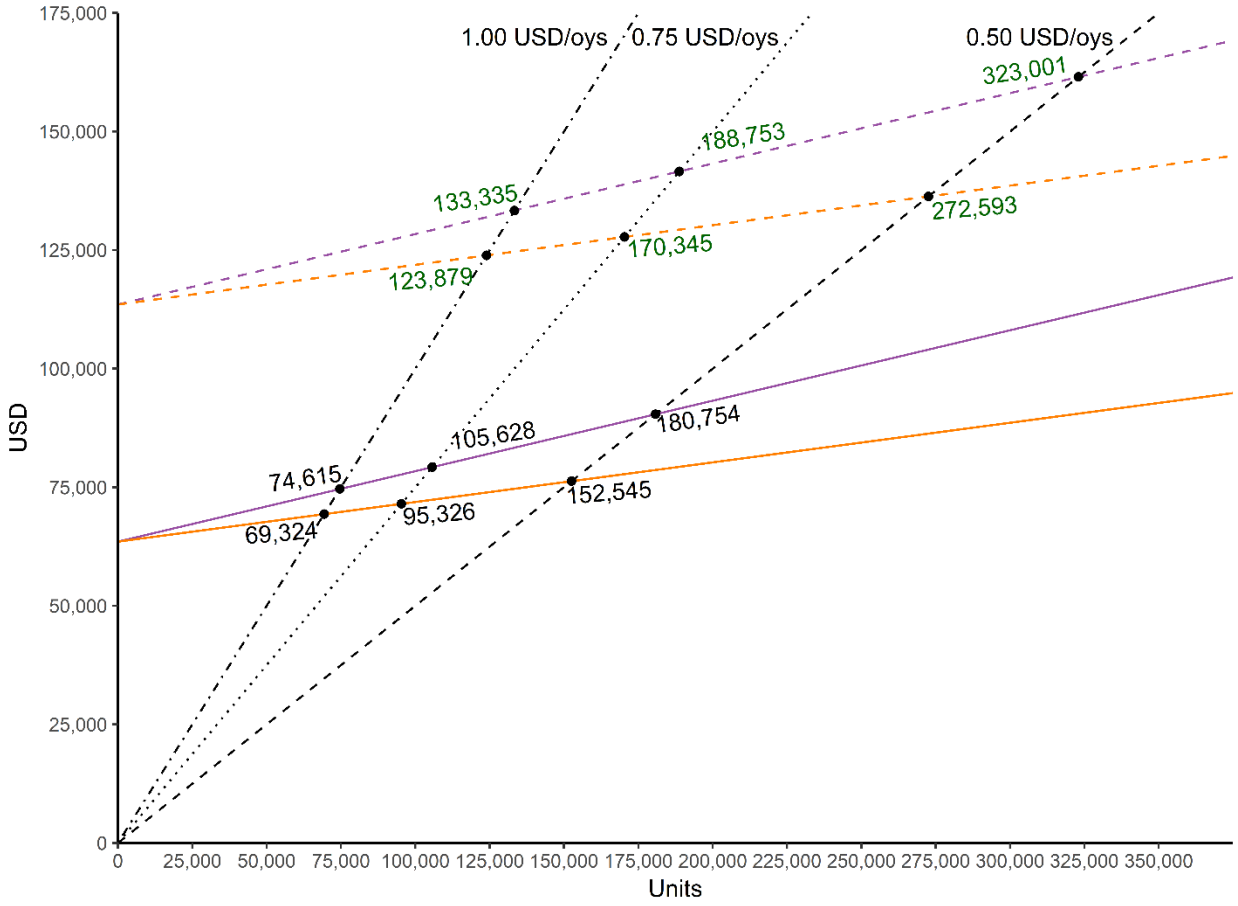


Figure 6.5. Volume-cost analysis of floating-cage oyster culture representing several combinations of unit price (0.50 USD – dashed line; 0.75 USD – dotted line; 1.00 USD – dashed and dotted line) and aerial drying regime (weekly drying – purple; biweekly drying – orange). Dashed purple and orange lines indicate target profit (50,000 USD). Unit production amounts required to break even are in black. Unit production amounts required to achieve target profit are in green.

