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Integrating Germplasm Repositories into Oyster Aquaculture Systems at the Pathway, Center, and Network Levels

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INTEGRATING GERMPLASM REPOSITORIES INTO OYSTER AQUACULTURE SYSTEMS AT THE PATHWAY, CENTER, AND NETWORK LEVELS

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Abstract

Germplasm repositories offer many potential benefits to commercial farm operations, including preserving genetic resources and increasing production capabilities. Aquaculture industries have yet to take advantage of repository storage despite decades of research evaluating cryopreservation protocols. Eastern oysters (*Crassostrea virginica*), for example, are a useful research species when addressing this problem. Oysters are an economically important aquaculture species and a high-throughput cryopreservation method for oysters already exists. To address the gap between protocol research and commercial application a different approach is necessary that can analyze the requirements of repository development at multiple organizational levels. The goal of this dissertation was to advance repository development in commercial aquaculture species by integrating repository activities into oyster aquaculture systems at the Pathway, Center, and Network Levels. Beginning at the smallest scale, the Pathway Level focuses on the steps of a particular process (e.g., cryopreservation) that takes place in a repository facility. The Center Level encompasses multiple processes within a repository facility and its community. At the largest scale, the Network Level outlines the relationships of multiple repositories and their surrounding communities. The chapters of this dissertation provided tools for repository development at each organizational level using concepts from different fields of study. Chapters 2 and 3 operated at the Pathway Level and used industrial engineering principles to create process maps and simulation models of the cryopreservation pathway. Chapters 4 and 5 operated at the Center Level and used aquaculture and physiology research to evaluate the types of data that can be collected about genetic lines stored in a repository. Finally, Chapter 6 operated at the Center and Network Levels. This chapter

used industrial engineering concepts to assess data management requirements and to outline the exchange of materials and information between a repository and the surrounding community. By understanding the complex organizational structure of repository networks and by analyzing the processes that take place within them, repositories can be incorporated and sustained in aquaculture industries.

Chapter 1. Introduction

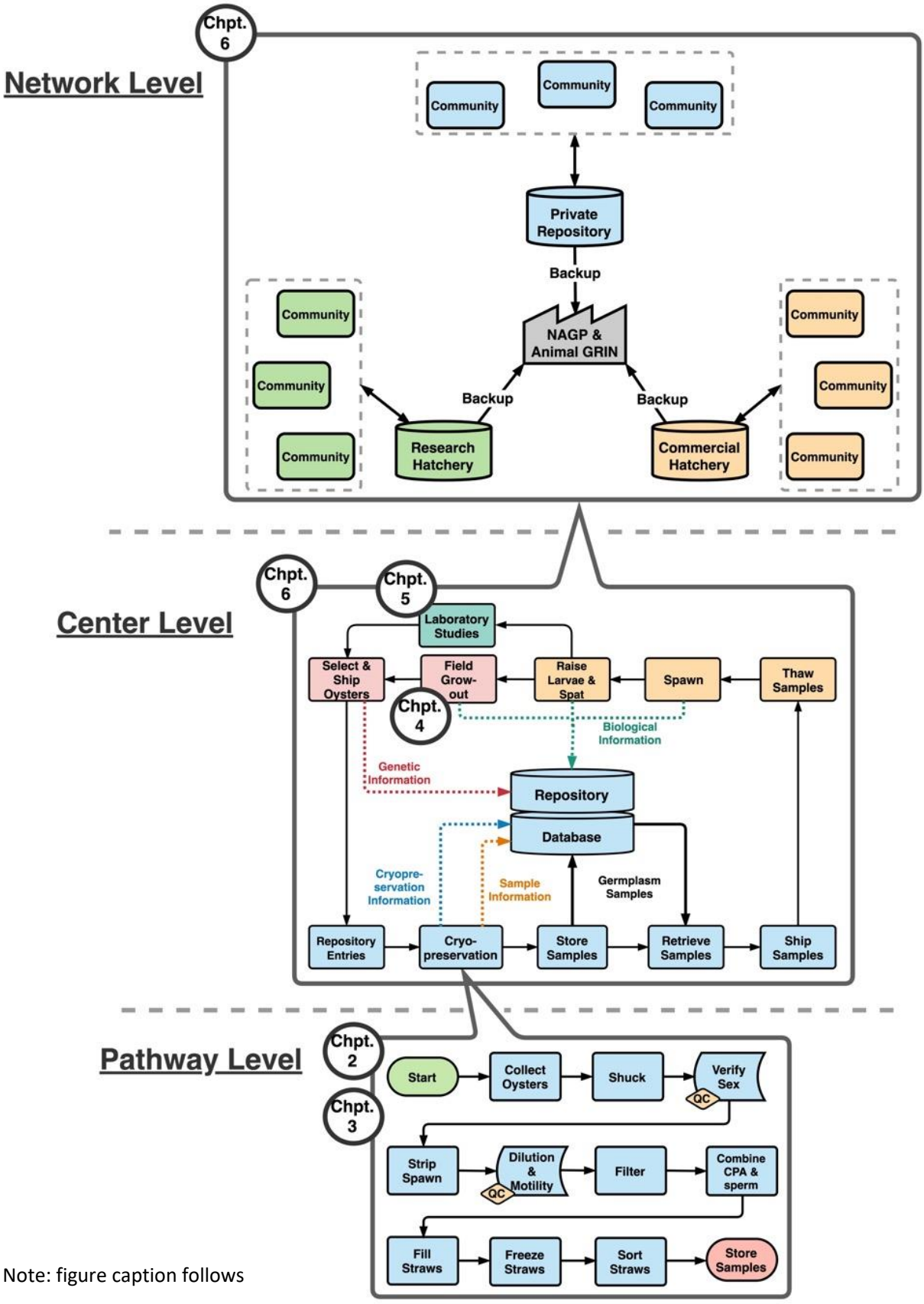
Germplasm Repositories and Dissertation Overview

In 1990, the United States Congress passed Public Law 101–624 which established the National Genetic Resources Program (NAGP) as part of the U.S. Department of Agriculture (USDA). The purpose of the NAGP was to collect, preserve, and distribute genetic resources of importance to American food and agriculture production (Food, Agriculture, Conservation, and Trade Act of 1990). To fulfill those objectives, the NAGP has established a germplasm repository and database. Germplasm repositories are collections of genetic resources that store physical germplasm samples, usually in form of cryopreserved sperm, eggs, embryos, and early life stages, as well as associated information (Torres et al., 2016; Wayman, 2003). Over the past 20 years, the NAGP established one of the largest animal germplasm repositories in the world, housing over 1.2 million units of germplasm from 46 species and 63,000 individual animals (https://agrin.ars.usda.gov/, NAGP, 2023). The majority of the collection (by units of germplasm) is made up of agricultural animals such as swine, dairy cattle, and beef cattle (Blackburn, 2009). Most of these samples were donated by commercial artificial insemination centers and breeders. Samples from important aquaculture species including freshwater and marine vertebrates as well as aquatic invertebrates have also been collected. Aquaculture species, however, constitute only 10% of the total number of stored germplasm samples. This points to the disparity of repository use between agriculture and aquaculture industries, and how aquaculture has yet to take advantage of the benefits of this technology.

Repositories can provide a ready source of genetic material that is easily transported and can be used to facilitate selective breeding programs. For example, repository storage and artificial insemination have led to rapid and large-scale genetic improvement in beef and dairy industries (Curry, 2000). Furthermore, storing germplasm in repositories is an alternative to maintaining live genetics lines, freeing up space at farms and hatcheries. Repository storage also prevents the loss of genetics from disease or natural disasters (Hu et al., 2011). Research to develop cryopreservation protocols for agriculture and aquaculture species each began in the 1950s (Tiersch et al., 2011). While injuries sustained during cryopreservation decrease sperm viability, fertilization rates can be compensated for by increasing insemination doses to contain a greater number of sperm (Watson, 2000). In cattle, fertility using higher doses of cryopreserved semen is similar to fertility using fresh semen (Vishwanath et al. 1996; Barbas & Mascarenhas, 2009). As a result, cattle industries quickly adopted cryopreservation and repository technology, possibly because of the existence of breeders clubs and the understanding of trait improvement through breeding (Torres et al., 2016). Conversely, in aquaculture industries cryopreservation efforts have been mostly limited to protocol development. Without studies focusing on how repositories can be integrated into commercial aquaculture systems and how they can expand (rather than disrupt) production, research will continue to stagnate at the protocol level.

Eastern oysters, *Crassostrea virginica*, are a useful research species for this type of work, both in their own right due to their cultural and economic importance, and as a model to demonstrate how to develop repositories for commercial aquaculture. Eastern oysters are currently one of the largest aquaculture industries in North America. There are more than 528 oyster farms and 37

hatcheries along the East and Gulf coasts of the US with an industry valued at over $130 million in 2018 (farm gate value, Perdue, 2019). In addition, high-throughput methods for cryopreserving oysters already exist that yield fertilization rates suitable for line recovery and broodstock development (Paniagua-Chavez et al., 1998, 2000; Yang et al., 2012). Currently, these methods require analysis to facilitate use at the hatchery production level. To develop repositories for oyster aquaculture, research should focus on understanding the flow of materials and information within a repository, between a repository and the surrounding community, and among other repositories and community members. These interactions operate at three different organizational levels: Pathway, Center, and Network Levels (Figure 1.1). Organizational levels such as these can be studied using the tools of industrial engineering, which specializes in examining the interactions of people, materials, and equipment to design and improve production systems (Salaam et al., 2012). Industrial engineering tools, such as process flow mapping and simulation modeling, allow for analysis of processes and the creation of frameworks of relationships among facilities. Work of this type is key to moving beyond protocol research and transitioning to developing systems to integrate repositories into aquaculture industries.



Note: figure caption follows

Figure 1.1. Process flow diagrams for the three different levels of repository organization: Pathway, Center Level, and Network. Chapter labels indicate the level at which that work applies. The Pathway Level encompasses all the steps of a particular process that take place in a repository facility, such as cryopreservation. Boxes indicate individual steps in the pathway, and diamonds indicate Quality Control (QC) steps. Arrows indicate the flow of materials. The Center Level encompasses activities that take place at a central repository facility and the immediate interactions between the repository and its surrounding community. Solid arrows indicate the flow of materials and dashed arrows indicate the flow of data. (green for Biological Data, blue for Cryopreservation Data, orange for Sample Data, and red for Genetic Data). The Network Level encompasses the interactions of multiple repositories and their communities which comprise the repository network. Arrows represent the exchange of materials and information.

Starting at the level with the smallest scale, the Pathway Level outlines all the steps of a particular process that take place in a repository facility, for example the process of cryopreservation (Figure 1.1). The process of cryopreservation at a repository is referred to as a pathway and not a protocol because quality management has been integrated, the process is reproducible using different equipment options (harmonization), and the process is scalable and therefore can operate at multiple scales of production (Liu et al., 2021; Torres et al., 2016). This is in contrast to a bench-scale cryopreservation protocol that often lacks quality management, relies on specialized equipment not available at other facilities, and cannot process samples at a scale applicable to commercial industries (Tiersch et al., 2011).

The next level is the Center Level which encompasses the multiple processes that can take place at a repository facility which include cryopreservation, shipping and receiving samples, and database management. (Figure 1.1). The Center Level also outlines the direct interactions, the exchange of materials and information, that take place between a repository and surrounding community. For example, a repository focused on aquaculture species may ship cryopreserved

sperm from selectively bred animals that can withstand high water temperatures to a hatchery in need of such genetics. The hatchery can use the sperm to spawn, raise larvae, and distribute juveniles to farms where they can be grown until ready for harvest. During harvest, the farm can collect new broodstock and send animals to the repository for cryopreservation and storage.

The final organizational level is the Network Level which outlines the relationships of a repository facility and the surrounding community members as well as interactions among other repositories and their communities (Figure 1.1). Together the repositories and community members form a repository network. This level tracks the flow of materials and information among the network members. For example, in a national repository network focused on aquaculture species, the central repository would be expected to interact with federal agencies (such as the USDA NAGP), research laboratories at universities, research and commercial hatcheries, and farms. These community members would exchange samples and data with the repository and collaborate with each other to reach aquaculture production goals.

By understanding the complex organizational structure of repository networks and by analyzing the processes that take place within a repository, these entities can be efficiently incorporated into aquaculture industries. The goal of this dissertation was to integrate germplasm repository development into oyster aquaculture systems at the Pathway, Center, and Network Levels. The specific objectives were to: 1) simulate the resources required to cryopreserve oysters at scales of production applicable to commercial industries *(Chapters 2 & 3),* 2) evaluate the data collected about genetic lines stored in the repository *(Chapters 4 & 5),* and 3) develop tools to assist with

database management and understanding of the exchange of materials and information between repositories and the surrounding communities *(Chapter 6)*. Studies performed in Chapters 2 & 3 operated at the Pathway Level and included time studies as well as simulation modeling to analyze cryopreservation at a commercial scale. Studies performed in Chapters 4 & 5 operated at the Center Level. These experiments were case studies that yielded relevant data for a repository database about genetic lines preserved in Chapters 1 and 2. Finally, the study performed in Chapter 6 operated at the Center and Network Levels. In this chapter, interviews were conducted to gain insight from oyster industry professionals, i.e. hatchery managers, as to how materials and information can be exchanged between repositories and communities.

Oyster Aquaculture Production and Genetic Resources

Crassostrea virginica is a relevant model for this dissertation because of its economic importance. Eastern oysters have been harvested in North America for centuries and are currently one of the largest aquaculture industries in North America (National Marine Fisheries Service, 2020). These oysters have traditionally been grown using on-bottom culture methods, however, off-bottom methods (also referred to as "alternative oyster aquaculture" in Louisiana) have become increasingly popular in recent years (Walton et al., 2013). Traditional on-bottom farmers rely on seed collected from natural spawning sites. Off-bottom production farmers rely on hatcheries to produce seed. Hatcheries spawn broodstock to produce larvae which are raised in large tank systems for approximately 14 days (Supan, 2002). After two weeks, hatcheries either sell larvae to nurseries or "set" the larvae themselves (allow larvae to settle and metamorphose into juvenile oysters) and continue to grow the oysters (Wallace, 2001). Oysters are raised in nurseries

or hatcheries until they are large enough (e.g., often 8 - 10 mm) to be grown at farm sites. At this stage juvenile oysters commonly are referred to as “seed” and off-bottom farmers purchase seed from hatcheries. Oysters are grown at the farm site (either off or on-bottom) for 18–24 months (in the northern Gulf of Mexico) until they reach market size, approximately a height of 76 mm although it varies by state (Galtsoff, 1964). Oysters are harvested and sold to seafood distribution companies, shucking houses, or directly to restaurants.

In the current production system for oyster aquaculture, the genetic resources that hatcheries produce and distribute are not always readily available and are not protected beyond maintenance of live populations. Farmers can choose to purchase and grow diploid (2N, two sets of chromosomes) or triploid oysters (3N, bred to have three sets of chromosomes). Triploids have become popular due to reduce gametogenesis which contributes to faster growth rates and higher meat quality than diploids, particularly in the summer months (Allen Jr. & Downing, 1986). Triploids, however, can be difficult to produce as male tetraploids (4N, four sets of chromosomes) parent are required, and access to tetraploids typically entails contractual arrangements. Furthermore, hatcheries (particularly research hatcheries funded by universities or government agencies) have produced genetic lines of oysters that are disease resistant (Casas et al., 2017; Haskin & Ford, 1979; Vrijenhoek et al., 1990) and able to survive in stressful water quality conditions such as low salinity (< 3 ppt, McCarty et al., 2020). Currently, as live populations, these lines are at risk from disease, natural disasters, and the limitations of hatcheries to continually maintain them. To safeguard these genetics, germplasm material from these oysters can be frozen stored in repositories similar to what is done in the beef and dairy industries (Purdy et al.,

2016; Wiggans et al., 2017).

Repositories also facilitate breeding programs because transferring genetics in the form of cryopreserved germplasm is often easier than shipping of live animals. For example, the ability to ship cryopreserved sperm from tetraploids would make spawning triploids easier in addition to protecting intellectual property of the tetraploid breeding facility. Breeding programs that involve multiple hatcheries would also benefit from this easy transfer as well as the possible streamlining of compliance biosecurity regulations when transporting samples across state lines. Multi-state breeding efforts for oyster aquaculture already exist, such as the Eastern Oyster Breeding Consortium that includes research hatcheries and state agencies along the Atlantic coast (CT, MA, ME, NC, NJ, NY, RI, VA; Malmquist, 2019) and the Gulf of Mexico Oyster Genetics and Breeding Research Consortium (hatcheries and agencies from AL, FL, LA, MS, and TX; Lucas, 2023).

The genetic material from wild oysters is also important to preserve. Oyster hatcheries often collect wild broodstock that may possess desirable traits as the foundation of breeding experiments (Bodenstein et al., 2023; Leonhardt et al., 2017). Broodstock are collected from multiple locations because eastern oysters from different regions across the country (e.g., Atlantic and Gulf coast states) as well as different estuaries within a state have been shown to constitute different genetic populations (Johnson & Kelly, 2020; Lowe et al., 2017). Preserving a wide selection of wild populations ensures that genetics resources native to a region will not be lost because populations can be reconstituted with genetics stored in the repository (Blackburn,

2018; Purdy et al., 2016). Furthermore, breeding programs that focus exclusively on trait advancement can cause a loss of genetic diversity through inbreeding contraction of genetic diversity. This can have deleterious effects on animals and in some cases can result in undermining of the genetic gains of the breeding program (Blackburn, 2018). Storing wild genetics and multiple broodstock generations ensures that a hatchery can breed diversity back into a line without losing genetic gains.

Along with storing physical genetic material, a repository also contains a database that stores information about the samples. The database is a crucial tool for managing genetic lines effectively and is what truly makes cryopreserved samples valuable (Tiersch et al., 2011; Varga, 2011). To create an useful database, however, huge amounts of information must be collected to characterize genetic lines (Tiersch et al., 2011). This includes information about the individual animals that were preserved, phenotypic and genetic data of the that line individual belonged to, and information about the cryopreservation protocol (i.e. Sample, Biological, Genetic, and Cryopreservation Data, discussed in detail in Chapter 6). Collecting, managing, and distributing these data requires the concerted efforts of many researchers, farmers, and hatchery personnel, as well as inclusion of an online database accessible to the public (Irwin et al., 2012; Tiersch et al., 2011).

A repository database is particularly critical for oyster aquaculture because a myriad of factors can affect oyster production (e.g., salinity, disease, food abundance) and oysters from distinctive genetic lines can respond differently in similar environmental conditions (genotype x

environment interactions) (Guévélou et al., 2019; Leonhardt et al., 2017; Sehlinger et al., 2019). Furthermore, research to identify the causes of mortality can take months and it is challenging to collect comprehensive measurements in every study (e.g., morality, growth, clearance rates, oxygen consumption, genotyping). An example of these challenges can be seen in research concerning triploid oyster mortality. Triploid mortality events (periods of elevated deaths, usually in late-spring or early-summer) have been observed along the East and Gulf coasts, and the causes remain undetermined (Guévélou et al., 2019; Matt et al., 2020; Wadsworth et al., 2019). Preserving genetic material (from the broodstock used to produce triploids) and storing data collected in a repository would allow researchers to integrate their work. Increased data sharing and the ability to recreate lines could expedite efforts to decrease oyster mortality and increase production. Repository databases also make breeding programs more efficient as comprehensive data allows for more accurate trait selection (Gadberry et al., 2016; Purdy et al., 2016). Thus, to create a successful germplasm repository and database, the facilities that supply and utilize the genetic material and information must be outlined. This is where industrial engineering tools can be used.

Industrial Engineering Tools

The field of industrial engineering is concerned with the design and analysis of people, materials, information, and equipment in production systems (Salaam et al., 2012). Industrial engineers generally work with commercial manufacturing facilities to identify and eliminate various forms of waste (inefficiencies) with the goal of improving production capabilities (Fernando & Cadavid, 2007). Industrial engineering work can involve creating or optimizing processes, production

control and scheduling, developing partial budgets, and motion studies. Two common industrial engineering tools are process flow diagrams and simulation models. Process flow diagrams (or maps) are applied in commercial-scale factory settings to help identify and eliminate wastes in service of streamlining the process (Damelio, 2011; Fernando & Cadavid, 2007). Flow diagrams generally outline the steps of a process in sequence to help understand the flow of materials and information and to identify bottlenecks and wastes (Damelio, 2011). These diagrams can also be used beyond the scope of individual processes and illustrate the interactions among suppliers, producers, and customers (Apaiah & Hendrix, 2005; Emelogu et al., 2019). Process flow diagrams that encompass multiple facilities or processes are sometimes referred to as relationship diagrams (Damelio, 2011). While common in manufacturing settings, these diagrams have also been used in the agricultural and biomedical fields. In the context of repository development, process flow diagrams can be used to outline activities at three levels of organization relevant to this dissertation: Pathway, Center, and Network (Figure 1.1).

Process flow diagrams are also the basis for a second common industrial engineering tool, simulation modeling. The specific type of simulation modeling used in this dissertation is discrete-event simulation (DES) modeling. These models simulate processes by modeling all the steps (in sequence) of a process throughout time. Models can include the resources required for a process to run such as the number of operators, the cost of equipment and supplies, and logic rules that reflect “real world” constraints (e.g., an operator can work on only one step at a time) (Schriber et al., 2013). With the use of DES models, industrial engineers can capture the complexity of commercial-scale production systems and inform decisions made about those systems (Gittins et

al., 2020). Models can be used to calculate the amount of time and what resources (e.g., operators, supplies, equipment) would be needed to meet certain production goals. Discrete-event simulation modeling has already been used in field of aquatic species cryopreservation to assist in facility planning and high-throughput cryopreservation for blue catfish (*Ictalurus furcatus*) (Hu et al., 2015). Therefore, these tools can also be useful for developing repository systems in oyster aquaculture and specifically for understanding the transfer of genetical material and data at the Pathway, Center, and Network Levels.

Chapter 2. Simulation Modeling of a High-throughput Oyster Cryopreservation Pathway

Introduction

Discrete-event simulation models are powerful tools capable of capturing the complexity of large-scale production systems and informing decisions made about those systems (Gittins et al. 2020). These types of models simulate the behavior of "real-world" processes by modeling all the events, or steps, that occur in a process throughout time (Schriber et al. 2013, Allen et al. 2015). Simulation models, specifically discrete-event simulation models, have historically been used in the manufacturing and service-industry sectors (Robinson 2005). Their utility in other sectors, particularly in agriculture and aquaculture, remains largely unexplored despite their potential benefits (Gittins et al. 2020). Most modeling pertaining to aquatic systems has been focused on nutrient dynamics, stock assessments, and hydrodynamic and benthic processes (McCausland et al. 2006; Tran et al., 2017). By also utilizing discrete-event simulation, the current state of aquaculture systems can be understood, and alternative scenarios of defined relevance can be modeled (Halachmi et al. 2005, Hu et al. 2015). These models can help identify constraints (bottlenecks) of current systems and enable predictions of system improvement should alternative approaches be put in place (Gupta & Boyd 2008, Hu et al. 2015).