6.4.5 Sensitivity analysis

Sensitivity analyses are used to estimate the relative contribution of all of the model parameters toward the total net revenue. The VCA above (see 6.4.4) shows only units produced to break even or to achieve a desired target income. While a VCA can incorporate various scenarios (like weekly vs. biweekly aerial drying), it does not quantify the relative contribution of the changes from one scenario to the next. Cage flipping regime (weekly, biweekly, and triweekly), price per unit (0.50, 0.75, and 1.00 USD), survival rate (0.75, 0.85 and 0.95), and harvest rate (ratio of a given crop harvested the same year: 0.50, 0.75, and 1) were used. Default values for parameters are: weekly flipping, 0.50 USD per oyster, 0.75 survival rate, and 0.50 harvest rate. These values were chosen because they are conservative estimates. Harvest rate changes, for this analysis will only influence total net revenue for the first year of operation. This is because the oysters not harvested in Year 1 may be harvested in Year 2. For example, if 75% of oysters are harvested in Year 1, the remaining 25% of Year 1 oysters will be harvested in Year 2 with Year 2's 75% harvest rate oysters (assuming crop size and survival are constant).

The price per oyster has the greatest influence on the total net revenue of a one-acre floating cage oyster farm in the Gulf of Mexico (Table 6.4). Increasing the price per unit from the default 0.50 USD per oyster to 0.75 USD per oyster increased total net revenue by 138%; increasing from 0.50 USD to 1.00 USD increased total net revenue by 276%. The cage flipping routine is another area where a farmer may affect change on the total net revenue. Changing from weekly aerial drying to biweekly increased estimated total net revenue by 48%. By spreading out how often cages are flipped, farmers can reduce labor and fuel costs, which are significant sources of variable costs (Table 6.2).

Table 6.4. Sensitivity analysis 1 acre floating-cage oyster farm in the Gulf of Mexico. Shown are the percent changes in total net revenue when a given parameter is changed from the default values: weekly cage flipping, 0.50 USD per unit, 0.75 survival rate, and 0.50 harvest rate.

Parameter	**Percent change in total net revenue**
Cage flipping	
Biweekly	48%
Triweekly	64%
Price per unit	
0.75 USD	138%
1.00 USD	276%
Survival rate	
0.85	37%
0.95	74%
Harvest rate	
0.75	16%
1.00	31%

The cost of reducing cage flipping frequency may negatively impact gear handling ease as well as oyster growth efficiency if cages become sufficiently fouled as to reduce water flow and thus food availability for oysters. The parameter with the next greatest effect on total net revenue was survival rate. A 10% increase in survival, from 0.75 to 0.85, can increase net revenue by 37%. Survival can be manipulated by ensuring that gear fouling is adequately controlled allowing for optimal water exchange through the cages and stocking density is appropriate. Harvest rate represents how many oysters were harvested and sold. It is not uncommon, regardless of demand, for a crop to have a small proportion that is not large enough to be sold as a market oyster (i.e., *runts*). Another aspect of harvest rate, independent of oyster growth, is that it may be beneficial to withhold from sale a portion of a given crop so that a farmer has marketable oysters on hand at any given time.

6.4.6 Analyzing uncertainty using Monte Carlo simulation

Scenario-based analyses (like the volume-cost and sensitivity analyses above) estimate revenue to importance of parameters under set conditions, or scenarios. Monte Carlo (MC) simulation, by contrast, is a resampling technique that simulates outputs for a given model based on predefined probability distributions for each input variable. MC simulations, therefore, simulate all possible combinations of input variable values (based on their predefined likelihood). Defining probability distributions for important model variables allows for approximating all conceivable combinations of input variables in proportion to their likelihood by simulating large numbers of outcomes. The output of MC is a probability distribution of outcomes simulated from a large number of combinations of the predefined variable distributions.

Hertz (1979) first described the use of MC simulation in risk analysis of capital investments. The general process was 1) define probability values for important model variables, 2) simulate, at random, values for each variable based on the chance of obtaining that value from the defined probability distributions, 3) determine revenue based on each simulated combination of variable values, and 4) repeat the simulation process many times to give a distribution of likely outcomes. Based on this process, the limiting factors for model accuracy are the selection of important input variables and realistically defining likely probability distributions for each variable.

One of the main difficulties in MC simulations is estimating density functions for model variables. One approach that is practical, requires minimal information, and easy for non-experts to determine is a triangular probability distribution (Figure 6.6). Variables needed are likely minimum, likely maximum, and most likely values, which are easily estimated generally (Geuze and Jolly, 1980; Kotz and van Dorp, 2004). The triangular probability density function is defined as the following:

$$P(x) = \begin{cases} 0 & for\ x < a, \\ \frac{2(x-a)}{(b-a)(m-a)} & for\ a \leq x < m, \\ \frac{2}{b-a} & for\ x = m, \\ \frac{2(b-x)}{(b-a)(b-m)} & for\ m < x \leq b, \\ 0 & for\ b < x \end{cases} \qquad \text{(Equation 6.7)}$$

where *a* is the minimum parameter value, *b* is the maximum value, and *m* is the most likely value.

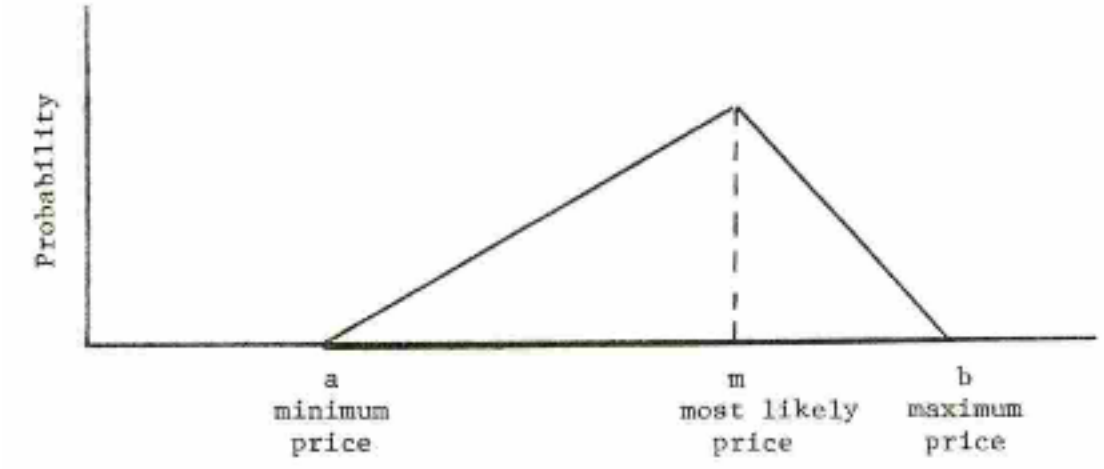


Figure 6.6. A hypothetical triangular distribution constructed by defining minimum (a), maximum (b), and most likely price (m). From Geuze and Jolly (1980).

To assess potential risk in off-bottom oyster cage culture triangle distributions for several of the variable costs were defined for MC simulation. Specifically, probability distributions for seed cost, survival to harvest, distance required to travel by vessel, vessel fuel cost, and price per oyster were defined (Table 6.5). Seed costs were estimated based on 2 mm seed prices from commercial nurseries from the southeastern Atlantic and northern Gulf of Mexico coasts. Survival to harvest ranged from very conservative survival rate (0.6) to high survival (0.9). Distance required to travel by vessel was based on current travel distances for oyster farmers utilizing the Grand Isle Oyster Farming Zone, though trip distance is highly specific for any farmer. The fuel price distribution was defined from the minimum, median, and maximum fuel prices reported on the Garmin Active Captain Community for Louisiana, Mississippi, and Alabama. Distributions were provided using the R package 'triangle' (Carnell, 2017).