The ability to efficiently plan, build, and maintain aquaculture systems is becoming increasingly important. Sustainable aquaculture, such as bivalve aquaculture, may be the key to global food

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security as global warming impacts traditional agricultural practices (Turan & Neori 2010, Willer & Aldridge 2019). In addition, the foundational genetic resources of important aquaculture species are threatened by anthropogenic factors such as climate change and overfishing (Sumaila & Tai 2020). Genetic resources are the product of millions of years of evolution, and in domestic populations that have undergone selective breeding, the result of millions of dollars of research investment (Byrne et al. 2018, Torres & Tiersch 2018). The genetic resources of aquatic species cannot easily be replaced if lost, and therefore safeguarding them should be recognized as a high priority. Currently, aquatic genetic resources are mostly maintained in the form of live populations, which is unnecessarily risky and expensive. Other options exist, for example collections of cryopreserved genetic material such as sperm can be stored alongside associated information (e.g., phenotypic, physiological, genetic) forming the basis of a germplasm repository (Yang et al. 2012, Byrne et al. 2018, Torres & Tiersch 2018).

Repositories can safeguard genetics and free up space on farms by reducing the number of lines (and males) that must be maintained as live animals. Repositories also facilitate selective breeding programs by allowing for easy management and transfers of genetic material (Torres et al. 2016, Byrne et al. 2018, Yang et al. 2021). Repository systems have long been used in the dairy cattle industry to provide breeders with genetic material to facilitate rapid genetic improvement (Torres et al. 2016). Aquaculture industries have yet to take advantage of germplasm repositories because they are complex systems with many logistical considerations, stakeholders, and interlocking activities, making their development difficult. Therefore, there is great opportunity to use discrete-event simulation modeling to facilitate growth in aquaculture through germplasm

repositories that can be used to protect, distribute, and improve the genetic resources of aquatic species.

In this study, the eastern oyster, *Crassostrea virginica*, was used as an example to demonstrate how discrete-event simulation can facilitate repository development. Eastern oysters are an important part of the culture and economy of the Gulf of Mexico; however, high oyster mortality is currently threatening the livelihood of oyster farmers (Matt et al. 2020, Bodenstein et al. 2021). Specific causes of this mortality remain unclear (Wadsworth et al. 2019, Matt et al. 2020) and underscore the need for genetic resources of farmed and wild oysters to be protected. Unfortunately, a commercial-scale repository system capable of serving the needs of the oyster industry does not yet exist, despite the existence of successful cryopreservation protocols.

Cryopreservation research on oysters has been ongoing for more than 30 y (Bougrier & Rabenomanana 1986, Paniagua-Chavez & Tiersch 2001, Yang & Huo 2021), but has not progressed into widespread industry adoption. The research often deals with dozens of samples at a time, limiting the translation of bench-scale work to the commercial scale (Torres et al. 2016). Even with development of high-throughput approaches, repositories have not been adopted in the industry (Yang et al. 2012). The goal of this study was to begin to address the gap between cryopreservation research and repository development by using simulation modeling of high-throughput oyster cryopreservation. The objectives of this study were to: (1) define the steps in high-throughput cryopreservation processing and create a process flow diagram; (2) create and validate a simulation model based on time studies of high-throughput oyster cryopreservation;

(3) identify bottlenecks and waste in the model, and (4) expand the utility of the model by evaluating different device options that address identified bottlenecks. The model developed in this study was able to simulate oyster cryopreservation accurately and yielded insights for production capacity, system costs, and production bottlenecks.

Materials and Methods

Process Mapping of the Cryopreservation Protocol

To create a model of high-throughput oyster cryopreservation, each step in the process was defined and all steps were compiled in a process flow diagram. The process was based on a previous protocol (Yang et al. 2012) that is representative of commercial-scale approaches. In total, 21 steps were defined and included in the process flow diagram. Within Steps 4–12 and 19–21, oysters were handled individually, whereas in Steps 13–18 oysters were handled in batches (groups) due to time-sensitive constraints of the cryopreservation process. Steps 1–3 did not involve direct handling of oysters or frozen germplasm units. In addition, Steps 1–3 and 20 and 21 did not need to take place on the same day as Steps 4–19 in any given cryopreservation trial. Thus, this study focused on the modeling of Steps 4–19 because they encompassed the main cryopreservation process. Future research can address how the preparation process (Steps 1–3) and post processing (Steps 20 and 21) interact with the main cryopreservation process. Steps 3–12 took place in a traditional laboratory setting whereas Steps 13–19 took place in a commercial-scale processing facility (Figure 2.1). Detailed explanations of each step are described below.

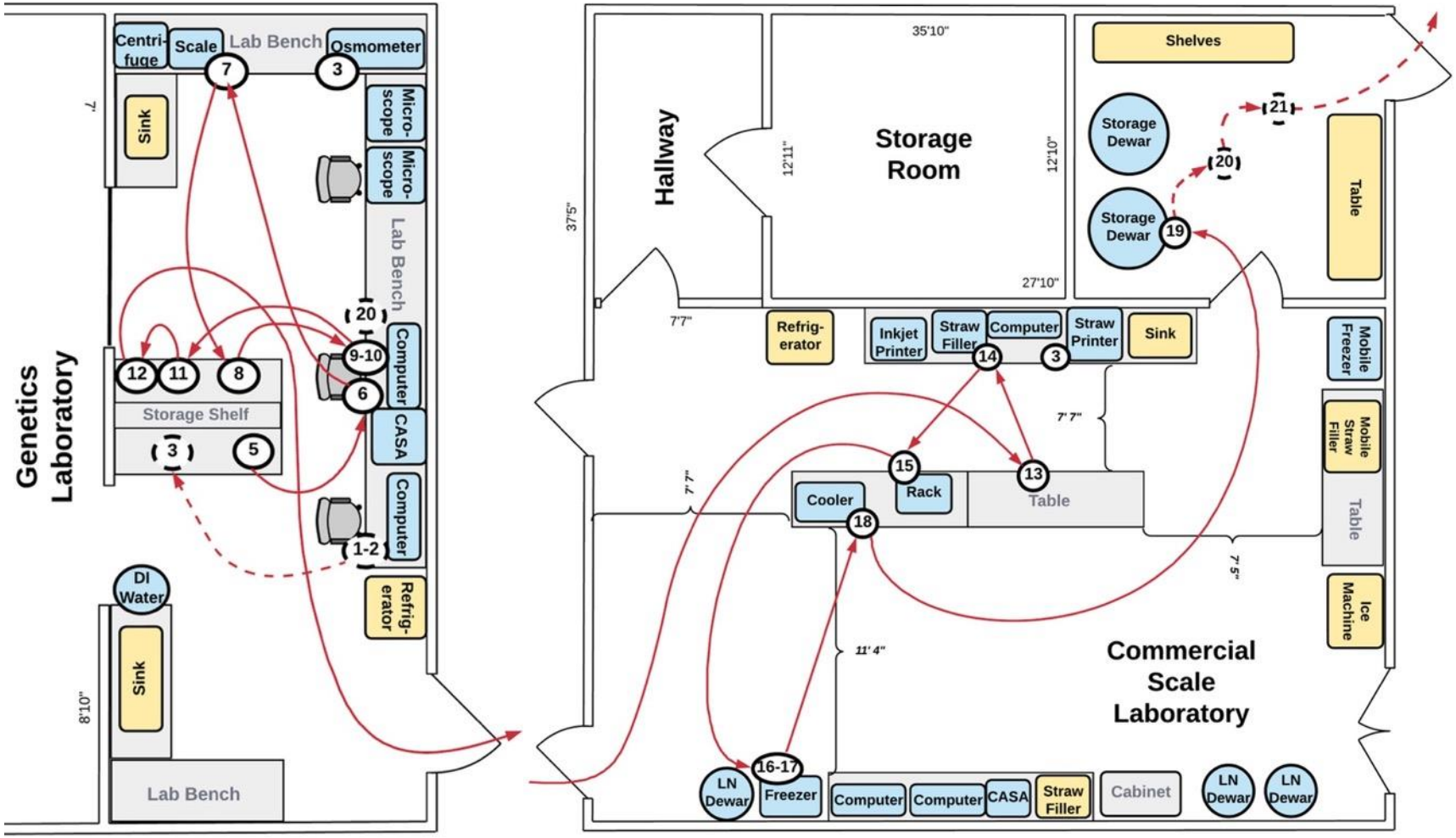

Figure 2.1. Blueprints indicating where each step in the cryopreservation protocol was physically performed. Such "spaghetti diagrams" are a visual representation of the flow of materials and operators through steps of a process. Solid arrows indicate operator movement from one step to the next. Dashed arrows indicate steps (Steps 1–3 and 20 and 21) that were not performed on the same day as the main cryopreservation process (Steps 4–19). Immoveable room features included: tables, shelves, sinks, and refrigerators. All other room features could be moved.

Steps 4–12 consisted of identifying and preparing samples for cryopreservation. First, in Step 4, 1- or 2-year-old oysters were collected from holding tanks in the LSU Animal and Food Science Oyster Wet Laboratory and transported to the Aquatic Germplasm and Genetic Resources Center (AGGRC). The salinity of each tank was held at 15 ± 1 ppt and the water temperature was 27 ± 1°C. After oysters were collected, they were shucked using a 4-in. oyster knife (Sani-Safe, Dexter Russell, MA) to remove the right valve. Sex was verified by taking a sample of the gamete tissue with a pipette tip (2−200 µL) and examining with a microscope at 100× magnification (Zeiss Axio Laboratory.A1, Jena, Germany) (Step 5). Step 6 was a "process oriented" quality assurance activity which is typically used to prevent defects and errors from occurring with the material being handled (Torres et al. 2016). In Step 6, only male oysters with ripe gonads (≥80% follicle coverage, Matt & Allen 2021) were selected for cryopreservation to prevent poor-quality sperm from being cryopreserved.

In Step 7, the height, length, width, and wet weight of each male oyster selected for cryopreservation were measured and recorded. Oysters were strip spawned by scraping the gonadal tissue with a disposable scalpel into a 50-ml centrifuge tube (Step 8). Next, sperm collected from each male was diluted with calcium-free, 650 mOsmol/kg Hanks' balanced salt solution (Ca-free HBSS650) to yield a sperm concentration of 2×10^8 cell ml^{-1} [Step 9, the first quality control (QC) measurement]. Quality control measurements are "material oriented" and focus on defect identification in the material being handled. Standardizing and recording the concentration of sperm in Step 9 increased the consistency of sperm quality after thawing (Dong et al., 2007). Sperm motility (ratio of moving sperm to total number of sperm in a defined volume)

was determined manually using the Zeiss microscope with a Makler® counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel) at 100× magnification (Step 10). To measure motility, the number of moving and stationary sperm in one square of the Makler® counting chamber (100 μm^2) were counted, and the percent of moving sperm was calculated. This was repeated for two more squares and an average motility from all three counts was calculated. After sperm concentration and motility from all individual males were recorded, sperm samples were each filtered through a 100-µm Nitex screen to remove debris (Step 11). The final volumes of diluted sperm solutions were recorded, and for each sample equal volumes of 20% dimethyl sulfoxide (DMSO, diluted in Ca-free HBSS650) were prepared in separate 50-ml centrifuge tubes (Step 12).

Within Steps 13–18, the sperm samples collected from multiple oysters were processed at the same time and were referred to as a batch. Batched samples were collected from individual oysters and were not combined, meaning that the sperm from “Oyster A” was stored in separate French straws than sperm from “Oyster B”. A batch consisted of samples from either 4 or 5 oysters depending on the total number of individuals being cryopreserved. First, in Step 13, the sperm solutions were combined with the cryoprotectant, 20% DMSO, in a semen container cone (IMV Technologies, France) yielding a final solution with a cell concentration of 1×10^8 cell mL^{-1} and a cryoprotectant concentration of 10% DMSO. The 20-min equilibration time began from the time the DMSO and sperm solutions were mixed, and ended when freezing began (freezer program cooled from 4°C to −80°C). During the equilibration time, sperm solutions from each male were transferred to an automatic straw filler (MPP Quattro, Minitube USA, Verona, WI) which filled 0.5-mL French straws with sperm from each male, then sealed, and labeled straws.

(Step 14). Sealed straws were visually inspected and placed by hand on horizontal metal racks in Step 15. After each rack was full, it was placed within a computer-controlled freezer (IceCube 14M-A, Minitube USA) and was held at 4°C until freezing began.

At Step 16 the freezing program was initiated to cool samples at 20°C min−1 until the chamber reached −80°C. Upon completion of the freezing program, the freezer temperature was held at −80°C for 20 min while straws were unloaded, one rack at a time, into a Styrofoam Cooler that contained approximately 15 cm of liquid nitrogen (Step 17). Straws were grasped individually with large stainless-steel forceps (LIUNA 304 Stainless Steel) and sorted into daisy goblets, cylindrical plastic containers (67 mm wide and 135 mm tall) with 12 radially arrangement compartments (IMV Technologies), for storage in Step 18. Each daisy goblet held as many as 240 French straws. After all straws were sorted, Steps 13–18 were repeated if there were additional sperm samples to be frozen. After all samples were frozen and sorted, goblets were transferred under liquid nitrogen to a high-capacity cryogenic tank (MVE 1500 Series 190) (Step 19).

Model Creation and Validation

i. Time Studies and Simulation Modeling

During each cryopreservation trial, the amount of time required to complete each step was measured using digital timers. Only the numbered steps shown in Figure 2.2 were measured during time studies. Due to COVID-19 working restrictions in place at the time of the study, a single operator performed all steps in each cryopreservation trial. The operator self-timed during Steps 4–12 and a collaborator observed and timed the operator via video conference call (due to

COVID-19 restrictions) for Steps 13–19. The times to complete each step were standardized to time per oyster (sec oyster^{-1}), even for steps that did not directly involve handling oysters. Six initial cryopreservation trials were performed in Spring 2020 and time distributions were generated in MATLAB (v7.10.0, The MathWorks, Natick, MA) for each step based on replicate time measurement data. Six distributions were tested against time data for each step using the Kolmogorov–Smirnov goodness of fit test: exponential, gamma, lognormal, normal, poisson, and Weibull. The distribution with the best fit was selected to represent the data. To validate the dataset collected in the first six trials, three additional trials were performed in Spring 2021 and separate distributions were generated for each step.

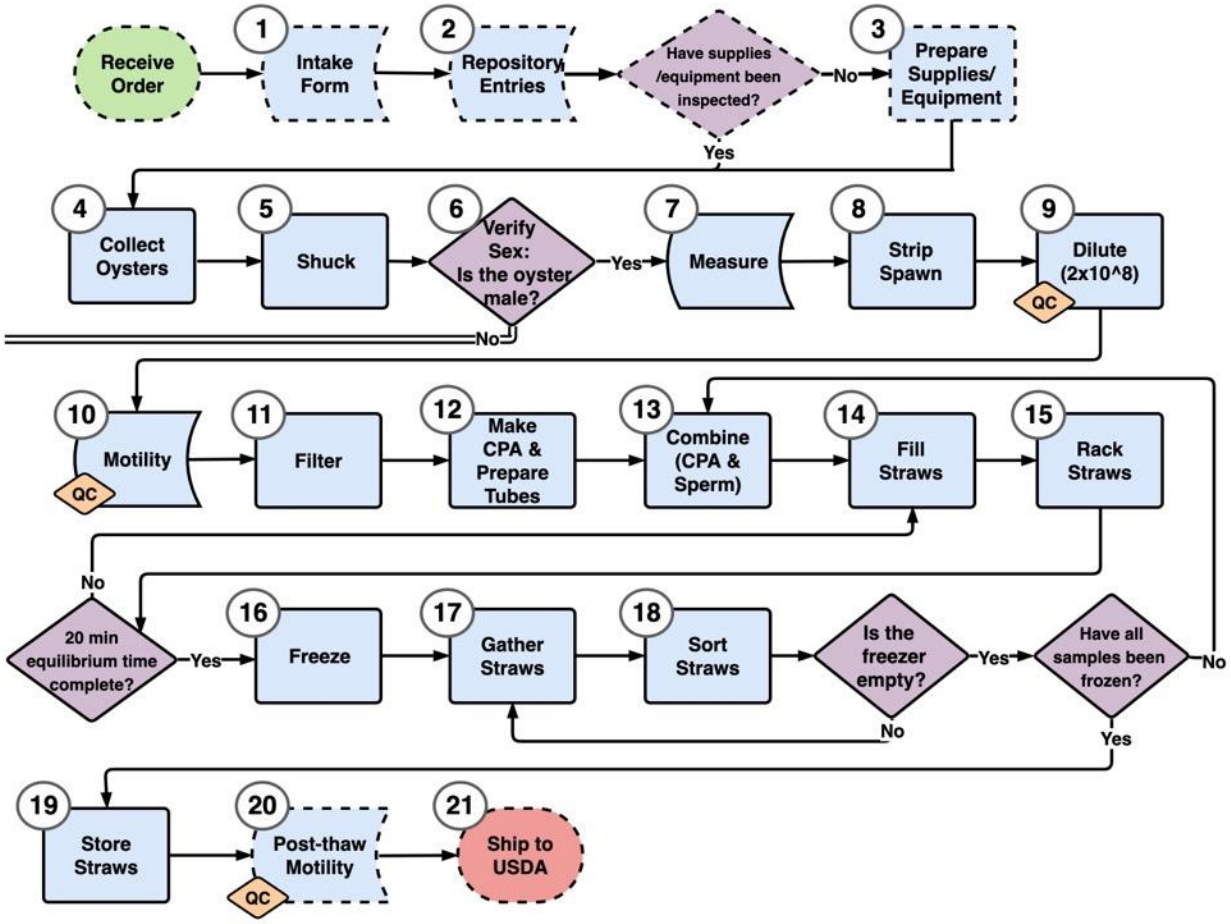

Figure 2.2. Process flow map of the high-throughput oyster cryopreservation protocol used in this study. Oval shapes represent the start and end steps in the protocol, rectangles indicate steps in the protocol, rectangles with curved sides indicate steps where data were recorded, large diamonds indicate quality assurance (QA) steps, and small diamonds indicate recorded quality control (QC) measurements. Steps with dashed outlines were not required to be performed on the same day as steps with solid outlines. Single-line arrows indicate the flow of material, whereas double-line arrows indicate mate- rial was removed from the system. Numbers indicate steps that were measured during time studies. This study addressed Steps 4–19. Steps in this map can be customized for any process. CPA is an abbreviation for cryoprotective agent.

The software Simio (v14.230, Simio LLC, Sewickley, PA) was used for simulation modeling. The original simulation model (Model A) used time distributions generated from cryo- preservation Trials 1 to 6. To simulate real-world conditions, the parameters of run time, number of operators, number of straws per oyster, and batch size were set to selected baseline values: 45 straws per

oyster, a batch size of five, one operator, and an 8-h runtime. Resources and associated costs were also inserted into the model (Table 2.1). Each simulation run represented a single workday (8 h) for one operator. Each simulation was run 10 times with the same settings to obtain replicate data. The major outputs of interest for the model were *Throughput, Time in System (TIS), Operating Cost,* and *Cost per Oyster*. Throughput was the number of oysters fully processed in the model during the run time. Time in System was the average amount of time each oyster required within the model (i.e., the average time it took to process each oyster). Operating Cost was the average cost of supplies and operator wages to process all oysters in a run, excluding Capital Costs (the one-time cost of equipment). Cost per Oyster was the average Operating Cost of processing one oyster. Total Cost was calculated as the cost to process all oysters in a run including Operating and Capital Costs.

Table 2.1. The time distributions (created from data collected in Trials 1–6), resources used, and costs (capital and operating costs) for each step in the cryopreservation protocol.

Step	Distribution (s/oyster)	Resources	Capital Cost	Operating Costs
Shuck	Weibull(1.291, 66.869)	Shucking Knife (×2)	$15 (×2)	–
Verify	Lognormal(3.906,0.443)	Pipettor tips (1–10 µL) (×2)	–	$0.03 (×2)
Measure	Lognormal(3.763, 0.218)	Ohaus Scale (EP612C)	$2,250	–
		Calipers (Pittsburgh 6” Digital Caliper)	$20.94	–
Strip	Normal(134.02,31.503)	Disposable Scalpel	–	$0.73
		Centrifuge Tubes (50 ml)	–	$0.51
Motility	Lognormal(5.421, 0.274)	Zeiss Axio Laboratory.A1 Microscope	$1,700	–
		Pipettor (1–10 µL)	$366.6	–
		Pipettor (10–100 µL)	$366.6	–
		Makler® Counting Chamber (IVF)	$811.57	–
		Pipettor tips (1–10 µL) (×2)	–	$0.03
		Kimwipes (×2)	–	$0.01
		HBSS-650	–	$0.12
Filter	Lognormal(3.805, 0.145)	Sterile Filters	$3.4	–
		Centrifuge Tubes (50 ml)	–	$0.51
Prep CPA	Lognormal(3.999, 0.232)	Balance (Mettler Toledo ME103TE)	$1,469	–
		Osmometer (5010 OSMETTE III)	$9,895	–
		Fisherbrand Electric Serological Pipet	$246.4	–
		Serological pipet (25 ml)	–	$1.08 (×2)
		HBSS-600 (100 ml)	–	$0.15
		DMSO (100 ml)	–	$5.0
Combine CPA	Lognormal(2.403, 0.448)	–	–	–
Fill	20	Straw Printer	$36,490	–
		Straw filler (CombiSystem MPP Quattro)	$73,774	–
		French straws (0.5 µL) (× # straws × batch size)	–	$0.06
		MPP Quattro Tubing (× batch size ×4)	–	$0.39
		Semen Cone (200 ml) (×batch size)	–	$0.38

Cont’d

Step	Distribution (s/oyster)	Resources	Capital Cost	Operating Costs
Freeze	8	SY-LABORATORY IceCube Controlled Freezer	$34,722	–
		LN transfer hose	$271	–
		Liquid Nitrogen Rental (110 L)(×0.25)	–	$16/batch size
Gather	Weibull(1.826, 0.550) × # straws × batch size	Cryogenic Gloves (×2)	$200.28 (×2)	–
Sort	Lognormal(1.148, 0.113) × # straws × batch size	Styrofoam Cooler (Miller Supply 12 ×8 ×8″)	$28	–
		Forceps (LIUNA 304 Stainless Steel) (×2)	$12.98 (×2)	–
		Daisy Goblets (×(# straws × batch size)/240)	$8.73	–
Store	Lognormal(2.932, 0.577) × batch size	Storage Dewar (Worthington, 35 L)	$1,573.99	–
		Roller Base for Storage Dewar	$241.77	–
		Low-level LN Alarm (115V)	$948.86	–
Clean	Lognormal(5.157, 0.349) × batch size	–	–	–
Motility with CASA	Lognormal(0.790, 0.392)	CASA (CEROS II Animal)	$30,000	–
		Pipettor (1–10 µL)	$366.6	–
		Pipettor (10–100 µL)	$366.6	–
		Makler® Counting Chamber (IVF)	$811.57	–
		Pipettor tips (1–10 µL) (×2)	–	$0.03
		Kimwipes (×2)	–	$0.01
		HBSS-650	–	$0.12
Sort with Funnel	Weibull(3.629, 0.227) × # straws × batch size	Three-dimensional printed sorting funnel	~$2	–
		Styrofoam Cooler (Miller Supply 12 ×8 ×8″)	$28	–
		Daisy Goblets (×(# straws × batch size)/240)	$8.73	–

Cont'd

The simulation model followed the structure of the process flow map. Model A contained four logic components to simulate real-world working conditions as closely as possible. The logic components (or rules) were as follows:

Logic Rule 1: All steps in the process required an operator to function. Operators are considered resources in Simio and can be “seized” and “released” by servers (steps) before and after processing. Each server in the model was required to seize an operator before processing could begin. After processing, the server released the operator, which could be seized by another server.

Logic Rule 2: The Fill and Freeze servers (Steps 14 and 16) had constant processing times rather than distributions. The Fill server had a processing time of 20 min because the cryopreservation protocol had an equilibration time of 20 min. The operator had 20 min to fill, seal, label, and place all straws on a rack within the freezer, and therefore the processing time for the Fill server was held constant. The Freeze server had a constant processing time of 8 min due to the cooling process which required 4.2 min to traverse from 4 to −80°C at a rate of 20°C min−1 and samples were held at −80°C for 3.8 min before removal from the freezer.