The use of MC simulation in enterprise budgets highlights uncertainty in revenues exposing possible risk in the success of an endeavor. The present MC simulation shows the uncertainty in revenue for Year 1 of the enterprise budget outlined in Table 6.3. To generate revenue values, values from each variable costs' triangle distributions (Table 6.5) were randomly chosen in accordance with their probability. For each of 10,000 simulations, one randomly generated value from each variable was added with the fixed costs (Table 6.1). Each of the 10,000 simulations were then combined to produce a probability distribution of Year 1 net revenue (Figure 6.7). MC simulations were performed for each of the fouling control regimes: weekly, bi-weekly, and tri-weekly aerial drying. Year 1 net revenues ranged from -45,641 USD to 10,251 USD for weekly aerial drying, -43,208 USD to 16,667 USD for bi-weekly aerial drying, and -42,816 USD to 18,853 USD for tri-weekly aerial drying.

Table 6.5. Minimum (a), maximum (b), and most likely prices (m) used to define triangular distributions for Monte Carlo simulation to assess risk in the enterprise budget for off-bottom oyster culture.

Variables	Minimum price (a)	Maximum price (b)	Most likely price (m)
Seed cost	12.00	13.50	13.50
Survival to harvest	0.60	0.90	0.75
Trip distance	1.00	3.65	2.65
Fuel cost	2.60	3.90	3.60
Unit price	0.50	1.00	1.00

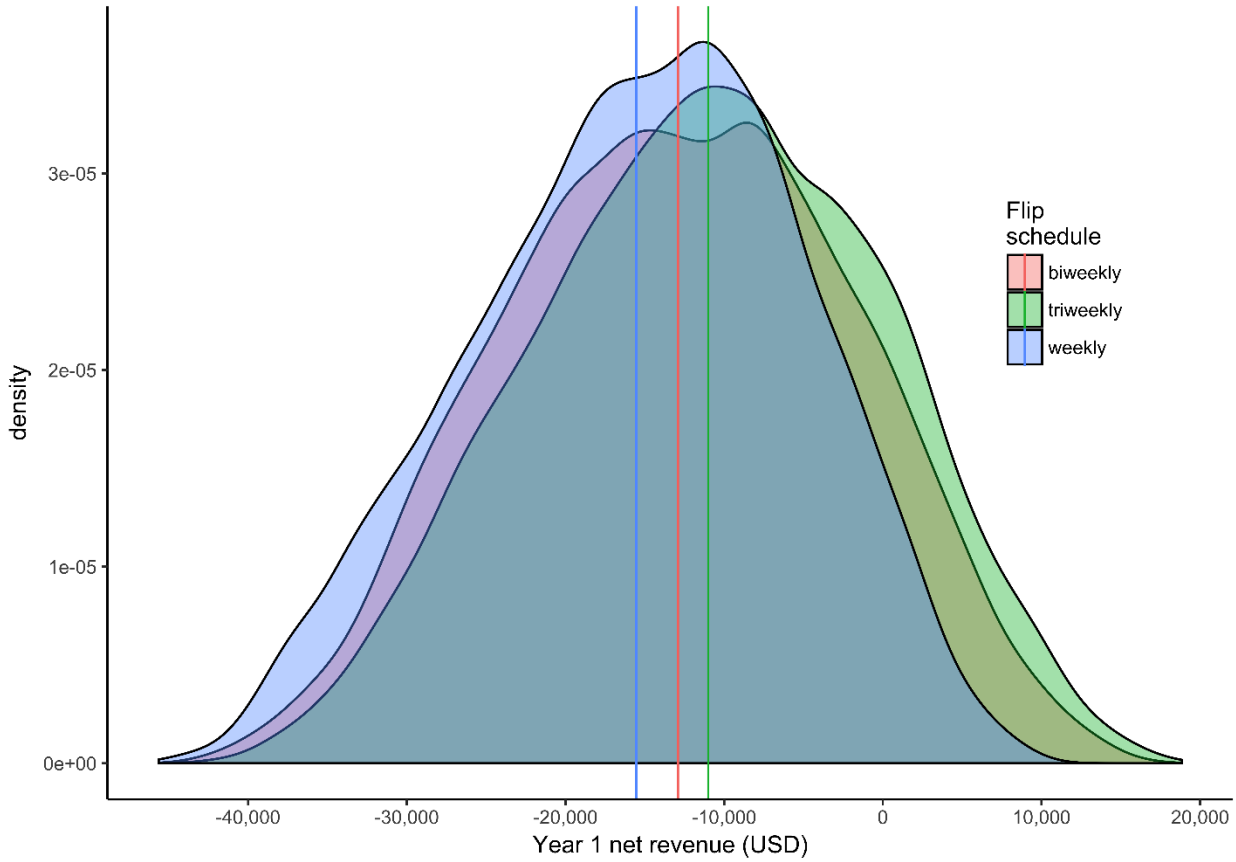


Figure 6.7. Probability distributions of MC simulations (n = 10,000) for Year 1 net revenue (USD) of an off-bottom floating cage oyster farm using three fouling control regimes: weekly, biweekly, and triweekly aerial drying.

Weekly aerial drying had the lowest mean net revenue for Year 1 at -15,545 USD, followed by bi-weekly at -12,913 USD, and tri-weekly aerial drying had the greatest mean Year 1 net revenue, -11,003 USD.

6.4.7 Net present value

Net present value (NPV) is a measure of the value of an investment over a given time period; in this case that investment is an off-bottom oyster farm. NPV is estimated by subtracting initial investment costs from discounted future cash flows (Equation 6.8).

$$NPV = \sum_{t=0}^{N} \frac{C_t}{(1+r)^t} - C_0 \qquad \text{(Equation 6.8)}$$

where *NPV* is the net present value, *t* is the time period, *N* is the total number of time periods, C_t is cash flow during period *t*, C_0 is the initial investment cost, and *r* is the discount rate. The NPV estimate discounts future cash flow to account for the time value of money: there is a greater benefit of acquiring money now rather than later. A NPV of zero indicates that the initial investment was repaid over the total of time periods (*N*) and a return on the initial investment at the predefined discount rate. A positive NPV indicates greater rate of return, while negative a lower rate.

The outputs of the MC simulation of Section 6.4.6 were used to estimate NPV. By using the MC simulation outputs, a distribution of likely NPVs was estimated for each of the three aerial-drying regimes: weekly, biweekly, and triweekly (Figure 6.8). All aerial-drying regimes had positive NPVs over a five-year period. NPVs were, for weekly aerial drying, 120,608 USD, 133,133 USD for biweekly, and 141,318 USD for triweekly drying.