Logic Rule 3: Before Step 13, individual oysters were associated with a specific number of straws using a “Combiner” server. The paired number of straws was a variable that could be changed and was set to a baseline value of 45. The combined oyster-straw units were passed to another Combiner server where they could be grouped in batches. Batch size

was another variable in the model and was set to a base- line value of 5. Batches (groups of oyster-straw units) were the units that entered Step 13. The combining process was instantaneous in both cases.

Logic Rule 4: Between Steps 13 and 18, only a single batch could be processed at a time. If the resource state of the Fill, Freeze, Gather, or Sort servers were equal to 1 (meaning one of the servers was processing a batch), the first server in the sequence (the Fill server) could not start processing another batch until all four servers had a resource state equal to 0 (meaning none of the servers were processing).

Model Validation and Optimization

To validate Model A, a second model, Model B, was created using time distributions generated from a dataset collected in Trials 7–9. Model B was otherwise identical to Model A. The average processing time (time to complete each step, s straw^{-1}) was compared between the models using a one-way ANOVA. The Throughput, TIS, and Costs (Operating Costs and Cost per Oyster) outputs of each model in an 8-h period were compared to verify Model A (Hu et al. 2015). In addition, a "face validity" test (Halachmi et al. 2005) was performed by comparing Throughput results from Models A and B to "real-world" throughput reported by experienced cryopreservation professionals who have worked at the AGGRC for more than 10 y.

In addition, the results of parameter optimization software built into Simio (OptQuest Engine, v14.230) were compared between the two models. The software was given ranges for three

parameters (number of straws, batch size, and number of operators) and was used to maximize Throughput and minimize TIS and Operating Cost. OptQuest, an internal capability of Simio, was used to simulate every combination of parameters and it presented the optimized combinations.

Process Bottlenecks and Waste

Bottlenecks were identified by analyzing which steps in each model had the highest Waiting Times (the average time model entities were held in the queue before being processed) and Processing Times (average time for a server to process an individual oyster). After a bottleneck was identified at a particular step, device options were selected to decrease waiting or processing times. The alternate devices are described below. The amount of starting material waste was also calculated by recording the number of oysters shucked but not processed (e.g., females) and the amount of sperm collected but not frozen.

Device Alterations

To assess the effect that alternate device options (tools or equipment) would have on the overall cryopreservation process, three alternative models were created, each with different device options. These models were identical to Model B except they incorporated the different device options. Model C used a new method to sort for Step 18 (Sort Straws). Instead of straws being picked up individually with forceps, a three-dimensional printed funnel specifically designed to work with a daisy goblet was used to sort 15 French straws at a time (Figure 2.3). This change was reflected in a new time distribution for the Sort server. Model D incorporated Computer

Assisted Sperm Analysis equipment (CASA, CEROS II Animal—Hamilton Thorne) to measure motility instead of using a standard microscope and Makler® counting chamber. This was reflected in a new time distribution for the Motility server. In Model E, the sorting funnel and the CASA were both used. Models C–E were com- pared with the original models by analyzing baseline model results (Throughput, TIS, Costs), bottlenecks, and optimized parameter recommendations.

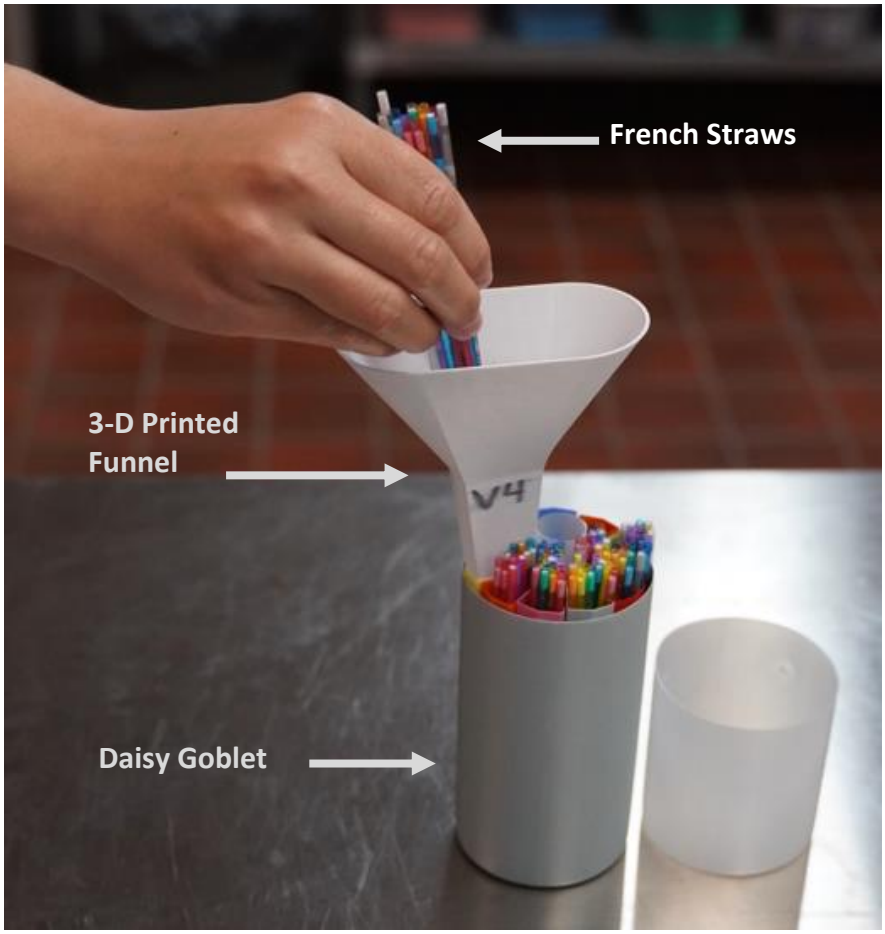

Figure 2.3. The three-dimensional printed funnel designed for sorting of 15 French straws into the compartments of a daisy goblet. This is a demonstration photo without liquid nitrogen for picture clarity. The goblet cap is shown on the right. The entire goblet and samples can be stored in liquid nitrogen or in vapor-phase nitrogen.

Results

Process Mapping of the Cryopreservation Protocol

A process flow diagram was developed based on a representative high-throughput cryopreservation protocol (Yang et al. 2012) (Figure 2.2). In total, 21 steps, five QA checkpoints and three QC measures were identified and described. Only the numbered steps shown in Figure 2.2 were measured during time studies, and only Steps 4–19 (the cryopreservation steps) were analyzed for the current study.

Model Creation and Validation

i. Time Studies and Simulation Modeling

In total, nine cryopreservation trials were performed across 2 y in Spring 2020 and Spring 2021 (Table 2.3). Trials 1–6 took place from March 20 to June 5, 2020. Trials 7–9 took place from April 5 to 14, 2021. A total of 3,444 straws from 89 males were cryopreserved (Table 32.). The time data collected for each step in Trials 1–6 were converted into time distributions (Table 2.1). The Operating and Capital Costs to perform each step was also calculated (Table 2.1). Finally, time distributions were calculated for data collected in Trials 7–9 (Appendix A, Table A.1). Three steps, Steps 12, 13 and 19, were only performed once or twice during each cryo- preservation trial. Therefore, there were not enough replicates across the nine trials to create two time distributions and the same time distributions were used in Models A and B.

ii. Model Validation

When the average time to complete each step (s straw−1) was compared between Models A and B, Steps 5 and 6 were significantly faster in Model B (one-way ANOVA, $P \leq 0.03$, for all comparisons). Step 5 (Shuck) took an average of 2.4 ± 1.8 s straw−1 in Model A and 1.3 ± 0.9 s straw−1 in Model B, whereas Step 6 (Verify Sex) took an average of 1.4 ± 0.7 s straw−1 in Model A and 1.1 ± 0.4 s straw−1 in Model B. All other steps (Steps 4 and 7–19) had similar average times when Models A and B were com- pared (one-way ANOVA, $P \geq 0.05$, for all comparisons).

Models A and B tend to yield similar results for Throughput, TIS, and Costs (Operating Costs and Cost per Oyster) despite Steps 5 and 6 having different average processing times. Under baseline conditions (Model A, 45 straws per oyster, batch size of five, one operator), 15 oysters could be processed in an 8-h day. The processing of 15 oysters produced 675 units (straws) of frozen germplasm that required three daisy goblets for storage. Models A and B had less than 5% difference in Throughput, TIS, and Costs results (Table 2.2). In addition, results from Models A and B corresponded with “real-world” results reported (number of oysters processed in a given amount of time: 12 – 13 oysters in 8 h) by experienced cryopreservation professionals (A. Guitreau and W. Childress, personal communication, cryopreservation professionals at the AGGRC for >10 y).

Table 2.2. Output statistics [Throughput, Time is System (TIS), Operating Cost, and Cost per Oyster] and identified bottlenecks (steps with the longest waiting and processing time) for Models A–E under baseline conditions (45 straws per oyster, batch size of five, one operator, 8 h runtime).

Model	Throughput	TIS (h)	Operating Cost ($)	Cost per Oyster ($)	Longest waiting time (step name and min)		Longest processing time (step name and min)	
A	15.0 ± 0.0	5.63 ± 0.33	559 ± 13	21.4 ± 0.8	Step 12	7.66 ± 1.05	Step 18	11.75 ± 0.44
B	15.0 ± 0.0	5.77 ± 0.33	539 ±9	21.4 ± 0.5	Step 12	7.86 ± 0.74	Step 18	12.13 ± 0.43
C	18.0 ± 1.9	6.00 ± 0.53	575 ± 11	21.4 ± 0.4	Step 12	7.57 ± 0.74	Step 10	4.00 ± 0.07
D	20.0 ± 0.0	5.69 ± 0.24	623 ± 11	21.2 ± 0.4	Step 7	6.90 ± 0.82	Step 18	12.00 ± 0.33
E	24.0 ± 1.5	5.80 ± 0.27	685 ± 15	21.1 ± 0.4	Step 13	6.58 ± 0.46	Step 8	2.72 ± 0.05

Bottlenecks and Waste

The bottlenecks identified in Models A and B were at Steps 12 and 18. Step 12 (Make CPA & Prepare Tubes) had the longest waiting times in each model (Table 2.2). The average waiting time of all other steps in both models was less than 6 min (Model A average, 5.24 ± 1.31 SD min; Model B average, 5.44 ± 1.33 SD min). When comparing processing times, Step 18 (Sort) had the longest average processing time for both models (Table 2.2). The average processing time of all other steps in both models was less than 2 min (Model A average, 1.40 ± 1.04 SD min; Model B average, 1.46 ± 1.11 SD min). Optimization results for Models A and B were less than 5% different. In each model, the parameter values recommended by the optimization software were five straws per oyster, a batch size of eight (sperm samples collected from eight oysters processed simultaneously), and three operators (Table 2.2).

On average, across all nine trials, 31 ± 15% of shucked oysters were female. Furthermore, across all nine trials, an average of 44 ml of sperm were collected from each oyster (after dilution to 2 ×

10^8 cell ml^{-1} in Ca-free HBSS650). On average, 71% of sperm was "wasted" (not frozen) due to the decision to limit the number of straws frozen per oyster (Table 2.3).

Table 2.3. Details of each cryopreservation trial including the date the trial took place, the number of individual oysters cryopreserved, the number of straws frozen per oyster, and the straw throughput (total number of straws successfully preserved).

Trial	Date	No. of Oysters Processed	Straws per Oyster	No. of Straws Processed
1	3/20/20	4	25	96
2	3/24/20	9	25	215
3	3/27/20	6	40	144
4	4/02/20	11	40	515
5	4/17/20	9	40	453
6	6/05/20	20	40	754
7	4/05/21	10	40	385
8	4/09/21	10	45	442
9	4/14/21	10	45	440

Device Options and Model Modification

Throughput, TIS, Operating Cost, and Cost per Oyster were calculated for Models A–E (Table 2.2). Model E had the highest Throughput, followed by Model D and Model C. Operating Costs increased in Models C–E as compared with Models A and B. Model E had the highest increase in Operating Costs, at 18%, when compared with Model A. Cost per Oyster decreased by less than 2% in Models D and E when compared with Models A and B. The average Total Cost increased substantially (an increase of over >$25,000) in Models D and E ($194,727 ± 12 SD) as compared with Models A and B ($168,619 ± 11 SD). The majority of bottlenecks identified in Models C–E were different from those identified in Models A and B (Table 2.2).

In Model C, Step 12 had the longest average waiting time (similar to Models A and B). The step with the longest average processing time in Model C was Step 10 (Motility) as opposed to Step 18 in Models A and B. Model C substituted a three- dimensional printed funnel specifically designed to funnel 15 French straws at a time into a compartment of a daisy goblet, as opposed individually sorting straws with the forceps. Using the sorting funnel decreased the processing time of Step 18 by 93%, from an average of 11.75 ± 0.44 min in Model A to 0.77 ± 0.11 min in Model C.

In Model D, Step 7, had the longest average waiting time and Step 18 had the longest average processing time. Using CASA decreased the processing time of Step 10 by 99%, from an average of 4.0 ± 0.07 min in (Model A) to 0.04 ± 0.01 min in (Model D). In Model E, Step 13, had the longest average waiting time and Step 8 had the longest average processing time. The optimized values for Number of Straws and Number of Operators for Models C–E were the same as in Models A and B (Table 2.4). Optimized batch size for Models C and D was slightly lower than in Models A, B, and E.

Table 2.4. Optimized parameter values (No. of Straws, Batch Size, and No. of Operators) and the resulting output statistics [Throughput, Time is System (TIS), Operating Cost, and Cost per Oyster] for Models A–E.

Model	No. of Straws	Batch Size	No. of Operators	Throughput	TIS (h)	Operating Cost ($)	Cost per Oyster ($)
A	5	8	3	66 ± 4	5.48 ± 0.31	1,221 ± 11	10.6 ± 0.5
B	5	8	3	64 ± 0	5.46 ± 0.21	1,196 ± 10	10.7 ± 0.3
C	5	7	3	63 ± 0	5.31 ± 0.31	1,205 ± 13	10.9 ± 0.3
D	5	7	3	80 ± 5	5.24 ± 0.22	1,390 ± 13	10.6 ± 0.3
E	5	8	3	80 ± 7	5.30 ± 0.28	1,398 ± 22	10.3 ± 0.7

Discussion

This study addressed the challenges of repository development in oyster aquaculture by simulation modeling of high-throughput sperm cryopreservation. Overall, previous discrete-event simulation modeling studies in aquaculture have been limited. Modeling was used to assist identification of optimal layout and management strategies for a recirculating aquaculture system (Halachmi et al. 2005). Work has also been done on modeling the logistics of a salmon aquaculture supply chain (Rørtveit & Lilienthal 2017), and emergency responses for pollution in aquaculture systems (Thunes 2018). In general, however, discrete-event simulation modeling has been underutilized in aquaculture and there are ample opportunities to use this tool in future work.

With specific relevance to cryopreservation, a discrete-event simulation model was built to analyze high-throughput processing of blue catfish (*Ictalurus furcatus*) to assist in facility planning (Hu et al. 2015) using a different modeling software (ARENA, Rockwell Automation, Inc. Milwaukee, WI). The present study extended this previous work by analyzing a cryopreservation process and adjusting that process based on identified constraints of the system. The steps of a high-throughput protocol were defined and mapped in a process flow diagram. A simulation model was created based using time study data and was validated. Sources of waste were identified in the model, and variations were created using alternate device options. Model A was found to accurately simulate the oyster cryopreservation process. Important parameters such as number of straws frozen per oyster, batch size, and number of operators affected how all models

(A–E) performed. Alternate device options in Models C–E changed production results, such as increasing Throughput, when compared with Models A and B.

Oyster Cryopreservation

Cryopreservation processing capabilities modeled in this study aligned with real-world, oyster processing capabilities, and also with previous catfish cryopreservation processing capabilities. In an 8-h day, 18 blue catfish could be processed using equipment similar to the current study (Hu et al. 2015). The protocol used for catfish processing, however, required four operators to process 18 animals and three of four operators were dedicated to testis dissection and cleaning (Hu et al. 2015). This is because collecting catfish gonads requires more a labor-intensive dissection and cleaning process than required for oyster gonads (Yang et al. 2012). Furthermore, the gamete collection strategies for fish species such as Atlantic salmon (*Salmo salar*), require only gentle pressure applied to the abdomen, and are less labor intensive (Yang et al. 2018). These examples show how specific reproductive biology characteristics of aquatic animals can affect the resources necessary to cryopreserve samples from a specific number of animals in a single workday.

Although gamete collection in oysters may be relatively straightforward, cryopreservation presents unique challenges, such as the inability to identify the sex of animals before removing the shell. Oysters are protandrous hermaphrodites, with 1- and 2-year-old animals generally spawning as males, and older animals spawning as females (Baghurst & Mitchell 2002). On average, 31% of the 1- and 2-year-old oysters processed in this study were female and their

gametes were not cryopreserved. Catfish cryopreservation in contrast, avoided this because fish were screened prior to processing and 100% of processed fish were males (Hu et al. 2013, 2014). The challenges and opportunities presented by the reproductive mode of various aquatic animals must be taken into consideration during cryopreservation protocol development, especially when predicting how the protocol can be integrated into a repository system.

Bottlenecks and Waste

The Theory of Constraints, an approach used to improve manufacturing processes, states that systems have at least one bottleneck which impedes the system from reaching its highest level of performance (Gupta & Boyd 2008, Şimşit et al. 2014). In the baseline model (Model A), Steps 12 and 18 were identified as bottlenecks. Step 12 (Make CPA & Prepare Tubes) had the longest waiting times because it was the last step before samples were grouped into batches for processing in Steps 13–18. Therefore, the operation was switching from one-piece flow to batch processing between Steps 12 and 13.

One-piece flow is generally considered a faster method of processing than batch processing (Millstein & Martinich 2014, Protzman et al. 2017). One-piece flow refers to a process where the operator of each step completes work on a single part or sample before sending it through to the next step. Batch processing refers to a process where the operator of each step completes work on a group of samples (a batch) before sending the batch on to the next step (Millstein & Martinich 2014). One-piece flow is faster under certain conditions because it limits the amount of queuing before a preceding or subsequent step (Protzman et al. 2017). In the cryopreservation

protocol described in the current study, batch processing was unavoidable due to time-sensitive constraints of cryopreservation (the equilibration time).

The 20-min time limit before freezing, which began after diluted sperm samples were mixed with cryoprotectant, was set to maximize equilibration phenomena (e.g., diffusion and dehydration) while minimizing toxicity (Tiersch et al. 2011, Yang et al. 2012). In that 20-min window, samples were packed in French straws that were sealed, placed on a freezing rack, and inserted into the freezer. This limited the number of samples that could be processed. After freezing, the batched straws were removed from the freezer and sorted into daisy goblets before the next batch could enter Step 13. Therefore, batch processing was implemented to ensure that all samples entering Step 13 would be ready for freezing at the 20-min mark. This resulted in an accumulation of samples in the step immediately preceding where processing switched from one-piece flow to batching. Thus, processing constraints demonstrate why simulation modeling and identification of bottlenecks are important when building and operating a repository.

The bottleneck identified at Step 12 was exacerbated by the bottleneck identified at Step 18 (Sort Straws) which had the longest average processing time for Models A and B. The step with the longest processing time was in the part of the model that used batch processing, compounding the time needed to complete Steps 13–18 and causing a build-up of samples waiting to be processed. Step 18 had the longest processing time because the original method (the forceps) used to sort frozen straws was inefficient and prone to error, causing movement waste. In addition, sorting required extra work because straws were not transferred directly from the

freezer to the daisy goblet but to an intermediary location (the bottom of the cooler) before being sorted into their final location, the daisy goblet (transportation waste, Joseph 2006). This exemplifies how waste identified in one step has consequences that affect waiting times of steps located upstream and can affect processing capabilities as shown in the following section.

Device Modification

Models C–E used alternate device options and consequently had different results and bottlenecks. When the three-dimensional printed funnel was substituted into Model C, Throughput increased and a major bottleneck (Step 18) was eliminated, whereas TIS and Operating Cost, Cost per Oyster, and Total Cost remained similar (<5% difference) to Model A. The decreased movement and transportation wastes when using the funnel allowed for increased Throughput capacity (Joseph 2006). One of the advantages of three-dimensional printed hardware is low manufacturing cost (Liu et al. 2021) which allowed Model C to benefit from increased Throughput without increasing the Total Cost to run the system. In addition, three-dimensional printed tools can be shared as open hardware, making devices with specific uses accessible to any- one with a three-dimensional printer (Childress et al. 2021, Liu et al. 2021). Despite the step with the longest processing time in Model C no longer being located in the batch processing section of the model, Step 12 still had the longest waiting times. This demonstrates that even with substantial improvements in processing times, batch processing can still create bottlenecks upstream in the manufacturing process.

Step 10 (Motility) had one of the longest processing times in Models A–C. Motility was analyzed manually using a Makler® counting chamber, which required an operator to count and record the number of moving and nonmoving sperm cells within grid lines (Torres et al. 2017). Manual measurement of motility was time consuming in Models A–C and was cumbersome for operators. An option explored in Models D and E was to automate Step 10 by using CASA which can rapidly and objectively report sperm motility (Agarwal et al. 2016). Automation is not always advantageous because it adds complexity to the system through machine downtime and maintenance, which can increase processing time (Edwards 1996). Conversely, in situations where automation alleviates repetitive manual work without causing frequent delays, the benefits outweigh the drawbacks and automation can be implemented (Edwards 1996, Hedelind & Jackson 2011), as was the case in Models D and E.

In both models, Throughput increased compared with Model A, and Step 10 had a greater than 95% reduction in processing time. Furthermore, Model E had the highest Throughput of any model and a 60% increase compared with the Model A. Incorporation of the three-dimensional printed funnel and the CASA provided the increased Throughput by eliminating the two largest processing bottlenecks at Steps 10 and 18. When these bottlenecks were removed Step 8 (Strip Spawn) became the step with the longest processing time in Model E. As a consequence of higher Throughput in Models D and E, Operating Costs also increased an average of 14% compared with Model A because of the increased supply costs from processing more oysters. The average Total Cost of each model increased by $26,000 as compared with Model A because the CASA was substituted into Models D and E. Investments in expensive, automated equipment that increase

model production capacity (such as CASA) may only make economic sense at higher scales of production (Caffey & Tiersch 2000, Hu et al. 2011, 2015). In addition, other benefits of automated equipment, such as increased standardization, should also be considered (Dong et al. 2007). Decisions on what devices should be used need to be balanced among several factors: project scale and goals, device costs, and the training needs for the incorporation of new devices.

Model Optimization

Models A–E had the same optimal values for the number of straws per oyster, batch size, and number of operators (except for Batch Size in Models C and D, Table 2.4). The optimal number of straws recommended for each model was the smallest number of straws permitted in the optimization program, five straws per oyster. Freezing fewer straws per oyster resulted in higher Throughput for all models (an increase of about 50 oysters) compared with the corresponding Throughput values with the baseline number of straws (45). The optimization program was instructed to prioritize maximized Throughput and therefore minimized the number of straws. Minimizing the number of straws frozen per oyster is not always advisable. Five French straws would contain 2.5 ml of sperm solution or 2.5×10^8 total sperm (each straw would contain approximately 5×10^7 sperm cells). This volume would be sufficient to fertilize eggs from a single female oyster (approximately 2×10^7 eggs) at a 15:1 sperm to egg ratio (Yang et al., 2012, 2013; Wallace et al., 2008), however, at least two frozen straws should be used to assess post-thaw motility (a QC measurement). This would leave three straws for breeding purposes, which may not be sufficient in many circumstances (Yang et al. 2012, Torres et al. 2017).