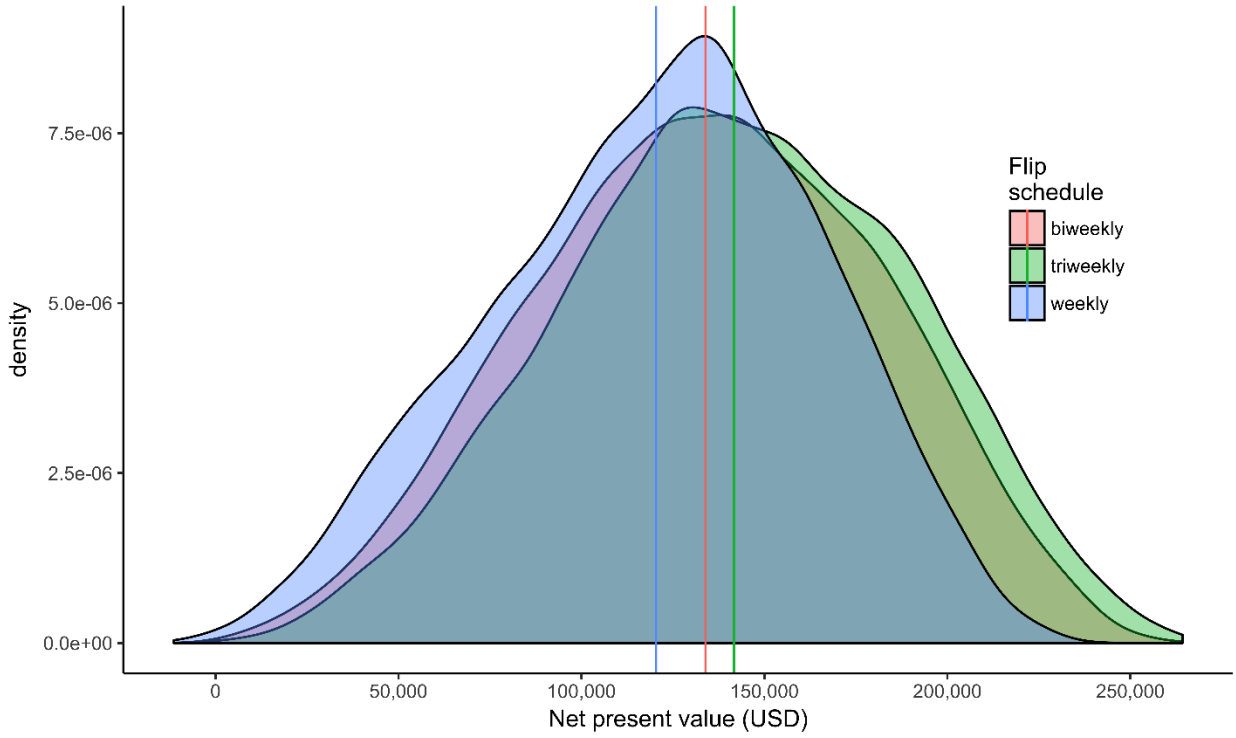


Figure 6.8. Probability distributions of MC simulations (n = 10,000) of a five-year net present value (USD) for an off-bottom floating cage oyster farm using three fouling control regimes: weekly, biweekly, and triweekly aerial drying.

6.5 Discussion

The success of floating cage oyster farms is driven by a variety of factors. Farmers can control some of these, such as stocking density, gear maintenance, harvest schedule. Other factors are outside farmer control: disease, theft, mortality events, unavailability of buyers, etc. Enterprise budgets and subsequent analyses that such budgets allow offer insight into how the factors farmers *can* control influence production and ultimately profit. Initial investment costs account for over 80% of the first-year costs. If a farmer is able to reduce these initial investment costs (e.g., not having to purchase a vessel), they can significantly reduce the time to profit. In addition to farmer inputs, consistent seed supply is imperative to a successful off-bottom oyster culture operation. Disruptions in seed supply lead to a disruption in oyster production and delay revenues.

The MC simulation presented in Section 6.4.6 illustrates the large range of possible net revenue outcomes resulting from the combination of farmer-controlled inputs and farmer-independent inputs that play critical roles in an off-bottom oyster farm. The MC simulation results show that a reduction of the high initial investment costs could result in breaking even during Year 1. A 23 percent reduction of initial investment costs is enough to put the most likely net revenue for Year 1 at a break-even value under the weekly aerial drying practice. Smaller reductions would be necessary if anti-fouling practices were altered to less frequent aerial drying.

The use of the MC simulation outputs to estimate NPVs over the range of possible revenue values showed that, regardless of aerial-drying regime, small-scale off-bottom oyster farms are likely to provide a return on the initial investment. The large range of NPVs is not unexpected given the large variation in some of the original input variable that generated the MC simulation data. Seven of the 10,000 estimated weekly aerial-drying NPVs were less than zero

indicating a possible poor investment. Negative NPV does not necessarily indicate a loss over the duration period, but rather that a return on the initial investment was less than the discount rate. Given that only 0.07% of the simulations resulted in negative NPVs, it is not a likely outcome.

This budget was designed as an aid for new floating cage oyster farm operations to accurately forecast costs and expected revenue. Many new farm operations may not have the capital required for the initial investment, and this budget can serve as a tool to help secure a loan to assist in the investment. As such, an electronic version of this budget, with outputs of the VCA, will be made accessible to the public through Louisiana Sea Grant. This will be in the form of an Excel workbook with accompanying instructions. The aim is to make budgeting as easy as possible so farmers can focus on securing loans, optimizing culture procedures, and maximizing profits.