Freezing this number of straws would also increase the amount of sample waste generated in the system. An average of 44 ml of diluted sperm was collected per oyster (88 ml when cryoprotectant was added) and 97% of collected sperm solution would be wasted if only five straws per oyster were frozen. In the only other studies of high-throughput cryopreservation in aquatic species, the total volume of sperm collected from blue catfish was frozen (Hu et al. 2014). In this study, if 100% of collected sperm was cryopreserved an average of 176 straws per oyster would be frozen. This, however, could affect Throughput and raise costs, particularly at larger scales of production. Therefore, the interactions among the number of straws, the Throughput capacity of the system, and production scale can be complex and warrant further study.

The optimal batch size recommended for each model version was the highest, or nearly the highest, value permitted to maximize Throughput. Batch size determined how many oysters could be processed as a group in Steps 13–18. It is important to note, however, that the optimization program could only select the largest batch size because the number of straws was set to the lowest permitted value (five). If the straw number was greater, there would not be enough time during equilibration to process larger batches of oysters. Decisions must be made to balance the total number of germplasm units produced with the amount of time required for production. Operators could decide on the number of straws needed per oyster and base the batch size on the maximum they can process in the equilibration window. Alternatively, operators could choose a larger batch size and limit the number of straws per oyster to an amount that would allow for that batch size. This would maximize the number of oysters that can be processed in a day, but would limit the number of germplasm units collected from each oyster.

Each scenario may be appropriate under different circumstances and factors should be balanced to account for the requirements of the repository system. It should be noted that all these scenarios can be evaluated with the simulation model.

One way to increase batch size, and overall production capacity, without sacrificing the number of straws was to increase the number of operators assigned to the system. Therefore, the optimization program recommended the maximum number of operators for all models. Increasing the number of opera- tors increased the Throughput and also increased the Operating Costs because of additional operator wages and increased sup- ply costs from higher production levels. Analyzing how Cost per Oyster (Operating Costs divided by oyster Throughput) changes with the number of operators more accurately reflects the effect that adding operators has on the system. Further analysis of the relationships among parameters, such as batch size and number of operators, and system outputs such as Throughput and Costs can be performed by simulation modeling. One final consideration when discussing operators is experience or skill level. Skilled operators can perform work more quickly, and there- fore replacing or training less-skilled operators could increase Throughput without requiring additional operators (Devotta 1983, Heneman et al. 1987, Hu et al. 2015).

Conclusions

The goal of this study was to address the gap between bench-scale cryopreservation research and repository development in aquatic species by simulation modeling of oyster cryopreservation. The complexity of the model offers the potential for specific recommendations

for a cryopreservation pathway that could integrate into a repository system when provided with additional information on the requirements of that system, such as specific production scale, equipment availability, cost targets, and personnel availability. The interconnected nature of cryo- preservation pathways with aquaculture repositories and user communities matches very well with the strengths of simulation modeling. Alternate models can be easily created and run, without actual investment in labor, management, construction, and equipment (Hu et al. 2015). This allows different scenarios to be analyzed rapidly to develop and maintain efficient operations. Simulation models can also be user-driven with input from stakeholders to more accurately represent community interactions.

Finally, the interdisciplinary nature of aquaculture, cryopreservation and repository development lends itself to simulation modeling. These models can incorporate variables from across disciplines involved in cryopreservation and offer a complete view of repository systems (Jones et al. 2017). This study can support high-throughput oyster cryopreservation and can serve as a model for other aquatic species. Simulation modeling can greatly facilitate repository development for all aquaculture species and help ensure that aquatic genetic resources are protected and available for efficient use. Simulation modeling offers a variety of opportunities for future research. Future research can make specific recommendations about parameters and device options for an oyster cryopreservation pathway by recognizing the scale and goals of production. Future research can also address the need for open, harmonized data and a centralized database. Proper and consistent data collection and storage can be a major constraint

on repository systems and simulation modeling provides a means to standardize and streamline these crucial aspects of germplasm repositories.

Chapter 3. Simulation Analysis of High-throughput Oyster Cryopreservation at Three Scales of Production

Introduction

Oyster aquaculture dates back centuries to the time of the Roman empire and remains an important industry today (Botta et al., 2020). Throughout that history technological advancement has substantially shifted the landscape of oyster aquaculture. The introduction of polyploid animals allowed farmers to harvest earlier in the season or harvest during spawning months (Allen Jr. & Downing, 1986). The popularization of off-bottom gear along the U.S. Atlantic coast allowed the industry to shift focus to the high-end, half-shell market (Petrolia et al., 2022). Now new technologies have emerged, and this study will attempt to anticipate the changes that cryopreservation and repository storage will have on oyster aquaculture.

Repositories are rigorously maintained collections of cryopreserved (often frozen) germplasm samples and associated information, such as phenotypic and genetic data (Hu et al., 2015). Through cryopreservation and repository storage, farms, hatcheries, and stock centers can safeguard valuable genetics from losses due to disease, accidents, and natural disasters while simultaneously reducing the number of live populations that must be maintained (Yang et al., 2021a). In addition, repositories enable selective breeding programs and rapid genetic improvement by providing ease of transport of germplasm (e.g., cryopreserved sperm) across hatcheries (Curry, 2000; Moore & Hasler, 2017; Yang et al., 2012). The dairy industry first began using cryopreserved semen from repository collections for artificial insemination in the 1950s and today the practice is established worldwide (Moore & Hasler, 2017).

In the United States alone, more than 17 million breeding units (typically packaged within 0.5-ml French straws) of dairy bull semen were sold in 2021 (National Association of Animal Breeders, 2022). The use of cryopreserved semen has enabled precise breeding practices that have facilitated significant increases in milk production, health, and fertility (Carvalho et al., 2022; Moore & Hasler, 2017). The development and integration of germplasm repositories for eastern oysters (*Crassostrea virginica*) into aquaculture operations could increase production in a similar way. Today, farmers face oyster mortality due to challenges such as disease and unsuitable water quality conditions including low salinity (Casas et al., 2018; Matt et al., 2020). Breeding of oyster lines that are tolerant to these challenges and distributing their cryopreserved sperm across hatcheries could decrease mortality and allow breeders to work collaboratively to advance genetic improvement in a process similar to the advances seen in the dairy industry (Moore & Hasler, 2017). Despite these potential benefits and more than 30 years of cryopreservation research, repositories are not widely used in oyster aquaculture or any aquaculture industry (Paniagua-Chavez & Tiersch, 2001; Yang et al., 2012, 2021).

Oyster aquaculture is an important part of the United States economy, with eastern oyster farmed along the coasts of the Atlantic and Gulf of Mexico. Oyster hatcheries produce larvae which are raised until becoming juveniles that are large enough to be sold to farmers and grown in field sites (Wallace et al., 2008). Oysters have traditionally been grown sub-tidally and “on-bottom”, but “off-bottom” aquaculture (where oysters are suspended in the water column using various types of equipment) has become increasingly popular, particularly along the Atlantic

coast (Walton & Swann, 2021). To offset the higher investment of using “off-bottom” equipment, many farmers have turned to growing triploid (3N) oysters (Petrolia et al., 2022). Triploids have three sets of chromosomes instead of the usual two (diploids, 2N) and as a result have reduced gametogenesis, faster growth, and better meat quality than diploids. Due to their inability to reproduce, hatcheries produce triploids by crossing tetraploid (4N) males with diploid females (Dong et al., 2005). The popularity of triploids and the resources required to produce tetraploid lines make tetraploid germplasm valuable to hatcheries. Hatcheries also have valuable germplasm in the form of selectively bred diploid lines, created to be disease resistant or resistant to low salinity (Casas et al., 2017; Haskin & Ford, 1979; McCarty et al., 2020). Thus, it is problematic that despite the value of these oysters, their genetic resources are not routinely stored in germplasm repositories.

Integration of repositories into an aquaculture system is a complex task that requires analysis of numerous interacting resources and communities. Understanding those interactions is crucial for repository development in aquaculture, and simulation models are a useful tool for doing so. A specific type of analysis, discrete-event simulation models, is capable of emulating large-scale production systems by modeling all the events (steps) and resources that occur or are used in a system throughout time (Allen et al., 2015; Gittins et al., 2020; Schriber et al., 2013). While this type of modeling has historically been used in the manufacturing sector (Robinson, 2005), it has also been applied to other industries such as healthcare (Oueida, 2016).

Discrete-event simulation models have utility to benefit aquaculture as well, particularly concerning the slow adoption of cryopreservation technology and germplasm repositories. The

goal of this study was to respond to the challenges of repository development in oyster aquaculture by evaluating cryopreservation needs at different scales of production. The objectives were to: 1) characterize two existing models of high-throughput oyster cryopreservation; 2) evaluate the effect of three parameters (Straws per Oyster, Batch Size, and Number of Operators) on the Throughput, Time in System, and Operating Cost of the models; 3) develop scaling and breakeven point analyses to analyze the models at the different scales; 4) make recommendations for high-throughput cryopreservation pathways at each scale of production, and 5) describe the relative values of broodstock, juvenile oysters sold by hatcheries (oyster "seed"), and oyster sperm to provide context for uses of germplasm resources.

Methods

Introducing the Cryopreservation Models

The two cryopreservation models, Models A and E, used in this study were initially developed in a previous study (Chapter 2, Bodenstein et al., 2022). Time study data of the steps comprising the cryopreservation protocol (Yang et al., 2012) were used to generate discrete-event simulation models in the software Simio (2021, v14.230, Simio LLC, Sewickley, PA). To simulate real-world conditions four key parameters were set to baseline values: the Workday (run time) was 8 h, the Number of Operators was one, the Straws per Oyster was 45, and the Batch Size was five. These values were chosen to reflect the working conditions and constraints of a single operator cryopreserving oyster sperm. Batch Size refers to the number of oysters for which sperm samples were being processed simultaneously during Step 13 – 18, the steps which contained time-sensitive constraints of the cryopreservation process. When diluted sperm

samples were mixed with cryoprotectant, a 20-min equilibration window began that was set to maximize chemical and physical phenomena (e.g., diffusion and dehydration) and minimize toxicity (Tiersch et al., 2011; Yang et al., 2012). During that 20-min window, samples had to be packaged into French straws, placed on a freezing rack, and inserted into the freezer unit before time expired. This limited the number of samples (i.e., Batch Size) that could be frozen at one time. Associated costs were also inserted into Models A and E.

Five models, Model A-E, were created and evaluated in Bodenstein et al. (2022b), each with different equipment or devices implemented that altered model production efficiency (throughput) and cost. Models A and E were selected for analysis in the current study because they represented the cryopreservation pathway under baseline conditions (Model A) and the pathway with all device improvements incorporated and enhanced Throughput (Model E). Models A and E contained the same steps, logic rules, and baseline conditions. They also used the same devices (tools and equipment) to process samples except for the specific options used in the steps where sperm motility was measured and the step where straws were sorted into storage containers after freezing. Computer-assisted sperm analysis equipment (CASA, CEROS II™ Animal - Hamilton Thorne Ltd, Beverly, MA) was used to quantify sperm motility in Model E instead of the microscope and Makler® counting chamber used in Model A. Additionally, a custom designed 3-D printed funnel was used to sort 15 French straws at a time into daisy goblets after freezing was completed in Model E instead of individually sorting straws with forceps (Model A). These changes were reflected in new time distributions for the steps in Model E where motility assessment and sorting of straws took place. These alternate devices were substituted

into Model E to improve throughput because previous work identified these steps as major process constraints (Bodenstein et al., 2022). In the current study, Model A will be referred to as the “Baseline Model” and Model E will be referred to as the “Enhanced Model”.

The major outputs analyzed for both models were Throughput, Time in System (TIS), Operating Cost, Cost per Oyster, and Total Cost. Throughput was the number of oysters fully processed during the 8-h workday. Time in System was the average amount of time it took to process an individual oyster (i.e., the average each oyster spent in the model). Operating Cost was the average cost of supplies and operator wages to process all oysters in a single batch, excluding Capital Costs (the one-time cost of equipment). Cost per Oyster was the average Operating Cost of processing of each oyster. Finally, Total Cost was the cost to process all oysters in a single batch including Operating and Capital Costs.

Evaluating Parameter Effects

Linear regressions were used to determine relationships among three parameters (Straws per Oyster, Batch Size, and Number of Operators) and three output statistics (Throughput, Operating Cost, and Time in System) for the Baseline and Enhanced Models. The workday remained consistent at 8 hours for all comparisons.

Scaling and Break-even Point Analyses

To assess how the Baseline and Enhanced Models would perform at different scales of production, a series of scenarios was created. Scenario 1 required that 50 oysters be processed in one year. Scenario 2 required that 1,000 oysters be processed in one year, and Scenario 3 required that 10,000 oysters be processed in one year. In addition, the Number of Operators in each scenario was tested at 1, 2, 3, 5, or 10 to analyze the effect varying this parameter had on results, such as Percent Processing Failure, required Processing Time, Breakeven Price, and Breakeven Times (all results defined in the next paragraph). Each scenario required that 45 straws would be frozen per oyster and the Baseline and Enhanced Models were run using each scenario. These scenarios were not run within an 8-h workday, but rather continued to run until all required oysters from a given scenario had been processed.

The Percent Processing Failure statistic represented the number of oysters per year that were not successfully cryopreserved. Oysters “failed” and were discarded from the system if freezing had not begun before the 20-min equilibration time ended. At the end of the simulation, the ratio of failed oysters and oysters that were fully processed was calculated. To calculate Percent Processing Failure a timer was placed at the beginning of the step where equilibration time began in both models. This timer was triggered each time a batch of oysters entered the step. A second timer was in the step where freezing began (after the 20-min equilibration time had ended). If longer than 20 min passed between a batch triggering the first and the second timers, the batch was discarded and all oysters within that batch were considered “failed”. The required Processing Time was the number of 8-h workdays needed to process all oysters under a given scenario.

The Cost per Straw, Breakeven Price, and Breakeven Times were also calculated to perform a breakeven point analysis. Such analyses are commonly used in business to determine the amount of revenue needed to cover Total Costs, which includes Operating and Capital Costs (Childress et al., 2018; Gandonou et al., 2006). The Cost per Straw was the cost needed to cover the Operating Costs to produce a certain number of straws and was calculated by dividing the Operating Costs by the total number of straws produced. The Breakeven Price of straws, the Cost per Straw needed to cover Total Costs, was calculated using the equation $BeP = (CC + (OC \times t))/(NS \times t)$, where BeP is the Breakeven Price of straws, CC is the Capital Cost, OC is the Operating Cost, t is the time in years (a one-year time period was allotted), and NS is the total number of straws produced. A one-year time period was selected when calculating Breakeven Price to demonstrate a "worst case" scenario where profits are required by the second year.

Breakeven Price could also be viewed as the cost to produce 1 straw, including initial Capital Costs. The Breakeven Time was the time, in years, required to pay off initial Capital Costs and Operating Costs for each year of production based, on the price of a straw. The Breakeven Time was calculated using the equation $BeT = (CC/(SP - CP))/NS$, where BeT is the Breakeven Time, CC is the Capital Cost, SP is the straw price, CP is the average cost to produce a straw, and NS is the total number of straws produced. There currently are no industry pricing structures for frozen oyster sperm, therefore, three fixed straw prices were selected based on average prices of semen from dairy bulls. The average minimum price reported was $14, the average price for "non-sexed" (does not guarantee female offspring) semen was $27, and the average price of all dairy

bull semen was $57 (Carvalho et al., 2022). Based on these values, the three fixed straw prices selected for this study were $15, $30, and $60.

In addition to fixed straw prices, the potential Gross Straw Value was calculated. The Gross Straw Value was calculated using the equation: Gross Straw Value = (number of oyster seed per straw × oyster seed price point). The number of 6 – 10 mm oyster seed that could be produced from a single straw of frozen sperm was calculated using actual oyster fertilization and survival rates, and the number of straws required to fertilize a specific number of eggs based on discussions with hatchery operators (B. Callam pers comm; F.S. Rikard pers comm; Wallace et al., 2008; W.C. Walton pers comm; Table 3.1). The average price to purchase a single diploid, 6 – 10 mm oyster seed from a hatchery was calculated to obtain the straw price point (Oyster Seed Holdings Inc., 2021; University of Maryland, 2022). Finally, the Net Straw Value was calculated by subtracting the Breakeven Costs (production costs) from the Gross Straw Value.

Table 3.1. For each major life stage of an oyster, the Time After Fertilization, Percent Fertilization, Percent Survival, estimated number of that life stage, and estimated number that could be produced from one straw of cryopreserved semen was calculated. The number of eggs reported (2×10^8) were from 10 females each producing 2×10^7eggs.

Life Stage	Time After Fertilization	Percent Fertilization	Percent Survival	Estimated Number at Each Life Stage	Number Produced from One Straw
Eggs	NA	20%	---	2×10^8	---
Larvae	1 d	---	16%	4×10^7	6.6 × 105
Pediveligers	14 d	---	33%	6.4×10^6	1.1×10^5
Spat	14-17 d	---	80%	2.1×10^6	3.5×10^4
Seed (R8)	3-4 months	---	70%	1.7×10^6	2.8×10^4
Adults	1 y	---	---	1.2×10^6	2.0×10^4

Relative Values of Broodstock, Juvenile Oysters, and Sperm

A table was generated to describe the relative values of broodstock oysters, juvenile oysters sold by hatcheries (oyster "seed"), and oyster sperm. Oyster seed order forms from commercial hatcheries were analyzed to compare the prices of different types of seed offered, such as seed from diploid, triploid, "wild" (the line had undergone no breeding program), and selectively bred lines. The relative values of seed were assessed from the perspective of commercial oyster producers, where triploid seed would be more valuable (compared to diploids) due to faster growth rates and selectively bred lines would be more valuable (compared to wild lines) due to valuable characteristics, such as disease resistance. Next, the relative values of broodstock oysters and frozen sperm were assessed based on the perspective of hatchery managers who would sell the resulting seed to producers. A hatchery manager with more than 5 years of experience was consulted (B. Callam, pers. comm.) and the previously defined seed value also influenced broodstock and sperm value.

Results

Parameter Effects

The Straws per Oyster, the Batch Size, and the Number of Operators affected the Throughput, Time in System (TIS), and Operating Cost in the Baseline and Enhanced Models (Figure 3.1). As the Batch Size and the Number of Operators increased so did the Throughput and Operating Cost in both models (Figure 3.1 B, C, E, & F). As the Straws per Oyster increased, Throughput decreased while Operating Cost increased in both models (Figure 3.1 A & D). While the relationship trends between the three parameters (Straws per Oyster, the Batch Size, and the Number of Operators)

and the Throughput and Operating Cost were the same in the Baseline and Enhanced Models, the magnitude of their effects (the slopes) were higher in the Enhanced Model (Figure 3.1 B-F). The exception being the effect of Straws per Oyster on Throughput, where the effect was stronger in the Baseline Model than the Enhanced Model (Figure 3.1 A). When examining how TIS was affected by the three parameters, the Baseline Model produced a larger magnitude of effect than the Enhanced Model (Figure 3.1 G-I). In fact, as Straws per Oyster increased, the TIS of the Enhanced Model was not affected while the TIS of the Baseline Model increased an average of 0.6 min for each straw added ($P < 0.001$, Appendix B, Table B.1).

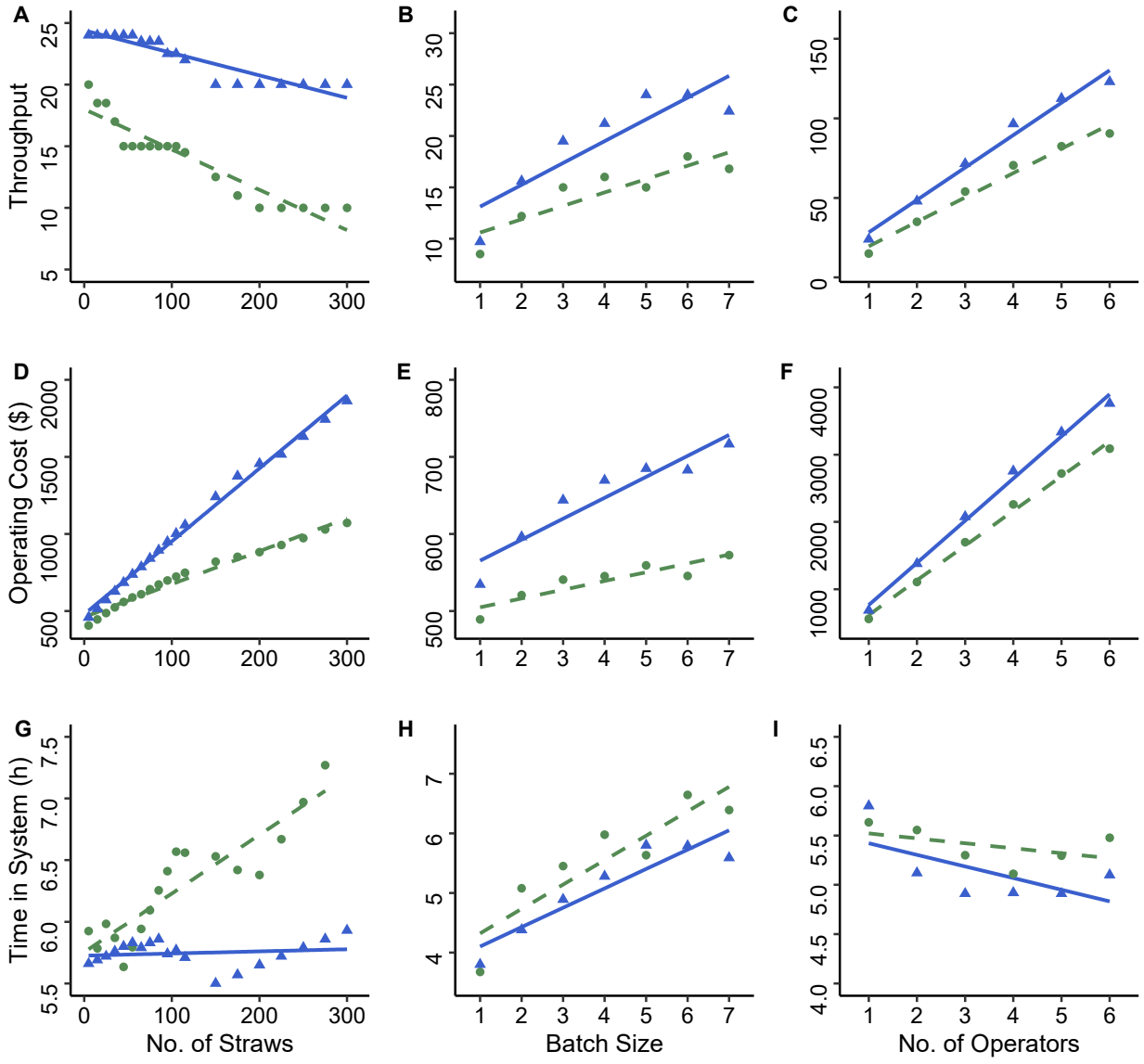

Figure 3.1. Linear regressions analyzing the effects Number of Straws per Oyster, Batch Size, and Number of Operators (arrayed along the X-axis) on the Throughput, Operating Cost, and Time in System (arrayed along the Y-axis) in Models A (dashed lines) and E (solid lines). Circles represent data points from the Baseline Model and triangles present data points from the Enhanced Model. Shaded areas indicated confidence limits for the Baseline Model and Enhanced Model regressions.

Scaling and Break-even Point Analyses

i. Baseline Conditions

Under baseline conditions (45 Straws per Oyster, Batch Size of five, one operator), the Percent Processing Failure values were similar (< 10% difference) for the Baseline Model (mean of 25% ± 0.7) and the Enhanced Model (mean of 11% ± 0.4) across all scales of production (Table 3.2). The required Processing Times (number of 8-h workdays) increased as the scale of production increased (when the Number of Operators was held constant) for both models. The Enhanced Model, however, had shorter Processing Times than the Baseline Model compared within the same production scales with the same Number of Operators.

Table 3.2. Scaling analysis for the Baseline and Enhanced Models analyzing the effect of Operation Scale (oysters processed per year) and the Number of Operators on Percent Processing Failure, required Processing Time (number of 8-h workdays), Cost per Straw, and Breakeven Price. The Batch Size for all scenarios was five.

Oysters per Year	Model	No. of Operators	Percent Processing Failure (%)	Processing Time (d)	Cost per Straw	Breakeven Price
50	A	1	24.3	7.3	$0.51	$85.27
		2	0.0	4.2	$0.51	$83.63
		3	0.0	3.1	$0.51	$84.45
		5	0.0	2.0	$0.54	$79.81
		10	0.0	1.2	$0.70	$88.96
	E	1	10.7	4.7	$0.48	$93.91
		2	0.0	3.2	$0.49	$94.82
		3	0.0	2.6	$0.48	$94.82
		5	0.0	1.9	$0.48	$98.64
		10	0.0	1.3	$0.50	$97.67
1,000	A	1	25.6	148.2	$0.51	$4.62
		2	0.0	80.2	$0.51	$4.62
		3	0.0	56.0	$0.51	$4.65
		5	0.0	35.6	$0.52	$4.63
		10	0.0	19.3	$0.56	$4.68

Cont'd

Oysters per Year	Model	No. of Operators	Percent Processing Failure (%)	Processing Time (d)	Cost per Straw	Breakeven Price
1,000	E	1	11.4	101.5	$0.49	$5.18
		2	0.0	59.5	$0.49	$5.19
		3	0.0	43.5	$0.49	$5.19
		5	0.0	29.3	$0.49	$5.18
		10	0.0	17.2	$0.53	$5.21
10,000	A	1	25.0	1479.5	$0.51	$0.92
		2	0.0	800.6	$0.51	$0.92
		3	0.0	559.0	$0.51	$0.92
		5	0.0	355.5	$0.52	$0.93
		10	0.0	192.4	$0.55	$0.96
	E	1	11.1	1021.1	$0.49	$0.95
		2	0.0	593.2	$0.49	$0.96
		3	0.0	431.7	$0.49	$0.96
		5	0.0	289.3	$0.49	$0.96
		10	0.0	168.1	$0.53	$1.00

Cont'd

Breakeven Prices (price required for a unit of germplasm to pay off Capital and Operating costs in one year) decreased at larger scales of production (Table 3.2). Breakeven Times (years to pay off Capital and Operating costs based on fixed Straw Price) followed the same pattern, decreasing at larger scales of production (Table 3.3). In addition, as the Straw Price increased, the Breakeven Time decreased for both models at all production scales. The Baseline and Enhanced Models has similar (<5% difference) Breakeven Times across all scales of production for all Straw Prices.

Table 3.3. Results for the Baseline and Enhanced Models analyzing the effect of production scale (oysters processed per year) on Breakeven Time (reported in years or in days). Breakeven Times were based on fixed Straw Prices (SP, $15, $30, and $60 per straw). The Batch Size for all scenarios was five and the Number of Operators was one.

Model	Oysters per Year	Breakeven Time (SP $15)	Breakeven Time (SP $30)	Breakeven Time (SP $60)
A	50	5.9 y	2.87 y	1.42 y
	1,000	103 d	51 d	26 d
	10,000	11 d	4 d	4 d
E	50	5.7 y	2.79 y	1.38 y
	1,000	104 d	51 d	26 d
	10,000	11 d	4 d	4 d

ii. Adjusting the Number of Operators

When more operators were assigned to the Baseline and Enhanced Models, the Percent Processing Failure values and required Processing Times decreased (within the same production scale), while the Breakeven Prices increased (Table 3.2). When more than one operator was assigned to a model, the Percent Processing Failure values dropped to zero at all scales of production. Within the same the same scale of production, as the Number of Operators increased, the Processing Time decreased. A power function was found to explain this relationship (Figure 3.2). Initially, increasing the Number of Operators decreased the Processing Time at a faster rate. For example, Processing Time decreased by 1 d when the Number of Operators increased from one to two in the Baseline Model at the smallest scale of production. However, as more operators were assigned, the rate at which Processing Times decreased was slower. For example, Processing Time decreased by 0.03 d when the Number of Operators increased from nine to ten in the Baseline Model at the smallest scale of production. The slopes of the power functions were higher in models operating at the medium and large scales of production (1,000 and 10,000 oysters per year) than in models operating at the smallest scale

(50 oysters per year, Figure 3.2). When comparing the Baseline and Enhanced Models within the same scale of production, the Baseline Model always had higher slopes. Therefore, increasing the Number of Operators had a stronger effect on Processing Times at higher scales of production across both models and a stronger effect on the Baseline Model across all scales of production.

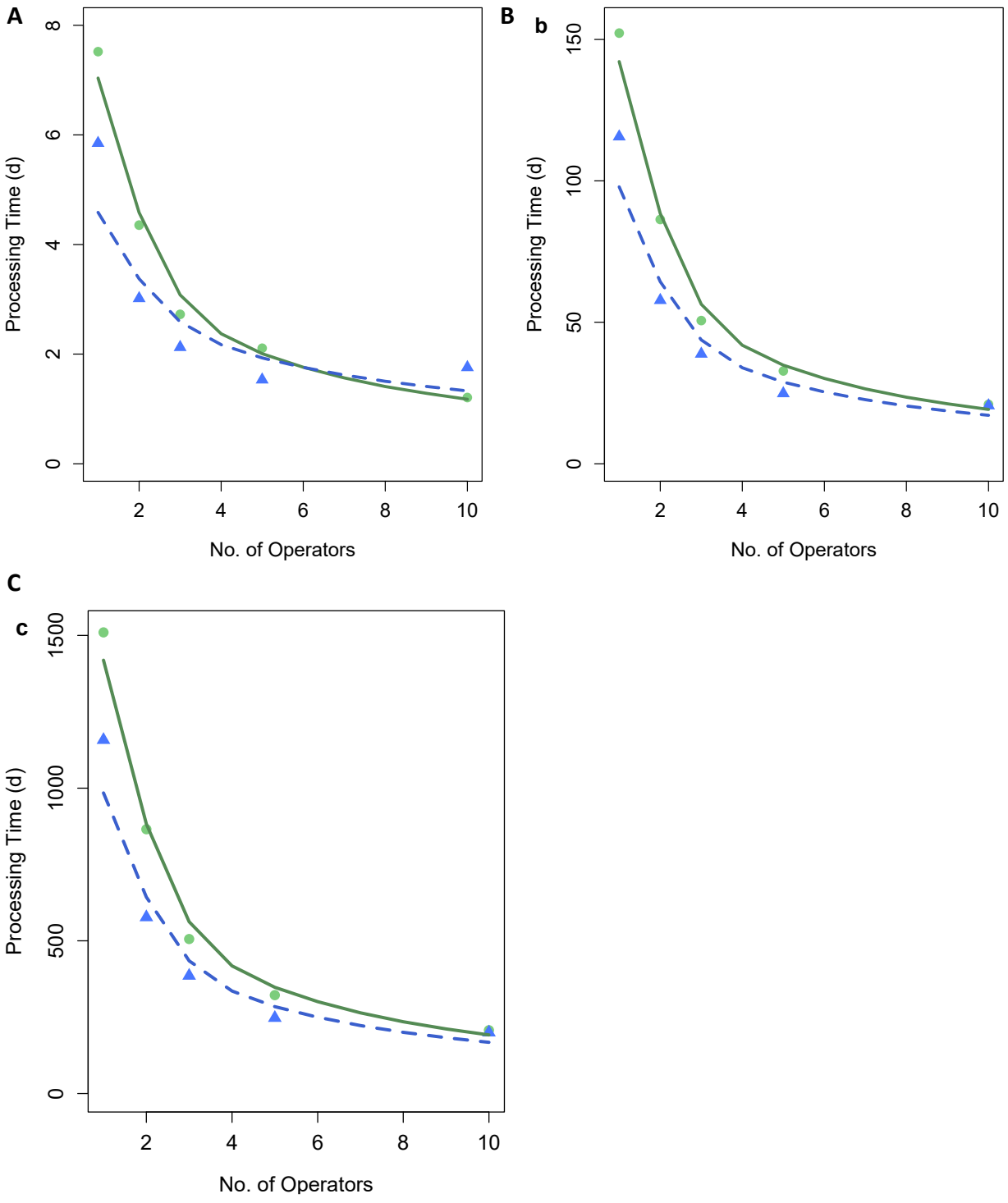

Figure 3.2. Plotted power functions of the relationship between the Number of Operators and the required Processing Times at three scales of production (A: 50 oysters per year, B: 1,000 oysters per year, C: 10,000 oysters per year) for the Baseline Model (solid line) and the Enhanced Model (dashed line). Circles represent data points from the Baseline Model and triangles represent data points from the Enhanced Model.

For the Baseline and Enhanced Models at medium and large scales of production, the Number of Operators had an effect on the Cost per Straw and the Breakeven Price of a straw, although the effect was small (general linear model, $P = 0.04$ or less for all cases). As the Number of Operators increased, so did the Cost per Straw and Breakeven Price, however, the price increase was < \$0.1 as the Number of Operators increased by one (Table 3.2). For the Baseline Model at the small scale of production, as the Number of Operators increased, the Cost per Straw increased by \$0.02 (general linear model, $P < 0.001$), however, the Number of Operators did not affect Cost per Straw for the Enhanced Model, at the small scale (general linear model, $P = 0.05$). Across all scales of production, the Costs per Straw values were similar (< 5% difference). When looking at Breakeven Price at the small scale of production, no significant relationship was found between the Number of Operators and the Breakeven Price for the Baseline and Enhanced Models (general linear model, $P = 0.05$ or greater for all cases). Finally, it was calculated that a single straw could potentially produce 2.8×10^4 oyster seed (Table 3.1) and the average price for 6 – 10 mm seed (diploid) was \$20 for 1,000 seed or \$0.02 per seed oyster (Oyster Seed Holdings Inc., 2021; University of Maryland, 2022). Therefore, the calculated Gross Straw Value was \$568 (2.8×10^4 x \$0.02).

Discussion

Parameter Effects

This study addressed the challenges of repository development in oyster aquaculture by evaluating cryopreservation needs at three different scales of production. When looking at the effect of the three parameters on both models, there was a trade-off where Throughput improved by increasing the Number of Operators and the Batch Size, but Operating Costs also increased. Operating Costs increased because of higher labor expenses from adding more operators and from increased supply costs from processing more oysters (higher Throughput). To construct a productive system, these two critical factors, Throughput and Costs, must be balanced; Costs should be minimized while still meeting the required Throughput demands (Russell & Meller, 2003). In this study, when the effects of the parameters were analyzed, no Throughput requirements were set because the objective was to discover the relationships between the three parameters and the three key outputs (Throughput, Operating Costs, and TIS). By setting minimum Throughput requirements (discussed further in Section 4.2), decisions can be made about how many operators or what Batch Size is necessary to meet Throughput demands while minimizing Costs.

The three parameters affected the Throughput and Operating Costs of the Baseline and Enhanced Models similarly, either having positive or negative relationships (Figure 3.1 B, C, E, & F). However, in general, the magnitude of these effects was larger in the Enhanced Model. This could be attributed to the alternate devices implemented in the Enhanced Model that eliminated two major bottlenecks. These devices resulted in higher Throughput and therefore higher Costs

in the Enhanced Model when compared to in the Baseline Model, even when all other factors remained constant between the two models (Bodenstein et al., 2022). Therefore, the Enhanced Model had a greater maximum potential Throughput than the Baseline Model and changing certain parameters affected the Enhanced Model more than the Baseline Model.

Conversely, the Time in System (TIS) of the Enhanced Model was not affected by the change in Straws per Oyster while the TIS of the Baseline Model was affected (Figure 3.1 G). Time in System is the average amount of time it takes to process an entity moving through the system (in this study, an oyster). Industrial engineers commonly try to minimize TIS by eliminating wastes and bottlenecks to increase productivity (Baesler et al., 2003; Diego Fernando & Rivera Cadavid, 2007). The TIS of the Baseline Model increased rapidly (a 19 s increase for every additional straw) because one of the major bottlenecks discovered in the Baseline Model was sorting straws manually after freezing (Bodenstein et al., 2022). The Enhanced Model implemented a custom 3-D printed funnel which allowed 15 straws to be sorted at one time, therefore, the TIS of the Enhanced Model did not increase as the Straws per Oyster increased. The alternate devices implemented in the Enhanced Model allowed the model to handle a wider variety of situations (such as the need to process more Straws per Oyster) than the Baseline Model. Development of more flexible cryopreservation pathways, such as the Enhanced Model, could increase the sustainability of repositories as their needs change through time.

Small-Scale Recommendations

The smallest production scale, 50 oysters per year, represented a scenario where a research laboratory was cryopreserving their own samples for future use. In this scenario, the high-throughput devices used in the Enhanced Model were not recommended. Using the basic equipment found in the Baseline Model allowed for 50 oysters to be processed in just over a week (7.3 workdays) with one operator. This time dropped to under a week (4.2 workdays) if a second operator was added. Therefore, the required number of oysters were processed within the allotted time period without the need for high-throughput devices.

Using the high-throughput devices in the Enhanced Model resulted in shorter Processing Times than the Baseline Model (with 1-3 operators). This was because alternate devices (the funnel and CASA) were employed in the Enhanced Model to remove two major bottlenecks and increase model Throughput (Bodenstein et al., 2022). In fact, across all scales of production, the difference between the Processing Times for the Baseline and Enhanced Models remained consistent, when the Number of Operators was held constant. For example, with one operator, the Enhanced Model on average had Processing Times that were 27% lower than the Baseline Model, across all scales of production. Both models, however, were able to process 50 oysters within the one-year period at the smallest scale of production.

A facility that would only need to process 50 oysters a year could lower their Capital Costs by not purchasing high-throughput devices. This would slightly raise the Cost per Straw compared to a facility using high-throughput devices ($0.51 vs. $0.48 per straw) because of the increased

production time. The Breakeven Price of a straw (over a one-year period), however, would remain lower ($85 vs. $94, Table 3.2) for a facility not using high-throughput devices, therefore the high-throughput devices were not recommended. Of the two high-throughput device options, however, only the CASA caused a substantial increase in Capital Cost. The funnel was inexpensive and still increased Throughput, even when implemented without the CASA (Bodenstein et al., 2022). Therefore, the funnel could be implemented into a production pathway operating at any scale.

While the basic equipment was sufficient for the smallest production scale, employing two operators was recommended. Within the same production scales, adjusting the Number of Operators affected the Percent Processing Failure, required Processing Time, and Breakeven Prices of the Baseline and Enhanced Models. When only one operator was assigned to a model (either model), a certain percentage of oysters were not successfully processed and "failed" out of the production system at all scales of production. However, if one more operator was assigned to a model, the failure rate dropped to zero at all scales of production. The percentage of oysters that failed to be fully processed can also be recognized as the defect rate. Products that are defective in a manufacturing system constitute one of the seven major types of waste identified in industrial engineering. Defects cause additional work, inspections, production delays, poor quality products, and increase production cost (Arunagiri & Gnanavelbabu, 2014). Reducing the number of defects is imperative when creating an effective production system.

The Number of Operators also affected the Processing Times for both models and this relationship was best described by a power function. Due to the nature of power functions, as the Number of Operators increased from one to eight, the resulting decreases in Processing Times diminished. Adding one more operator (two total) had the largest effect on Processing Time, and adding additional operators required the same increase in labor costs without providing the same reductions in Processing Times. Therefore, using two operators is recommended at the small production scale to minimize Percent Processing Failure, Processing Times, and labor costs.

Medium-Scale Recommendations

The medium scale of production, 1,000 oyster per year, represented a scenario where a hatchery was cryopreserving samples to back up their own lines. In this scenario, it was recommended to use the high-throughput device options in the Enhanced Model. Without these high-throughput devices it took almost 150 days to process 1,000 oysters. With high-throughput devices it took just over 100 days (Table 3.2). This meant that a facility using the devices from the Enhanced Model would have faster Throughput, a greater yearly production capacity, and higher potential profits than a facility using the basic equipment from the Baseline Model (Duggan, 1998; Sullivan et al., 2002). Facilities, such as oyster hatcheries, interested in starting repositories should consider using higher-throughput cryopreservation pathways like the Enhanced Model to maximize their production capacity and profits.

At this scale of production for example, an operator at the hatchery would spend roughly a third of the year cryopreserving oyster sperm. This part of the year would align with the oyster spawning season in the region (spring to early summer for *C. virginica* in the northern Gulf of Mexico, Wallace et al., 2008). This would leave the operator with the rest of the year to focus on other hatchery duties such as producing algae, conditioning broodstock, managing the farm site. Furthermore, employing two operators at the medium-scale facility is recommended. Using an additional operator (two total) decreased the required Processing Time by almost half (150 to 80 days, Table 3.2) and decreased the Percent Processing Failure to zero. This decrease in Processing Time and defect waste would also provide operators with more flexibility to either cryopreserve a greater number of oysters or focus on other hatchery tasks.

Large-Scale Recommendations

The large scale of production, 10,000 oyster per year, represented a scenario where a commercial facility produces and cryopreserves broodstock lines to sell to laboratories and hatcheries. In this scenario, it was recommended to use the high-throughput device options and to employ six operators. The average number of workdays in a year is 260 (5 workdays x 52 weeks per year) and using high-throughput device options in combination with six operators resulted in a Processing Time of 251, 8-h workdays (Figure 3.2 C). In a facility using equipment found in the Baseline Model, eight operators were required to attain a Processing Time under 260 8-h workdays. A commercial facility with faster Processing Times would be able freeze their annual quota of oysters more quickly. This would leave time in the year to freeze other “in-season” aquaculture species based on an annual “spawning calendar” (Hu, 2012). Using high-throughput

device options would allow the facility shorten Processing Times and cryopreserve material from multiple species during a year providing sustained cash flow.

Processing Times can be improved with a variety of methods including increasing the number of operators, switching to automated equipment, or both (Chong & Ng, 2016; Edwards, 1996; Kato, 2000). However, labor costs can constitute the majority of Operating Costs in a system (Hu et al., 2015), making it uneconomical and inefficient to only increase the Number of Operators when attempting to decrease Processing Times. By implementing device improvements and additional operators, required Processing Times can decrease more quickly than when only increasing the Number of Operators. In addition, current operators could be trained to increase their skill and experience level. Experienced operators can perform work more quickly and efficiently (with less errors), therefore lowering Processing Times without adding more operators and increasing Operating Costs (Devotta, 1983; Heneman et al., 1987; Hu et al., 2015).

Incorporating a costly piece of automated, high-throughput equipment, such as the CASA, increased the Breakeven Prices of the Enhanced Model as compared to the Baseline Model across all scales of production, despite decreasing Processing Times. The differences between the Breakeven Prices of the models were highest at the smallest scale of production and lowest at the highest scale of production. Therefore, the greatest benefit from incorporating an expensive, automated device was seen at the higher scales of production, highlighting the economy of scale. This principle has also been observed in previous work analyzing the economics of cryopreservation, where hatcheries investing in cryopreservation equipment saw the greatest

benefits at the largest production scales (Caffey & Tiersch, 2000). Breakeven Times also followed economies of scale, with faster Breakeven Times the more oysters were produced per year (Table 3.3). Finally, as the proposed Straw Prices increased (from $14 to $60), the differences in Breakeven Times between the Baseline and Enhanced Models decreased until they were the same at the highest production scales.

This again points to the benefits of incorporating automated equipment for systems operating at high scales of production, even if initial Capital Costs are high. An important caveat, however, is that machine downtime (the proportion of time automated equipment is out of service) must be low. Frequent machine downtime creates high levels of waiting waste and decreases Throughput, particularly at high production scales (Kumar, 2014). Furthermore, to prevent machine downtime routine machine maintenance of automated equipment must be performed (Nwanya et al., 2017). Complex equipment may require time-consuming maintenance and therefore the additional labor costs must be considered. For example, the most expensive equipment used in this study was the CombiSystem MPP Quattro and Domino Printer (Minitube USA, Verona, WI). The Quattro can fill and seal four French straws simultaneously followed by labelling with the Domino Printer (note: the Quattro is now sold with a different printer). This equipment can fill, seal, and label 15,000 straws an hour, and allows for high-throughput cryopreservation. The machine downtime on the Domino Printer, however, was 33% with the printer not functioning properly when turned on and needing to be repaired, which took an average of 30 min. Therefore, while an automated straw filler and printer are recommended, equipment should work reliably and be easy to maintain.

Finally, device improvements can be incorporated into a system without high Capital Costs by using open-source, 3-D printed hardware (Liu et al., 2021). For example, the other high-throughput device used in the Enhanced Model was a 3-D printed funnel that increased Throughput without significantly increasing (< 5% difference) Capital Costs due to the low manufacturing cost of 3-D printed technology (Bodenstein et al. 2022b). Another benefit of 3-D printed hardware is the ease of file sharing, making high-throughput tools accessible to a wide user base (Childress et al., 2021).

Straw Value

The Gross Straw Value was calculated to be $568 per straw. In a scenario where oyster hatcheries cryopreserved and used sperm from 50 oysters (using the pathway in the Baseline Model), the Breakeven Price (Capital and Operating Costs to produce one straw) would be $85. Therefore, straws would have a net value of $483 to offset Capital and Operating Costs within one year. This example at the smallest production scale is a "worst-case scenario" with the highest Breakeven Prices and lowest net straw value. Due to economy of scale, Breakeven Prices decreased and Net Straw Value increased at greater scales of production. For example, at the large production scale, the Net Straw Value would be roughly $567 because the Breakeven Prices for both models were under $1.

Even the smallest Net Straw Value ($483) was 155% higher than the highest fixed straw price used in this study ($60). This value, however, was not as high as some of prices seen for dairy bull

semen. The maximum price (or value) for a dose of semen reported in 2002 was $2,019 (Carvalho et al. 2022), almost four times higher than the Net Straw Value reported in this study. It should be noted that this maximum price reported in Carvalho et al., (2022) was for a specific dairy bull (Gir), was from a proven bull (proven to produce offspring with desired characteristics relevant to milk production) and was processed to eliminate Y-chromosome-bearing sperm (increasing the probability of producing a female). These traits increased the value of the dairy bull semen and were therefore reflected in a higher price point. Even with just one of these traits, sexed semen, the price of a dose increased by more than 400% ($27 to $151, Carvalho et al. 2022).