Chapter 7
Conclusion

The purpose of this dissertation was to help address the commercialization of triploid *C. virginica* production and develop tetraploid *C. virginica* broodstock in Louisiana. Specifically, this dissertation addressed triploid commercialization and tetraploid broodstock development by: 1) developing regional breeding plans for diploid and tetraploid *C. virginica* targeted at the Gulf Coastal Region, 2) assessing the effect of chromosome set manipulation on gonadal development in diploid, triploid, and tetraploid *C. virginica*, 3) quantifying the variation of commercially important hatchery traits in triploid *C. virginica* larvae from different tetraploid parents, 4) developing an up-to-date remote setting manual for the Gulf Coastal Region for industry use, and 5) performing a basic economic analysis to produce an enterprise budget for floating cage oyster farming in the Gulf Coastal Region.

Oyster breeding programs have been established across the globe for a variety of commercially important species. A small sampling of such programs consists of: for *C. virginica* – Aquaculture Genetics and Breeding Technologies Center, VIMS; University of Maine; University of Rutgers, Haskin Shellfish Laboratory; for *C. gigas* – Molluscan Broodstock Program (Oregon State University); The Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER), France; several industry lead programs for *C. gigas* and *S. glomerata* under the guidance of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia; Hong Kong University for both *C. gigas* and *C. ariakensis*. While this list is not exhaustive, it illustrates the diversity of oyster breeding location and target species. These programs were created in response to major threats to commercial oyster production; these include disease, overfishing, and habitat loss. As breeding programs become established and the

original threat is mitigated, they often turn to improving other economically important traits (e.g., growth, meat yield, shell shape, etc.).

The oyster fishery in Louisiana is, perhaps, unique in that the wild oyster fishery continues to support the Gulf of Mexico as the leading region in U.S. oyster landings. Oyster landings and the fishery remain large, but over the past several decades, oyster seed availability from public areas has dropped to a 30-year low (Figure 6.1). The Louisiana oyster fishery is driven by transplanting wild seed to more productive waters allowing farmers to improve growth and flavor of oysters destined for market (MacKenzie *et al*., 1997; Supan, 2002). Relying solely on natural recruitment for seed supply means that oyster growers are bound to the rise and fall of wild oyster populations to maintain productivity on their private leases. Decreases in oyster populations on public areas means fewer seed oysters are available to farmers for transplanting to private leases, thus reducing their productivity. According to the Louisiana Department of Wildlife and Fisheries, as reported in the 2016 Louisiana State University AgCenter 2016 Louisiana Summary, oyster sales in Louisiana increased from $28.5 million in 2005 to $68.1 million in 2015. The oyster fishery in Louisiana continues to support a large number of fishermen and oyster landings.

It is for these reasons (large fishery supporting high landings) that Louisiana, and the Gulf of Mexico, are in an almost unique position to exploit selective breeding strategies to improve oysters while getting ahead of catastrophic failures in the fishery. In recent history, a series of events have contributed, at least in part, to a threatening of the traditional livelihoods of coastal Louisianans: namely a series of major hurricanes (Hurricanes Katrina, Gustav, Ike, and Isaac) and manmade events (e.g., the Deepwater Horizon Oil Spill and freshwater diversions). These events and the decline of seed-oyster populations in Louisiana serve as warnings of further

potential declines for the oyster fishery and reinforce the necessity of an oyster breeding program to mitigate future problems.

The lack of a catastrophic failure of the oyster fishery in Louisiana poses the problem of just what traits to select for to prevent such a failure. However, it is possible to employ breeding strategies that have worked well in other areas of the world in a more general way to hedge bets for the future. To this end, I designed two breeding programs for oysters in the Gulf of Mexico Region: a diploid oyster breeding program and a tetraploid oyster breeding program. These programs are complimentary to each other and work to improve aspects of growth and survival (both economically important traits). For diploids, the breeding program will seek to exploit genotype-by-environment interactions to create breeding lines for the varied environments oyster farmers may choose to utilize in the region. For tetraploids, the breeding program will first investigate combining abilities of tetraploid populations to determine if any effects inherited from the tetraploid parent are specific to a parental population or if tetraploids simply affect triploids equally.

Prior to achieving lofty breeding goals, a better understanding of the reproductive development of diploid, triploid, and tetraploid oysters was required. Understanding reproductive development of these three ploidy states is important not only for timing commercial spawns accurately (e.g., tetraploids and diploids for triploid production), but also to introduce new genetic material into tetraploid breeding lines as tetraploids are made from obtaining eggs from triploids (Stanley et al., 1981). Prior to this dissertation, various combinations of ploidy state were compared, but not all three (diploid, triploid, and tetraploid) concurrently.