A similar phenomenon can be seen currently in the oyster industry. Straws containing sperm from oyster lines with specific traits, such as selectively bred lines or tetraploid lines, would be even more valuable and their resulting seed could be sold at a higher price. Research hatcheries have bred lines of eastern oysters with increased resistance to major oyster pathogens such as, *Haplosporidium nelson* (MSX), Roseovarius oyster disease (ROD), and *Perkinsus marinus* (dermo) (Casas et al., 2017; Davis & Barber, 1999; Haskin & Ford, 1979). These pathogens have caused mass mortality events in affected areas but with the advent of disease-resistant lines, oyster aquaculture has been able to expand in regions prone to disease (Proestou et al., 2016). As a result, seed oysters produced from disease-resistant lines are priced on average of 20% more than wild-type oyster seed (Ferry Cove Oyster Seed Order Form, 2022; University of Maryland, 2022). While these practical limitations currently exist, the use of cryopreserved sperm can add value to existing hatchery operations by allowing or easing access to genetic resources, such as disease-resistant lines, that were difficult or costly to obtain previously (Table 3.4)

Table 3.4. Hypothetical relative values for broodstock, juvenile oysters sold at hatcheries (oyster "seed"), and oyster sperm (frozen in straws) possessing a variety of genetic traits. Provenance indicates if the location of origin or pedigree of the broodstock is known. Relative values of broodstock and frozen sperm were assessed based on the perspective of oyster hatchery managers (who would sell the resulting seed to producers), and relative values of seed were assessed based on the perspective of commercial oyster producers (i.e., farmers). "NA" (not available) indicates that triploid broodstock do not exist and that sperm of triploids (3 sets of chromosomes; 3N) is not cryopreserved because these animals are functionally sterile and are typically produced by crossing tetraploid (4N) and diploid (2N) broodstocks. "NA" also indicates that tetraploid seed are typically not made available to oyster producers. The "$" symbols only indicate relative value levels, i.e., a space with two "$" symbols have greater value but not twice the value of spaces with one "$" symbol.

Source & Genetic Traits	Known Provenance	Relative Value as Broodstock	Relative Value as Frozen Sperm	Relative Value as Seed
2N	No	$	$	$
3N	No	NA	NA	$$
4N	No	$$	$$	NA
2N	Yes	$$	$$	$$
3N	Yes	NA	NA	$$$
4N	Yes	$$$	$$$	NA
Disease-Resistant 2N	Yes	$$$	$$$	$$$
Disease-Resistant 3N	Yes	NA	NA	$$$$
Disease-Resistant 4N	Yes	$$$$	$$$$	NA
Low Salinity 2N	Yes	$$$	$$$	$$$
Low Salinity 3N	Yes	NA	NA	$$$$
Low Salinity 4N	Yes	$$$$	$$$$	NA

Tetraploids are another type of high-value genetic resource that are used to produce fast-growing triploids. Triploid oysters (3N) are in high demand for many US oyster farmers due to faster growth rates and better meat quality during the summer months (Walton et al., 2012). This makes the relative value of triploid seed and tetraploid broodstock higher than diploid seed or broodstock (Table 3.4). The most straightforward production of triploid oysters requires use of sperm from tetraploid (4N) broodstock, which are often difficult to condition for spawning (B. Callam, pers. Comm.) or are under proprietary control due to intellectual property constraints.

In addition, creating new tetraploid lines is time consuming and costly, making the available tetraploid genetics valuable. Cryopreservation could assist distribution of tetraploid sperm and make production of triploids easier for hatcheries. Tetraploid suppliers could distribute sperm without the downsides associated with transporting live animals and the risk of customers attempting to produce unauthorized tetraploid lines. Producing triploids results in a terminal cross, and tetraploid sperm and eggs are required for the production of new tetraploids. Therefore, cryopreservation technology would facilitate greater triploid production while protecting the intellectual property of the supplier.

Transfer of cryopreserved germplasm could also ease state regulations often imposed on broodstocks entering a given state (e.g., Tiersch & Jenkins, 2001). Cryopreserved germplasm could be tested for pathogens in concert with the freezing process, meaning that producers receiving the germplasm could use it immediately and would not need to order a pathology report. Furthermore, a batch of cryopreserved sperm need only be tested for pathogens once while broodstock must be continually tested to ensure biosecurity. Cryopreserved sperm can also open new markets for a hatchery by reducing the burden of a hatchery to maintain live adults either in coastal waters or in expensive recirculating aquaculture systems. Without the need to maintain all genetic lines as live animals, hatcheries would have the ability to introduce new genetics into breeding programs and produce a variety of selectively bred lines. Cryopreservation would also allow for easier distribution of genetic traits between hatcheries because cryopreserved samples can be shipped in high quantities with less risk than shipping live animals.

Finally, the service model for cryopreservation in hatcheries is yet to be determined. Hatcheries could contract a company (like the one mentioned in the large-scale scenario) to receive oysters and cryopreserve and store samples for them (referred to as a "custom collection" model in livestock). Another model would be for hatcheries to conduct cryopreservation in-house and store samples on-site or at a back-up facility, as mentioned in the medium-scale scenario. A hatchery could also offer cryopreservation services to interested parties, such as other hatcheries or researchers. Careful consideration must be given when choosing a service model as certain models would not be appropriate for all hatcheries. For example, a laboratory that only wants to cryopreserve 10 oysters once a year (similar to the small-scale scenario) would likely use a third-party company rather than investing in the equipment, personnel, and training for in-house cryopreservation. To determine the appropriate service model, future research could assess factors such as the resources, production scale, spawning schedule, biosecurity, regulatory compliance, and cryopreservation needs of hatcheries and their customers.

Conclusions

There are unique and persistent challenges for cryopreservation and repository development of aquatic species at all scales of production. Laboratories, hatcheries, and commercial cryopreservation facilities all have different requirements to be able to freeze samples within certain time and budget constraints. Simulation modeling facilitates understanding of various cryopreservation systems and how they can be affected by key parameters, such as device options and operator capacity. Results from these models can be used to make recommendations for operating repositories in a sustainable manner, allowing them to support

aquaculture enterprises in the future. Although commercial markets for cryopreserved oyster sperm are still in the early stages of development, demand will grow as frozen sperm becomes more available and cost-effective compared to traditional spawning methods (Caffey & Tiersch, 2000). We indicate here that germplasm repositories can be planned and operated from the start in cost-effective ways that best serve their communities by use of simulation modeling, hastening the proliferation of cryopreservation technology.

This study also attempted to anticipate how oyster aquaculture will change in response to the integration of cryopreservation and repository storage. Hatchery production cycles may change as broodstock conditioning (of females) and "on-demand" frozen sperm make spawning more possible outside of the traditional season (Chávez-Villalba et al., 2002). Through repository storage, less labor would be needed to maintain live animals, freeing hatcheries to dedicate more resources to producing more oyster larvae, growing more algae for feeding of larvae, or developing genetically improved lines. Farmers would be able to request oysters with genetic traits suited to specific production sites as hatcheries and laboratories develop, store, and transport such genetics more easily through frozen sperm (Moore & Hasler, 2017; Yang et al., 2012). With the integration of repository systems oyster aquaculture and aquaculture in general can sustain growth as production systems reach their limits, by improving the animals that are cultured, as seen in the dairy industry.

Chapter 4. Survival and Growth of Triploid Eastern Oysters, *Crassostrea virginica*, Produced from Wild Diploids Collected from Low-salinity Areas

Background

In this chapter, a year-long field study was performed to evaluate the growth and survival of three genetic lines of diploid and triploid oysters. While the concepts of cryopreservation and germplasm repository storage were not directly mentioned, the experiments performed in this chapter were a case study of the types of data that would be collected and stored in a repository database. The data were collected from oysters that belonged to the genetic lines cryopreserved in Chapters 1 and 2. After the field study was completed, the data were entered into the USDA National Animal Germplasm (NAGP) Animal GRIN repository database (NAGP, 2023). The work in this chapter served to provide data about samples stored in the repository, analyze the time requirements to enter data in to a database (discussed in Chapter 6), and evaluate what forms of data that would be necessary to collect for samples stored in a repository (discussed in Chapter 7). In addition, this work operated at the Center Level and assisted with outlining the exchange of information that would occur between repositories and communities (discussed in Chapter 6).

Introduction

Eastern oysters (*Crassostrea virginica*) have been harvested from U.S. Gulf of Mexico (GoM) and Atlantic estuaries for centuries. Today, oyster farming is one of the highest valued sectors of

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aquaculture in the United States, with the Gulf region producing the most oysters (by volume) in 2017 (National Marine Fisheries Service, 2020). *Crassostrea virginica* has traditionally been grown sub-tidally on-bottom in GoM estuaries but production is being increasingly supplemented by off-bottom aquaculture (Walton et al., 2013; Walton and Swann, 2021). Triploid oysters (3N), bred to have three sets of chromosomes instead of the normal two sets (2N), have been adopted in off-bottom aquaculture partially to offset the higher initial investment (Petrolia et al., 2022). Triploids can grow faster and have better meat quality, especially in the summer months, than do diploids because of reduced gametogenesis of triploids (Allen Jr. and Downing, 1986; Dégremont et al., 2012). However, triploids suffer greater mortalities in low salinity conditions (<5) than do diploids, although the reasons remain unresolved (Callam et al., 2016; Matt et al., 2020; Wadsworth et al., 2019). Preventing high triploid mortality is an urgent goal considering that periods of low salinity are becoming more frequent and sustained in the most productive areas of the GoM (i.e., Louisiana). This is due to a combination of river and coastal management strategies and increased precipitation due to the changing climate (Powell and Keim, 2015; Soniat et al., 2013; Swam et al., 2022; Wang et al., 2017).

Although eastern oysters are tolerant to a wide range of salinity, long periods of low salinity (<5 ppt) can result in oyster mortality, especially in conjunction with high water temperatures (> 28°C) (La Peyre et al., 2013; Rybovich et al., 2016). There is however increasing evidence of divergence in salinity tolerance among GoM oyster populations even across a narrow geographic range (Leonhardt et al., 2017; Marshall et al., 2021a; Swam et al., 2022). In Louisiana, diploid progeny of broodstock collected from a low-salinity site had higher survival when exposed to low

salinity conditions (5.1 ± 3.0 ppt) than the progeny of broodstock from higher salinity regimes (Leonhardt et al., 2017). It remains to be determined whether breeding populations of diploid broodstock with higher tolerance to low salinity can enhance the tolerance of triploids to low salinity.

Using selected diploids to improve the performance of triploids has been demonstrated in past studies (Callam et al., 2016; Dégremont et al., 2010; Hand et al., 2004). As early as 2004, it was showed that the growth of chemically induced (cytochalasin B) triploid Sydney rock oysters (*Saccostrea glomerata*) was improved when using a line of diploids selected for enhanced growth compared to unselected diploids (Hand et al. 2004). In another study, the growth and survival of triploid Pacific oysters (*Crassostrea gigas*), produced by crossing diploid females selected for resistance to summer mortality with unselected tetraploid males, were shown to be greater than triploids produced from unselected diploid females (Dégremont et al., 2010). Additional support for additive gains in triploid *C. virginica* was recently observed as diploid parental contributions to the performance of triploid offspring were found to be significant (Callam et al. 2016). This suggests that improved traits such as increased growth rate or disease resistance can be transferred from desirable diploids to triploids.

The goal of this study was to investigate the effect of ploidy, broodstock parentage, and hatchery cohort on the field performance of oysters in moderate and low salinity conditions. The objectives were to: 1) collect wild oysters from three native populations in Louisiana: Calcasieu Lake (CL), Sister Lake (SL), and Vermillion Bay (VB); 2) produce six crosses of diploid and triploid

oysters (CL2N, SL2N, VB2N, CL3N, SL3N, and VB3N) at two hatcheries (the AU and LSU cohorts) using the native oyster populations as broodstock; 3) monitor the growth and mortality of the crosses monthly at two field sites (one moderate and one low salinity site) for one year; 4) measure sex ratio, gametogenic stage, gonad-to-body ratio, condition index, and *P. marinus* infection intensity of crosses grown at both field sites, and 5) evaluate the effects of ploidy, broodstock parentage, and hatchery cohort on the field performance of oysters grown at both field sites. Triploid and diploid crosses produced with broodstock collected from sites with lower mean salinities were predicted to have higher survival and growth in the low-salinity field site than crosses produced using broodstock from sites with higher mean annual salinity.

Methods

Oysters

In January and February 2019, about 300 wild oysters were collected for broodstock from each of three Louisiana public oyster grounds that had different salinity regimes: Calcasieu Lake (CL, 29° 51’2.34”N, 93° 16’59.81”W) with an annual mean salinity of 16.2 ± 2.8 ppt [± standard deviation (SD), n = 10, 2009 – 2018] (Louisiana Department of Wildlife and Fisheries [LDWF] hydrological data, Marshall et al., 2021b), Sister Lake (SL, 29° 14’45.0”N, 90° 54’35.0”W) with an annual mean salinity of 11.2 ± 5.5 ppt [n = 10, 2009 – 2018] (United States Geological Survey [USGS] 07381349 water-quality monitoring station), and Vermilion Bay (VB, 29° 34’47”N, 92° 2’4”W) with an annual mean salinity of 7.4 ± 1.6 ppt [n = 10, 2009 – 2018] (LDWF hydrological data, Marshall et al., 2021b) (Figure 4.1). Oysters from each broodstock population were placed in baskets suspended on long lines (BST Oyster Co., Cowell, South Australia) for conditioning at

the Louisiana Sea Grant Oyster Research Farm (LSURF) a moderate salinity site, adjacent to the Louisiana Sea Grant Oyster Research Laboratory and Mike C. Voisin Oyster Hatchery (LSUL) in Grand Isle, LA. In addition to the wild diploid broodstocks, the tetraploid broodstocks LSU 4DGNL17, maintained at LSURF, and AU 4MC18, maintained at the Grand Bay Oyster Park, Alabama, were used to produce the triploids. These tetraploid broodstocks were originally part of the 4MGNL13 line developed by LSUL, and both hatcheries had advanced the line two generations prior to their use in this study (Figure 4.2).

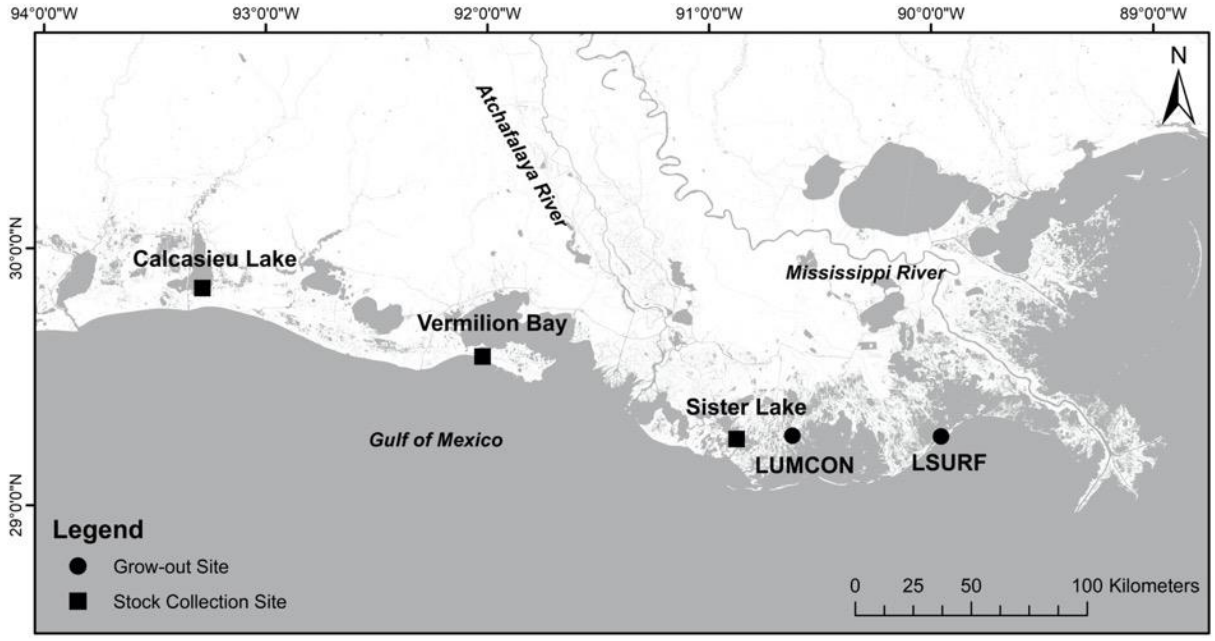

Figure 4.1. Map of Louisiana indicating the locations of the three broodstock collection sites (Calcasieu Lake, Vermilion Bay, and Sister Lake) and the two field sites (LUMON and LSURF). LUMON is an abbreviation for the Louisiana Universities Marine Consortium. LSURF is an abbreviation for the Louisiana Sea Grant Oyster Research Farm.

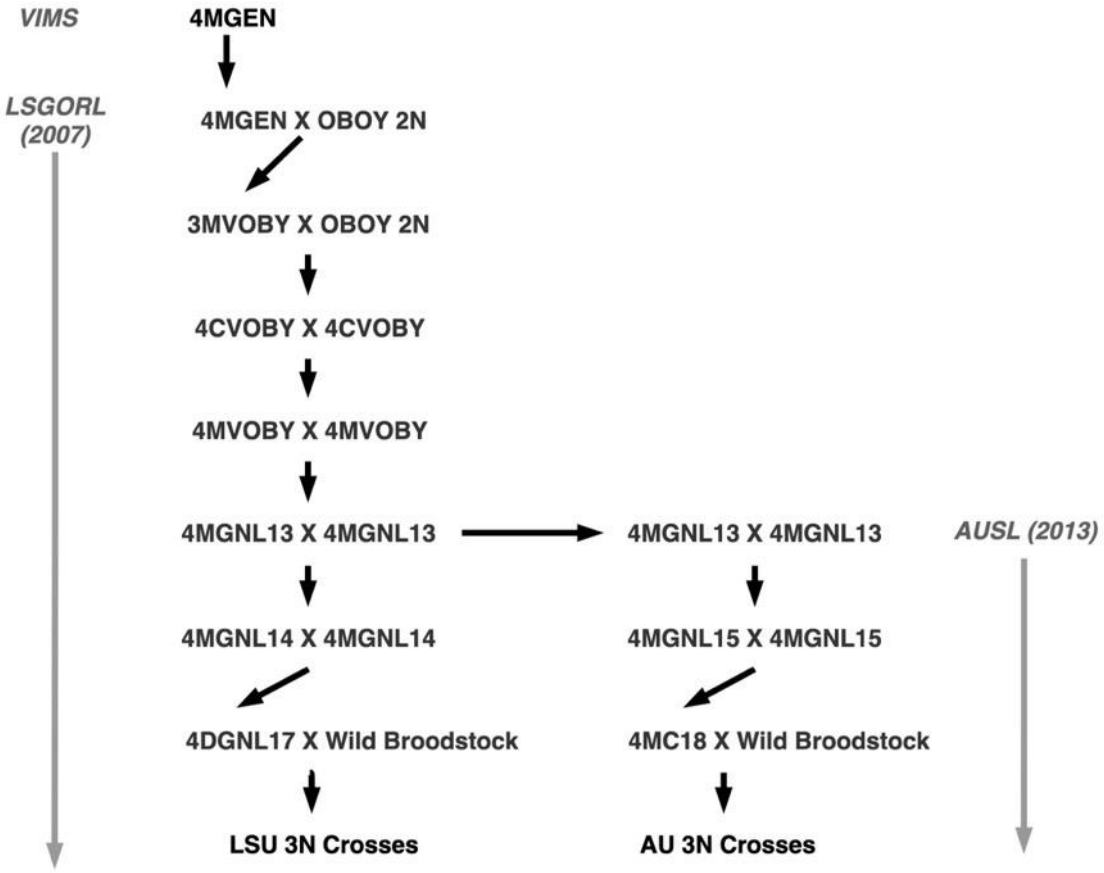

Figure 4.2. Pedigree of tetraploid oysters used to produce F_1 LSU and AU triploid oyster crosses for this study. The number "3" indicates triploid lines, "4" indicates tetraploid lines, "C" indicates chemically induced lines, "M" indicates mated lines, "GEN" refers to a generic or general lines, and the last two numbers indicate the spawning year. VIMS is the abbreviation for the Virginia Institute of Marine Science. LSUL is the abbreviation for the Louisiana Sea Grant Oyster Research Laboratory. AU is the abbreviation for the Auburn University Shellfish Laboratory., Grand Isle Oyster Research Laboratory).

Spawning

Oysters were spawned at LSUL in June 2019 and at the Auburn University Shellfish Laboratory (AUSL) in Dauphin Island, AL immediately after transfer in July 2019. The F_1 progeny were designated as LSU (Louisiana State University) or AU (Auburn) cohorts depending on which

hatchery they were produced (Table 4.1). For spawning, the oysters were placed in individual containers and induced to release gametes via thermal shocking by alternating ambient and warm (5°C above ambient) water flowing into each container (Wallace et al., 2008). Eggs collected from wild diploid females (~1×10^6 eggs per female) were pooled based on broodstock parentage and allocated based on intended ploidy breeding (i.e., based on cross). The eggs were aliquoted prior to fertilization with sperm from wild diploids or tetraploids. The number of aliquots was equal to the number of males used to fertilize female broodstock from each cross. Female and male oysters from each wild broodstock population (CL, SL, and VB) were used to produce three diploid F_1 crosses at each hatchery (six crosses total): AU CL2N, AU SL2N, AU VB2N, LSU CL2N, LSU SL2N, and LSU VB2N (Table 4.1). In addition, female oysters from each wild broodstock were crossed with males from two tetraploid lines 4DGNL17 at the LSU hatchery and 4MC18 at the Auburn hatchery) to produce three triploid crosses at each hatchery (six total): AU CL3N, AU SL3N, AU VB3N, LSU CL3N, LSU SL3N, and LSU VB3N (Table 4.1). Sperm were verified to be from tetraploid males by flow cytometry prior to fertilization (Allen and Bushek, 1992). At the Auburn hatchery, diploid and triploid crosses were produced from the same females and were therefore half-siblings.

Table 4.1. Diploid broodstock collection site and number of diploid females and diploid or tetraploid males used to produce F_1 oysters at the LSU and Auburn affiliated hatcheries (AU or LSU hatchery) in 2019. Tetraploid line name is listed next to the number of tetraploid males used. Full F_1 Cross Name (used to identify the different crosses in each cohort) are listed in addition to their abbreviations (F_1 Stock Abbr.).

Collection Site	No. 2N Females	No. 2N Males	No. 4N Males	Tetraploid Lines	Hatchery/ Cohort	F_1 Cross Name	F_1 Cross Abbr.
Calcasieu Lake	14	7			AU	Auburn Diploid Calcasieu Lake	AU CL2N
Calcasieu Lake	14		10	4MC18	AU	Auburn Triploid Calcasieu Lake	AU CL3N
Calcasieu Lake	5	3			LSU	LSU Diploid Calcasieu Lake	LSU CL2N
Calcasieu Lake	15		3	4DGNL17	LSU	LSU Triploid Calcasieu Lake	LSU CL3N
Sister Lake	51	33			AU	Auburn Diploid Sister Lake	AU SL2N
Sister Lake	51		8	4MC18	AU	Auburn Triploid Sister Lake	AU SL3N
Sister Lake	3	4			LSU	LSU Diploid Sister Lake	LSU SL2N
Sister Lake	6		2	4DGNL17	LSU	LSU Triploid Sister Lake	LSU SL3N
Vermilion Bay	40	46			AU	Auburn Diploid Vermilion Bay	AU VB2N
Vermilion Bay	46		8	4MC18	AU	Auburn Triploid Vermilion Bay	AU VB3N
Vermilion Bay	14	19			LSU	LSU Diploid Vermilion Bay	LSU VB2N
Vermilion Bay	7		2	4DGNL17	LSU	LSU Triploid Vermilion Bay	LSU VB3N

At the LSU hatchery diploid and triploid crosses were not half-siblings and in some instances were produced on different days (Table 4.1). Larvae were reared and set on micro-cultch material to produce single oyster spat using standard hatchery techniques (Wallace et al., 2008). Spat were grown in upwelling nursery systems at each hatchery from July to September until oysters were

large enough to be deployed in 6-mm mesh baskets at LSURF for further grow out before deployment at the study field sites. The F_1 crosses produced at AU were transported to Grand Isle in September following authorization from the Louisiana Department of Wildlife and Fisheries. Ploidy verification by flow cytometry was performed on oysters from both cohorts at the LSUL as described by Callam et al. (2016).

Field Sites and Experimental Design

Oysters from both cohorts were deployed at two study sites in Louisiana with different salinity regimes: a low-salinity site, near the Louisiana Universities Marine Consortium (LUMCON) in Cocodrie, LA, with an annual mean salinity of 9.3 ± 5.0 ppt [n = 11, 2010 – 2020] (LUMCON, 2021), and LSURF a moderate salinity site, with an annual mean salinity of 19.4 ± 6.7 ppt [n = 11, 2010 – 2020] (USGS 073802516 water-quality monitoring station). Oysters from the Auburn cohort were deployed in November 2019 at both sites while oysters from the LSU cohort were deployed at LSURF in December 2019 and at LUMCON in January 2020. Four replicate baskets containing 80 oysters each were deployed at each site for each of the six crosses of each ploidy in the two cohorts for a total of 48 baskets per site by January 2020. Eighty oysters (~40 mm shell height) filled less than one third of the total basket volume and were below the maximum stocking density of a long-line basket (Comeau et al., 2011; Davis, 2013), eliminating overcrowding as a potential stressor. The baskets were suspended on long-lines beneath the water surface and off-bottom to reduce the chance of predation. Growth and mortality were assessed monthly until November 2020, with the exception of May due to Covid-19 restrictions accessing the field site.

Because of COVID-19, there were six weeks (instead of four weeks) between sampling in mid-March to the end of April and the end of April to mid-June (Appendix C, Table C.1).

Data Collection

Mortality was evaluated by counting the number of live and dead oysters in each basket. Dead oysters were discarded after each sampling. For each basket at both sites, interval, adjusted interval, and cumulative mortality of oysters were calculated following procedures from Ragone-Calvo et al. (2003).

Growth was evaluated by measuring the shell height (from umbo to furthest shell edge) of 25 oysters from each basket by use of digital calipers (Mitutoyo 500–171-30, Mitutoyo Corp., Japan). Overall growth rates were calculated for each basket at both sites by subtracting the mean starting shell height from the mean final shell height and dividing that by the number of days the bags had been deployed.

During June 2020 sampling, ten oysters were also collected from each cross (2–3 per basket) to be cross-sectioned and processed by standard histological technique (Howard et al., 2004) to determine sex and gametogenic stage and to measure gonad-to-body ratio. The stages of gametogenic development were determined using methods described in Matt and Allen (2021) as Inactive (I), Very Early Active (VEA), Early Active (EA), Active (A), Late Active (LA), Ripe (R), Spawning (S), Advanced Spawning (AS), and Spawned Out (SO). The Inactive stage had the least amount of gonad development (follicles) continuing to increase until the Ripe stage (≥ 80% follicle

coverage) at which point follicle coverage began to decrease when spawning occurred. Gonad-to- body ratio was measured using the image analysis software ImageJ (Quintana et al., 2011; Schneider et al., 2012).

Additionally, in June and September 2020, 20 oysters from each cross (5 per basket) at each site were collected and processed to analyze condition index and *P. marinus* prevalence and infection intensity. Condition index [whole oyster dry wt * 100 / (whole oyster wt - wet shell wt)] and infection intensity (parasite number per g wet tissue) were assessed using standard methods (La Peyre et al., 2019). *Perkinsus marinus* prevalence was calculated by dividing the number of infected oysters by the number of oysters sampled multiplied by 100 and the mean infection intensity of infected oysters was determined. Oysters were further classified as uninfected, lightly infected ($<1 \times 10^4$ parasites g^{-1} wet tissue), moderately infected ($1 \times 10^4 – 5 \times 10^5$ parasites g^{-1} wet tissue) or heavily infected ($> 5 \times 10^5$ g^{-1} wet tissue) (Bushek et al., 1994; La Peyre et al., 2019).

Statistical Analysis

All statistical analyses were performed in RStudio (R Core Team, 2020). General linear models were used to assess water temperature and salinity differences between the two field sites. The Shapiro Wilkes test was used to test normality of interval and cumulative mortality, overall growth rate, gonad-to-body ratio, condition index and *P. marinus* infection intensity. Log transformations were used to restore normality to interval and cumulative mortality, gonad-to-body ratio, and infection intensity. General linear models were used to analyze the effects of ploidy, cohort, and broodstock parentage (called stock) on each measurement (interval and

cumulative mortality, overall growth rate, gonad-to-body ratio, condition index and infection intensity). The data for each measurement were organized by site. This allowed focus to be directed on the potential differences between ploidy and cohorts, and among stocks. In addition, for interval mortality, *posteriori* tests were conducted by selecting intervals at each study site (LUMCON June – August; LSURF: April–July) that showed the most obvious peaks in mortality. Finally, Chi-squared analysis was used to assess potential differences in the stages of gametogenic development and prevalence of *P. marinus* infection level among oyster stocks and between cohorts.

Results

Environmental Data

Mean salinity at LUMCON over the year-long study period was lower than at LSURF (general linear model, t = -22.7, $P < 0.01$) (Figure 4.3 A). The mean daily salinity at LUMCON was 9.0 ± 4.5 ppt while the mean daily salinity at LSURF was 17.4 ± 5.8 ppt from mid-November 2019 to mid-November 2020. A long period of low salinity (2.4 ± 1.2 ppt) at LUMCON was recorded between the mid-June and mid-July samplings. The lowest salinity at LSURF (10.4 ± 3.5 ppt) was recorded between the mid-March and end of April samplings. The salinity difference between the two sites was smallest between the mid-March and end of April samplings. Starting toward the end of April, the salinity at LUMCON started to fall below that of LSURF.

Water temperatures between the two sites were similar (general linear model, t = 0.35, $P = 0.73$) (Figure 4.3 B). LUMCON had a mean water temperature of 23.1 ± 5.9°C while LSURF had a mean

water temperature of 23.0 ± 5.6°C from mid-November 2019 to mid-November 2020. Water temperatures at LUMCON and LSURF were highest between the mid-July and mid-August samplings (general linear models, $P \leq 0.05$ for all cases). Water temperature had a daily mean of 30.3 ± 1.6°C at LUMCON and 30.2 ± 1.6°C at LSURF during this interval. From May to the end of September at both sites, water temperatures trended above 28°C, averaging 28.7 ± 2.4°C.

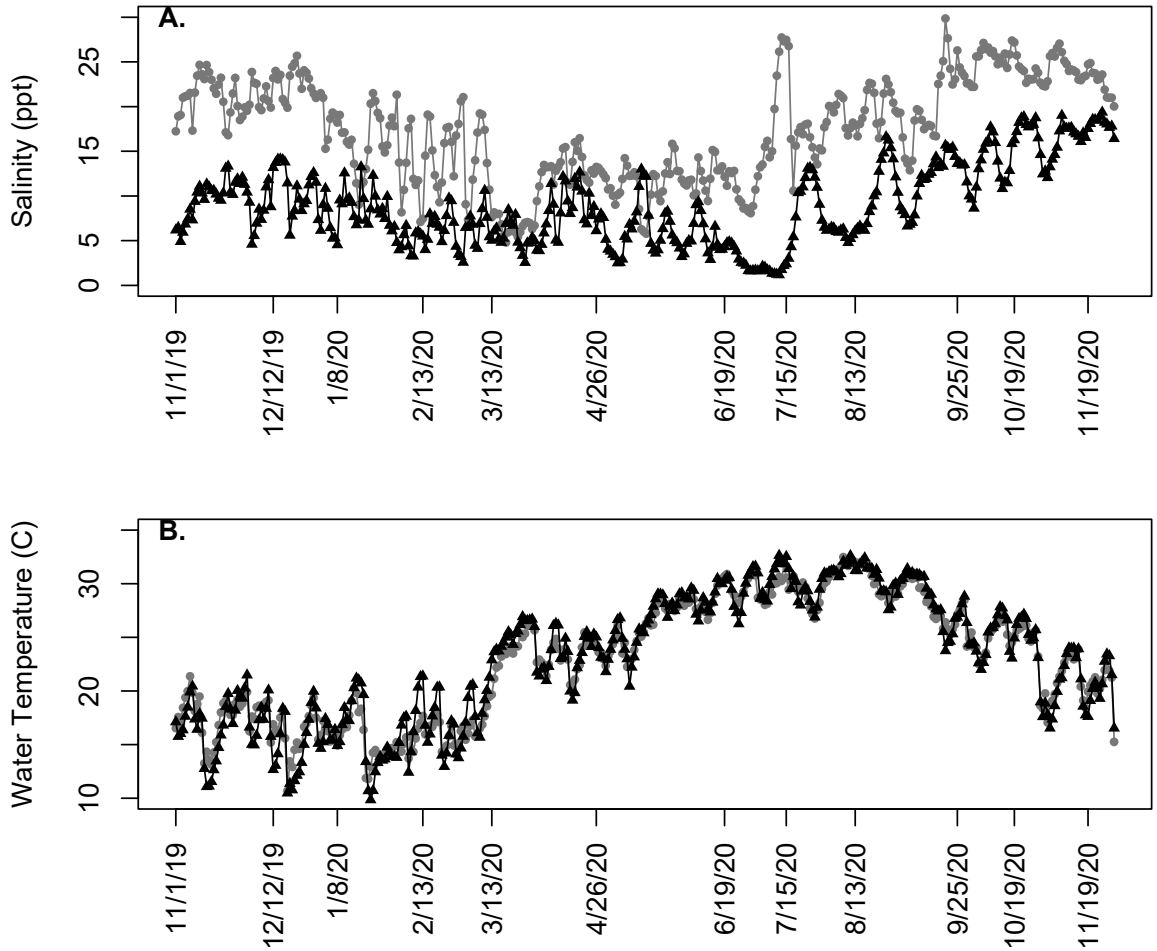

Figure 4.3. Daily salinity (A) and water temperatures (B) at LUMCON, black triangles, and LSURF, grey circles, from November 2019 to November 2020. Dates indicate sampling dates of the study.

Interval and Cumulative Mortality

Cumulative mortality of triploids was higher than diploids at LUMCON, the low-salinity site (general linear model, t = 11.89, $P < 0.01$). Stock did not affect triploid cumulative mortality; triploid crosses across cohort (CL3N, SL3N, and VB3N) at LUMCON had similarly high cumulative mortalities (general linear model, t < 1.94, $P > 0.07$ for all cases). However, there was a cohort effect; cumulative mortality of LSU triploids was slightly higher (4%, 95% CI 0–10%) than AU triploids (general linear model, t = 2.35, $P = 0.04$) (Figure 4.4 A). Cumulative mortality was 89% in LSU triploids (95% CI 86–93%) and 85% in AU triploids over the course of the study (95% CI 81–88%) at LUMCON. Mortalities for triploids at LUMCON peaked from mid-June to mid- August (the selected interval for the a posteriori test) (Figure 4.4 A).

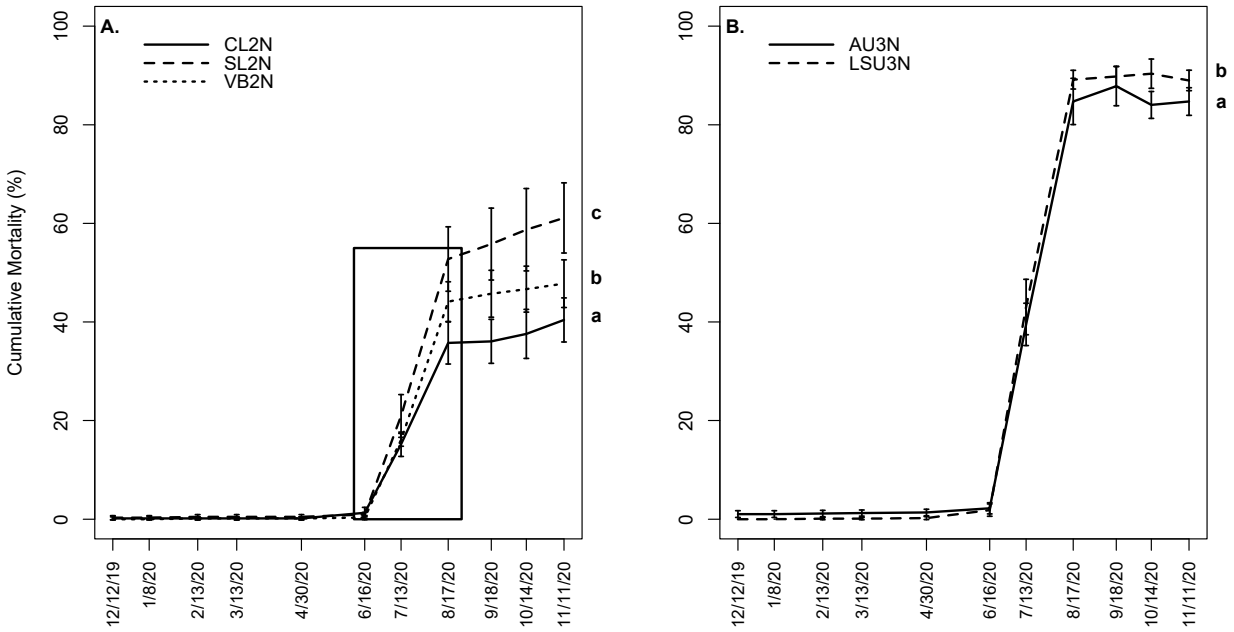

Figure 4.4. Cumulative mortality for diploids (A) and triploids (B) at LUMCON from Nov 2019 to November 2020. Error bars denote 2 standard errors. The box in Figure 4.4 A highlights the interval mortality from the period selected for a posteriori analysis. Dates indicate sampling dates of the study. Letters indicate significant differences ($p < 0.05$) between groups.

Stock affected the cumulative mortality of diploids at LUMCON (Figure 4.4 B). At the end of the study, cumulative mortality was higher in the SL2N, 61% (95% CI 55–67%), and VB2N, 48% (95% CI 4–54%), crosses than in the CL2N cross, 40% (95% CI 35–46%) (general linear model, $t \geq 2.12$, $P \leq 0.04$, for all cases). Peak mortalities for LSU and AU diploids were from mid-June to mid-August, similar to triploids. The SL2N cross exhibited higher interval mortality during this period than the CL2N cross (general linear model, $t = 2.88$, $P < 0.01$) (box in Figure 4.4 B). No differences in interval mortality were observed between the CL2N or SL2N crosses and the VB2N cross. Additionally, no cohort effect was observed for diploids at LUMCON.

At LSURF (the moderate salinity site), triploids again had higher cumulative mortality than diploids (general linear model, $t = 5.37$, $P < 0.01$). The cumulative mortality of all triploids at LSURF, across cohort and stock, were similar (general linear model, $t \leq 1.35$, $P \geq 0.20$ for all cases) (Figure 4.5 A). However, from the end of April to mid-July, LSU triploids had higher interval mortality than AU triploids, across stock (general linear model, $t = 3.32$, $P < 0.01$) (box in Figure 4.5 A). After this interval, mortality of triploids was less severe but continued to gradually increase until the end of the study.

Stock and cohort interacted to affect diploid cumulative mortality (Figure 4.5 B). The CL2N and VB2N crosses in the AU cohort had 14% (95% CI 1–27%) higher cumulative mortality than the AU SL2N cross (general linear model, $t \leq -2.32$, $P \leq 0.03$ for all cases) at the end of the study. However, all crosses in the LSU cohort (CL2N, SL2N, and VB2N) had similar cumulative mortalities to each other and to the SL2N cross in the AU cohort (general linear model, $t \geq -2.40$, $P \leq 0.03$ for all

cases). Diploid cumulative mortalities at LSURF started to rise at the end of April and increased largely between mid-June and mid-July. Additionally, unlike at LUMCON, from mid-October to mid-November 2 crosses (CL2N and VB2N) in the AU cohort experienced another marked increase in mortality (Figure 4.5 B).

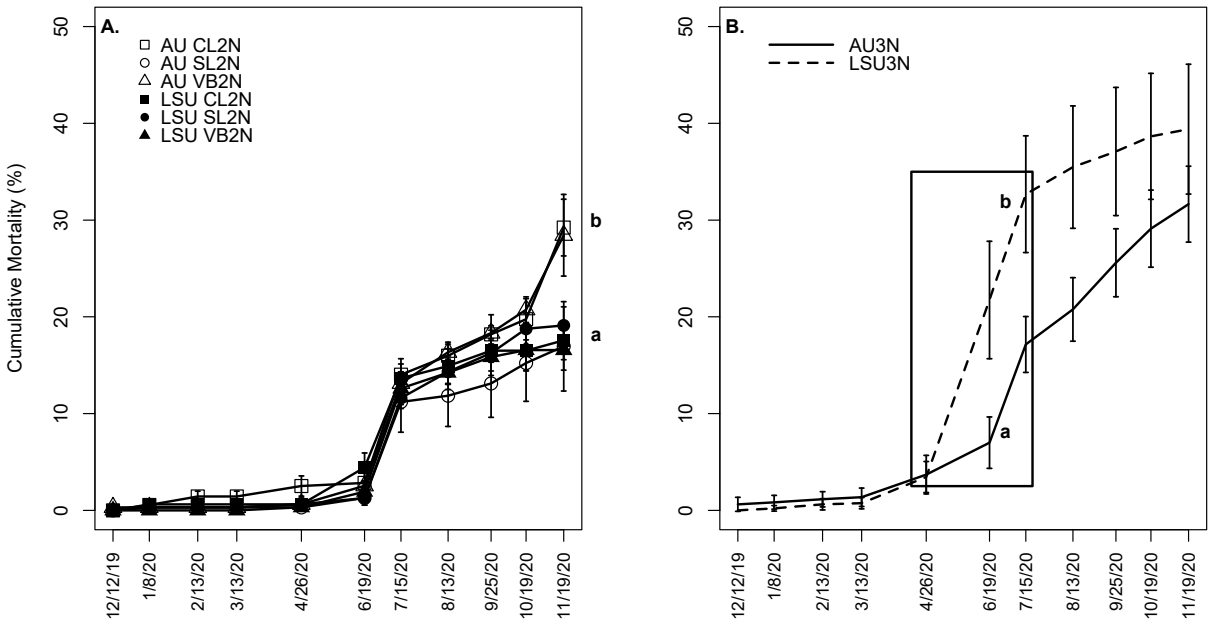

Figure 4.5. Cumulative mortality for diploids (A) and triploids (B) at LSURF from Nov 2019 to November 2020. Error bars denote 2 standard errors. The box in Figure 4.5 B highlights the interval mortality from the period selected for a posteriori. Dates indicate sampling dates of the study. Letters indicate significant differences ($p < 0.05$) between groups.

Overall Growth Rate

At LUMCON, triploids did not exhibit faster growth rates than diploids over the study (general linear model, t = 1.67, *P* = 0.10). Oysters grew throughout the study period except from mid-June to mid-August for triploids and mid-June to mid-July for diploids. During these periods, the mean shell heights of diploids and triploids decreased (Figure 4.6 A & B) presumably due to differential

mortality of larger oysters. Stock and cohort interacted to affect triploid growth rates at LUMCON. During the study, the SL3N cross of the AU cohort grew faster than any other cross and grew 60% (95% CI 30–80%) faster than the next fastest growing cross, LSU CL3N (general linear model, t ≤ -2.42, $P \leq 0.03$ for all cases). Among diploids, overall growth rate was affected by cohort. Although diploids from both cohorts had similar shell heights at the end of the study (general linear model, t = -0.43, $P = 0.66$), AU diploids, which were smaller at the time of deployment, grew 23% (95% CI 4–49%) faster than LSU diploids over the study (general linear model, t = -2.41, $P = 0.03$).

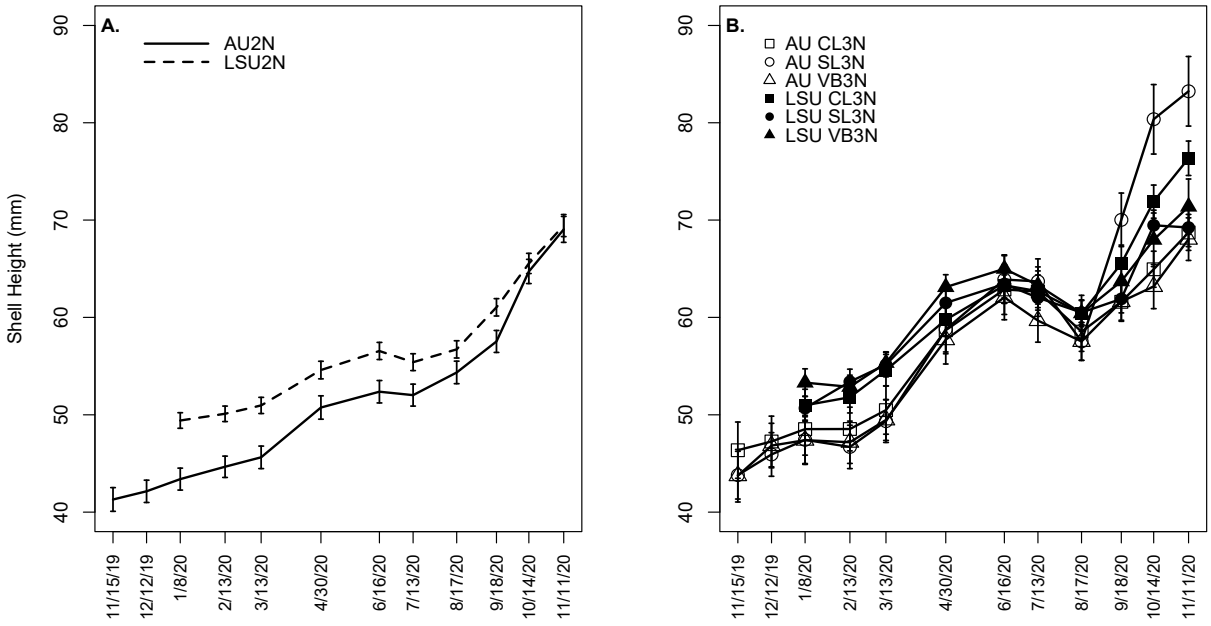

Figure 4.6. Shell heights in mm of diploids (A) and triploids (B) at LUMCON from Nov 2019 to November 2020. Error bars denote 2 standard errors.

Triploids and diploids at LSURF had faster growth rates than oysters at LUMCON over the course of the study (general linear model, t = -36.67, $P < 0.01$). Additionally, triploids exhibited faster

growth than diploids at LSURF (general linear model, t = 4.99, $P < 0.01$). Triploids at LSURF grew on average 4.1 ± 0.2 mm every 30 days and were on average 10.5 ± 0.5 mm longer than diploids at the end of the study (Figure 4.7 A). Stock and cohort affected triploid growth rate. The CL3N cross had 6.1% (95% CI 0.6–11%) faster growth than the SL3N and VB3N crosses, across cohort (general linear model, t ≥ 2.59, $P \leq 0.02$ for all cases). Additionally, triploids of the AU cohort had 6.4% (95% CI 2.1–11.2%) faster growth rate than triploids of the LSU cohort (general linear model, t = 3.69, $P < 0.01$). Diploids at LSURF grew an average of 3.7 ± 0.3 mm every 30 d throughout the course of the experiment (Figure 4.7 B). Stock affected the overall growth rate of diploids at LSURF. The SL2N and VB2N crosses had averaged 10% (95% CI 2–18%) faster growth than the CL2N cross, across cohort (general linear model, t ≥ 2.59, $P \leq 0.02$ for all cases).