Using diploid oysters as the standard for normal reproductive development, triploids were, in fact, abnormal. The majority of triploids observed (76%) exhibited abnormal and retarded gonadal development; abnormally developing triploids were classified as β triploids. This approximately 3:1 ratio of abnormal gonadal development to normal gonadal development was observed for *C. gigas* (Jouaux et al., 2010). The trend of 3:1 abnormally developing triploids to normally developing triploids breaks down in the literature when expanding to studies that include chemically-induced triploids. This study (and the Jouaux et al., 2010 study) used triploids from tetraploid-diploid matings rather than chemically-induced triploids. Tetraploid, by contrast, followed diploid gonadal development with the only exception being a temporal delay. I hypothesize this delay is either due to an increased metabolic cost of tetraploid oyster gamete production or due to the geographic origin of the founder population from which these tetraploids were created.

The better understanding of diploid, triploid, and tetraploid gonadal development in the Gulf of Mexico Region will undoubtedly improve conditioning regimes for commercial triploid oyster production in the region. Beyond making conditioning more efficient, these findings have uncovered new questions about the effects of chromosome set manipulation on reproductive development. Future research questions will address whether the proportion of β triploids is heritable, what mechanisms drive the locking behavior observed in β triploids, and if the temporal delay of tetraploid gonadal development is simply a metabolic cost issue.

Oyster hatchery operations carry large expenses in labor and infrastructure and it is for these reasons that all efforts to maximize hatchery production should be explored. Chapter 4 of this dissertation began investigating possible pathways for improving triploid oyster production inside the hatchery by exploring potential larval traits that can be exploited through selective

breeding. The four triploid brood types revealed that the tetraploid parent contributes to hatching success rate, survival to pediveliger harvest, time to harvest, and duration of harvest. These are all traits that can affect the profitability and efficiency of a hatchery operation and that are novel routes of triploid improvement. The diploid parent role in triploid larval performance follows trends observed in important adult oyster traits furthering the possibilities of ways that triploid oysters may be improved upon (Callam et al., 2016; Hand et al., 2004).

The work presented in this dissertation was undertaken in conjunction with activities at the Louisiana Sea Grant Oyster Research Laboratory. As a Sea Grant affiliated laboratory, work conducted therein should fall under at least one of the three key services Sea Grant institutions are tasked with providing: education, research, and extension services. The educational component is outside the scope of a dissertation, but the research component was met with the research presented above. The final two chapters of this dissertation address, in part, an extension component designed to transfer knowledge and technologies to practical end users.

Inherent in the creation of Land Grant institutions, and later Sea Grant programs, is the value of the agrarian, or maritime, tradition of small family or community run farms/business. Research, following this tradition, sought to enhance small business operations. As researchers began to incorporate economic analyses, technologies and techniques developed sought to maximize economic value per unit effort. This often lead to larger operations and cooperatives that could take advantage of scaling effects. Stakeholders that adopt outputs from Land and Sea Grant work, therefore, increase in size, and are the users that are likely actively engaging with Land and Sea Grant researchers. Since these are the people engaging researchers, research topics can then be influenced by these very users as they are likely providing feedback. Paarlberg (1981) argues that this creates a situation where Land and Sea Grant extension and research is

based on topics derived from these larger constituent groups and when a small family farm or fisherman comes and sees the work that may not be directly applicable to their operations they would be less likely to continue engaging with Land and Sea Grant personnel. This cycle creates outputs from Land and Sea Grant institutions that are biased toward larger more engaging constituents. In an attempt to combat this programmatic inertia, the final two chapters of this dissertation are targeted at small scale operations. Chapter 5, "Using Remote Setting to Produce Seed Oysters in Louisiana and the Gulf Coastal Region," was written in lay terms and for a scale of either a small existing oyster farming operation or a small start-up farming operation. The budget and accompanying economic analysis presented in Chapter 6 are at scales recommended for start-up farmers to get experience operating an oyster farm.

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Vita

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