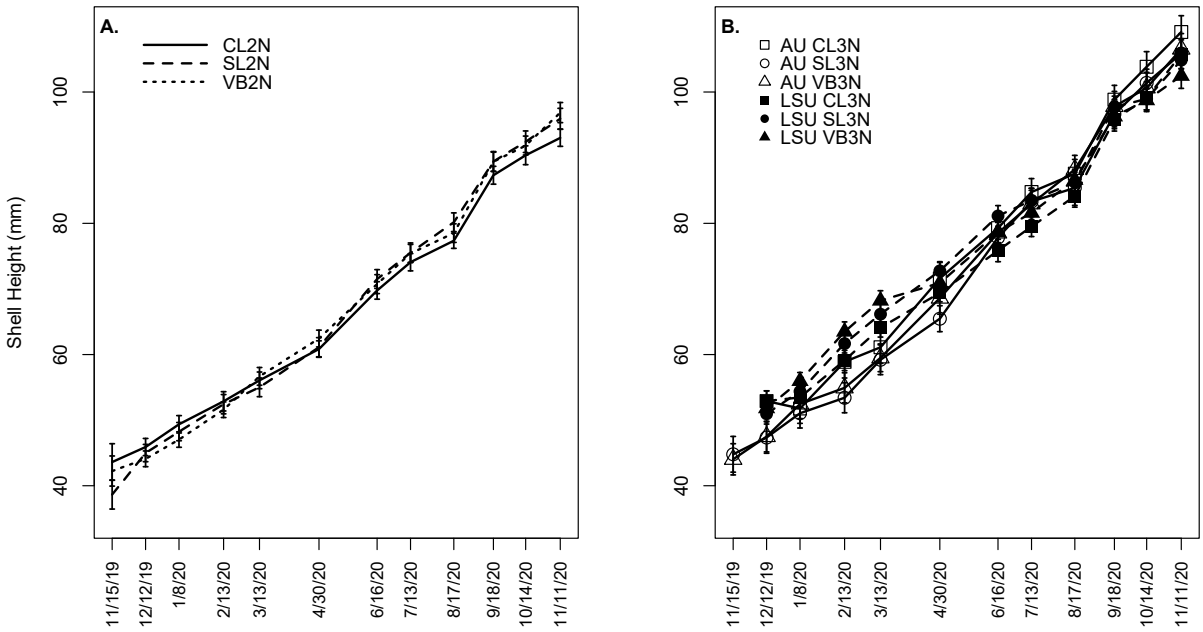

Figure 4.7. Shell heights in mm of diploids (A) and triploids (B) at LSURF from Nov 2019 to November 2020. Error bars denote 2 standard errors.

Sex, Gametogenic Stage, and Gonad-to-Body Ratio

Of the 240 oysters sampled in June, 58.8% were male and 41.2% were female. There was a difference in the sex ratio between sites (Pearson's chi-squared, $P = 0.02$). At LUMCON the sex ratio was 79:41 (M:F) and at LSURF the sex ratio was 62:58 (M:F). Diploids and triploids were at different stages of gonadal development (Pearson's chi-square, $P < 0.1$). Diploids had more advanced gonadal development (95% in the LA, R, and S stages) than triploids (79% in the VEA, EA, and A stages). Furthermore, diploids from the two cohorts were at different stages of gonadal development (Pearson's chi-square, $P < 0.1$) as more LSU diploids were at later stages of development (63% in S stage) than AU diploids (76% in LA and R stages, 15% in S stage). While cohort did not affect the gametogenic stage of triploids, stock did have an effect (Pearson's chi-square, $P = 0.01$). The oysters in the VB3N cross were in earlier gametogenic stages (47.5% in the VEA stage) than oysters in the CL3N or SL3N crosses (65% in EA and A stages). Triploids had lower gonad-to-body ratios across site, cohort, and stock than did diploids (general linear model, $t = 7.5$, $P < 0.01$). At LUMCON in June, Sister Lake triploids at LUMCON had lower gonad-to-body ratios than CL and VB triploids (general linear model, $t \geq 2.94$, $P \leq 0.01$ for all cases). There was no effect of stock or cohort on gonad-to-body ratio for diploids (general linear model, $t \geq -1.63$, $P \geq 0.11$ for all cases). At LSURF in June, there was no effect of stock or cohort on gonad-to-body ratios for triploids or diploids (general linear model, $t \geq -1.59$, $P \geq 0.12$ for all cases).

Condition Index

Condition index of triploids was only affected by stock in June at LUMCON. The SL3N and VB3N crosses had lower condition indices than the CL3N cross (general linear model, $t < -2.08$, $P < 0.04$

for all cases, Appendix C, Table C.1). The SL3N oysters had 12% (95% CI 1–28%) lower condition indices than did CL3N oysters, and VB3N oysters had 18% (95% CI 5–35%) lower condition indices than did CL3N oysters. Diploids of the LSU cohort had 16% (95% CI 6–30%) lower condition indices than those of the AU cohort (general linear model, t = -3.52, $P < 0.01$). Additionally, SL2N and VB2N crosses had lower condition indices than the CL2N cross (SL: 16%, 95% CI 4–32%; VB: 14%, 95% CI 2–29%), across cohort (general linear model, t ≤ -2.40, $P \leq 0.01$ for all cases). At LSURF in June, only cohort affected the condition indices of diploids (general linear model, t = -5.77, $P < 0.01$). Diploids in the LSU cohort had 36% (95% CI 6–30%) lower condition indices that diploids in the AU cohort.

In September, condition index was affected by site and ploidy only. Oysters at LSURF had 21% (95% CI 11–29%) lower condition indices across cohort and stock than did oysters at LUMCON (general linear model, t = 3.54, $P < 0.01$) (Appendix C, Table C.1). In addition, diploids across cohort and stock had 40% (95% CI 32–45%) lower condition indices than did triploids (general linear model, t = 6.61, $P < 0.01$).

Perkinsus marinus Prevalence and Infection Intensity

Average *P. marinus* prevalence (% infected oysters) was higher at LSURF (47%) than at LUMCON in June (22%, Pearson's chi-squared, $P < 0.01$). The prevalence of *P. marinus* was higher in oysters in the LSU cohort than AU cohort across ploidy and stock in September (AU average: 7%, LSU average: 97%, Pearson's chi-squared, $P < 0.01$).

The majority of *P. marinus* infection intensities were light (< 10^4 parasites g^{-1} wet tissue) for all oysters at both sites (Appendix C, Table C.1). Across both sites in June, oysters had similar mean infection intensities regardless of ploidy, cohort, and stock (general linear model, $t \leq 1.84$, $P \geq 0.07$, for all cases). Across ploidy, oysters of the AU cohort had higher mean infection intensities than did oysters of the LSU cohort (intensities: general linear model, $t = -2.38$, $P = 0.02$). At LSURF, triploids had higher mean infection intensities than did diploids across stock and cohort (general linear model, $t = 3.14$, $P < 0.01$). In September, across both sites, 16% of all oysters in the LSU cohort were heavily infected (> 5×10^5 parasites g^{-1} wet tissue), while 26% were moderately infected ($10^4 – 5 \times 10^5$ parasites g^{-1} wet tissue). No oysters in the AUSL cohort collected for *P. marinus* infection measurements in September had heavy infection intensities.

Discussion

The goal of this study was to examine the effect of wild broodstock parentage on the tolerance of triploid progeny to low salinity. Triploid crosses produced with broodstock from lower-salinity estuaries (SL and VB) were predicted to have higher survival and growth in a low-salinity field site than progeny produced with brood- stock from a higher-salinity estuary (CL). Diploid crosses were also produced using wild broodstock parents to verify expected differences among diploid progeny and between ploidy levels (i.e., higher triploid mortality at low salinity). Overall, diploid parentage (stock) had the smallest effect on the performance (growth and survival) of triploid progeny, while ploidy level, followed by cohort, had the largest effect. The near 100% cumulative mortality of all triploid crosses at the low-salinity site precluded analysis of the effect of broodstock parentage on triploid low-salinity tolerance, and this underscores that ploidy drove

this result. Broodstock parentage affected diploid field performance, although the field performance of diploids did not match with pre-dictions based on broodstock origin; for example, CL diploids had the lowest mortality at LUMCON. Ploidy had the largest effect on oyster performance, with triploids having higher cumulative mortality rates than diploids at both field sites and faster growth rates than diploids only at the high-salinity field site. Finally, hatchery cohort affected oyster performance at both sites.

Triploids experienced higher cumulative mortality than diploids at the low-salinity site in agreement with results from previous studies (Callam et al., 2016; Matt et al., 2020; Wadsworth et al., 2019). Interval mortalities peaked during an extended period of low salinity (2.4 ± 1.2 ppt) from mid-June to mid-July. During this period water temperatures were high, averaging 30 ± 1.6°C. Previous studies in the GoM have also observed disproportionately high triploid mortality when compared to diploid mortality (Wadsworth et al., 2019), and high diploid oyster mortality has been associated with periods of low salinity (<5 ppt) and high water temperatures (> 28°C) (La Peyre et al., 2009, 2013; Rybovich et al., 2016). In addition to increased mortality, triploids in this study did not have the expected faster growth rates than diploids at the low-salinity field site. Other studies have also observed that the triploid growth advantage (over diploids) is "site-dependent" and triploids in unfavorable growing conditions, such as low salinity, did not exhibit faster growth rates than diploids (Callam et al., 2016; Davis, 1994; Wadsworth et al., 2019). Higher triploid mortality and slower growth at the low-salinity site may have been caused by polyploid gigantism, an aspect of triploid cellular architecture.

In polyploid gigantism, polyploid animals, which acquire one or more additional sets of chromosomes, have a greater amount of cellular DNA contained within a larger cell nucleus, and larger cell volumes (Cavalier-Smith, 1982; Child and Watkins, 1994; Guo and Allen Jr, 1994). The increase in cell volume of a triploid animal without a reduction in cell number may partly explain why triploids are larger than diploids of the same age (Guo et al., 1996; Guo and Allen Jr, 1994; Piferrer et al., 2009; Wang et al., 2002). An increase in cell volume, however, lowers the cell surface-to-volume ratio of triploids compared to diploids, and likely slows the cellular response to changing environmental conditions in osmo-conforming and poikilothermic organisms. As oysters depend on cell volume regulation to deal with fluctuating salinity (Shumway et al., 1996), triploids may be at a disadvantage especially at low salinity when cellular metabolism, as well as intra-cellular ion and acid-base regulation, are being negatively affected (Ballantyne and Berges, 1991; Paparo and Dean, 1984; van Winkle, 1972). Further studies are needed to compare the ability of triploids relative to diploids to osmoconform and regulate the volume of their cells when exposed to low salinity.

At the moderate-salinity site, triploids also experienced higher cumulative mortalities than did diploids. Interval triploid mortalities increased in late spring while an uptick of diploid mortalities was most noticeable in early summer. The causes of the triploid mortalities are unknown and none of the environmental conditions nor *P. marinus* infection intensities (which were light, <104 parasites per g wet tissue and similar in triploids and diploids) would be considered lethal (Bushek et al., 1994; La Peyre et al., 2019). Diploid mortalities were slightly higher than expected as monthly summer mortalities in GoM estuaries are generally not excessive (<5%) unless

associated with low salinity (<5 ppt) or heavy *P. marinus* infection intensity (La Peyre et al., 1995, 2018, 2019; La Peyre et al., 2013; Wadsworth et al., 2019). Mortalities of diploids are generally attributed to stress from the intense physiological changes associated with gonad development and spawning (Huvet et al., 2010; Samain et al., 2007).

Interestingly, a positive correlation between reproductive effort and summer mortality in Pacific oysters (*Crassostrea gigas*) has been repeatedly reported (Cotter et al., 2010; Huvet et al., 2010; Koganezawa, 1974; Samain et al., 2007). Moreover, Pacific oysters which only partially spawned and retained unspawned gametes displayed greater mortality (Samain et al., 2007). The triploid mortalities in our study may be linked to advanced gametogenesis and unspawned gametes as almost 35% of triploids had gonads in an advanced stage of development (≥ 50% follicle coverage, Matt and Allen, 2021) while their condition index remained high indicating no spawning. It is possible that elevated metabolism for developing and maintaining gonadal tissues over an extended period at elevated temperature in triploids lead to oxidative stress and eventually cell death (Lesser, 2006); higher respiration rates have been observed in oysters with higher investment in gametogenesis (Bayne and Widdows, 1978; Casas et al., 2018a) and could cause an imbalance between reactive oxygen species production and elimination, resulting in oxidative stress. Higher energy expenditure has long been observed to be predictive of natural mortality with the oxidative stress theory the most generally accepted explanation (Speakman et al., 2002; Hulbert et al., 2007). Differences or changes in the fatty acid composition of cell membranes which underlies variation in metabolic activity have also been linked to natural mortality including of oysters (Hulbert et al., 2007; Pernet et al., 2010). A third possible explanation could

include a disruption of cellular energy homeostasis leading to death of the oysters (Sokolova, 2013; Sokolova et al., 2012). The processes of energy storage and release are dependent on highly integrated systems that may be dysregulated or insufficient to provide the energy and nutrients required by cells to support their high metabolism even in the presence of adequate stored energy. The bioenergetics of diploid and triploids at the organismal and cellular levels (such as oxygen consumption, mitochondrial function, fatty acids composition) will need to be compared in future studies to test these hypotheses.

Triploid mortalities may also be the result of a lack of genetic diversity. It is possible that some inbreeding of the tetraploid line could have occurred since its creation because of the high genetic load of oysters (Plough, 2016). The loss of genetic diversity in the tetraploids would be especially detrimental because they contribute a greater amount of genetic material than diploid parents to the triploid progeny and could explain the greater mortality of triploids compared to diploids in this and other studies that used the same line of commercial tetraploid oysters (Wadsworth et al., 2019). Moreover, the combination of reproductive effort and high temperature may act as a tipping point triggering mortality as environmental stress has generally been shown to increase the expression and magnitude of inbreeding depression (Plough, 2012). The genetic diversity and performance of the tetraploid line used in our study will need to be evaluated. More emphasis on monitoring genetic diversity in selected hatchery stocks could help in avoiding reduced allelic diversity and lowered heterozygosity and accompanying inbreeding depression (Boudry et al., 2002; Varney and Wilbur, 2020).

Triploids of the LSU cohort had higher peak interval mortality at the moderate salinity site, higher cumulative mortality at the low-salinity site, and lower growth rates at both sites than did AU triploids. Cohort differences observed in triploids could be due to genetic differences resulting from different levels of heterozygosity or from selection events caused by hatchery conditions where each cohort was produced. Associations have been made between higher heterozygosity and increased weight, growth rate, and survival in eastern oysters (Britten, 1996; Rodhouse and Gaffney, 1984; Zouros and Foltz, 1983). Auburn triploids could have had higher heterozygosity because a larger number of parents were used to produce AU triploids than LSU triploids (Aho et al., 2006; Hughes et al., 2019; Lind et al., 2010). Another mechanism for generating cohort genetic differences could be hatchery conditions. Oysters raised in hatcheries are exposed to stressors constantly present at the hatchery, such as water quality. Differential survival may occur due to stressors acting on larvae or spat, causing selection events that would occur before oysters are placed in the field, and could influence field performance (Nascimento-Schulze et al., 2021).

While high triploid mortality at the low-salinity site impeded analysis of the effect of broodstock parentage on triploid low-salinity tolerance, there were no differences in mortality rates among triploid crosses during the die-off between June and August. This suggests that broodstock parentage had little effect on triploid survival at low salinity. The field performance of diploids at the low-salinity site was however affected by broodstock parentage. The CL diploids at the low-salinity site had lower cumulative and interval mortalities than did SL and VB diploids. Additionally, CL diploids, across cohort, had the slowest overall growth rate at the moderate

salinity site. In a related study, CL diploids (produced at the Auburn hatchery) also displayed lower peak interval mortality rates than did SL diploids at a low salinity field site (Eastburn, 2021).

Furthermore, Calcasieu Lake diploids in Eastburn (2021) had slower growth than other stocks (SL) at a higher salinity site (19.2 ± 5.42 g/L Oct. 2019 – Sept. 2020). All of these results were unexpected because crosses produced with broodstock from the historically higher salinity estuary (CL) were predicted to perform poorly at lower salinity field sites (Casas et al., 2017; Leonhardt et al., 2017). However, mean salinity in Calcasieu Lake has decreased in recent years from 19.5 ppt for the 2009 to 2014 period to 15.2 ppt for the 2015 to 2019 period (Swam et al., 2022). The recent increase in freshwater entering the estuary combined with overfishing has contributed to a 90% loss of the CL oyster population (LDWF, 2020). Therefore, it is possible that the genetic structure of CL broodstock has changed, altering the survival of CL crosses in low-salinity conditions and illustrating how natural and anthropogenic variability can shift the multidirectional selection pressure oysters face routinely in estuarine environments.

Conclusions

The effect of parentage on the salinity tolerance of triploid progeny was predicted at the onset of the study. However, prolonged low-salinity conditions at LUMCON caused high mortality levels in triploids and prevented proper analysis of broodstock parentage on triploid low salinity tolerance. The influence of broodstock parentage was observed in diploids, although results in this study did not always align with predicted growth and mortality based on broodstock origin. However, other studies in the region have observed progeny survival that matched predictions

based on broodstock origin although estuaries had more extreme regimes (high or low) than those used in the current study (Marshall et al., 2021a; Swam et al., 2022). In future work, wild diploid broodstock should be collected from estuaries with salinity regimes that are historically and currently more extreme and different from one another. Alternatively, diploid broodstock that have been selectively bred to be more tolerant to low salinity could be crossed with unselected tetraploids to test whether triploid progeny would inherit low salinity tolerance. Survival at low salinity has recently been shown to be a trait with moderate heritability ($h^2 = 0.4$) (McCarty et al., 2020). While there is some evidence that traits from diploid broodstocks can be passed to triploid progeny (Dégremont et al., 2010), improvement of triploid survival and growth may also require selectively breeding a line of low-salinity tolerant tetraploids as proposed by Callam et al. (2016).

Chapter 5. Energetic Budget of Diploid and Triploid Eastern Oysters During a Summer Die-off

Background

In this chapter, physiological parameters of diploid and triploid oysters were measured to evaluate energy intake and expenditure. These were measured to gain insight into potential causes of the triploid mortality event observed during the field study addressed in Chapter 4. Furthermore, Chapter 5 continued the Center Level work presented in Chapter 4 because the experiments also provided a case study of the forms of data that could be stored in a repository database. As with the previous chapter, data collected in Chapter 5 facilitated analysis of the time requirements to enter database information (discussed in Chapter 6), evaluated what kinds of data would be necessary to store in a repository (discussed in Chapter 7), and visualized the exchange of information that would occur between repositories and communities (discussed in Chapter 6).

Introduction

During the last decade, 2012-2022, off-bottom aquaculture has been promoted to supplement traditional on-bottom farming of eastern oysters (*Crassostrea virginica*) within U.S. Gulf of Mexico (GoM) estuaries (Walton et al., 2013). Off-bottom culture involves placing single-set, hatchery-produced seedstocks in some form of container that raises the oysters above the seafloor. This protects oysters from predation and sediment burial, allowing them to be grown in areas that would otherwise be unsuitable for oyster aquaculture (Moroney & Walker, 1999; Walton et al., 2013). Off-bottom farming, however, requires higher initial investments (i.e., in

time, labor, and money) than on-bottom culture (Walton et al., 2013). To offset those costs triploid oysters (3N) are commonly deployed because they can grow faster and be harvested throughout the summer months when diploid oysters (2N) have spawned and have poor meat quality (Allen Jr. & Downing, 1986; Walton et al., 2012). Triploid oysters have these advantages because of reduced gonadal development compared to diploid oysters (Allen Jr. & Downing, 1986; Dégremont et al., 2012). However, in recent years, oyster farmers of GoM and Atlantic USA estuaries have reported unexpected die-offs in late spring or early summer depending on the region, particularly of triploids (Bodenstein et al., 2021; Guévélou et al., 2019; Matt et al., 2020; Wadsworth et al., 2019).

The causes of these late-spring or early-summer die-offs, often referred as “triploid mortality events”, remains unresolved. These events have not been associated with unfavorable environmental conditions such as abrupt changes of temperature, salinity, pH, dissolved oxygen or sediment concentration, nor any known pathogen infections (Guévélou et al., 2019; Matt et al., 2020). The triploid die-offs are often accompanied by more limited mortalities of diploid oysters that occur when diploids have ripe gonads (e.g., advanced gametogenesis) and are ready to spawn or have spawned (Guévélou et al., 2019; Matt et al., 2020; Wadsworth et al. 2019). For diploid eastern oysters in GoM estuaries, mortality rates during this period of reproduction are generally small (<5% per month), unless also associated with long periods (>1 month) of low salinity (<5 ppt) or heavy *P. marinus* infection intensity (Casas et al. 2017, Wadsworth et al. 2019).

In other oyster species such as in Pacific oysters (*Crassostrea gigas*), however, summer mortalities can be substantial and positively correlated with reproductive efforts (Cotter et al., 2010; Huvet et al., 2010; Jouaux et al., 2013; Koganezawa, 1974; Perdue et al., 1981; Samain et al., 2007). It has been proposed that "metabolic disturbances" from the high energetic costs of developing and maintaining gonadal tissues in oysters may divert energy resources away from basic cellular maintenance resulting in mortalities, especially when co-occurring with other stressors such as high temperature (Huvet et al., 2010; Samain et al., 2007). Stress from gametogenesis could be a factor in the high triploid mortalities of eastern oysters, however, the specific causes remain unclear. Identifying physiological and metabolic changes accompanying triploidy may be useful in explaining differential mortalities of triploids relative to diploids during late spring and early summer.

Previous studies have investigated differences in diploid and triploid eastern oyster survival, growth, and pathogen resistance (Dégremont et al., 2012; Guévélou et al., 2019; Matt et al., 2020; Wadsworth et al., 2019). Only one study has directly compared triploids and diploids in relation to some aspects of their energetic physiology (filtration, feeding and basal metabolism variables) (Mizuta et al., 2021). The goal of this study was to gain insight into triploid mortality events by measuring energy intake and expenditure to calculate scope for growth (i.e., the energy available for growth) of triploids as compared to diploids. The objectives were to: 1) collect diploids and triploids from the farm site during an observed "triploid mortality" event; 2) measure the physiological parameters of clearance rate, valve movement, absorption efficiency, oxygen consumption rate, ammonia excretion rate, scope for growth, pathogen (*Perkinsus marinus*)

infection intensity, and mortality for diploid and triploid (2N and 3N) oysters produced at two hatcheries, and 3) determine whether triploid mortalities were associated with differences in net energy balance between diploid and triploid oysters. We hypothesized that triploid mortality would be explained by greater metabolic demands (higher oxygen consumption and ammonia excretion rates) and decreased feeding behavior (lower clearance rates or absorption efficiencies resulting in lower scope for growth values.

Methods

Broodstock Collection and Spawning

In January 2019, about 300 wild broodstock oysters were collected from Sister Lake (SL; 29°14'45.0''N, 90°54'35.0''W), a public oyster seed ground in Louisiana with an annual mean (± SD) salinity of 11.2 ± 5.5 ppt [n = 10, 2009-2018] (USGS 07381349 water-quality monitoring station) (Marshall et al., 2021). These oysters were placed in longline bags for conditioning at the Louisiana Sea Grant Oyster Research Farm (LSURF) in Grand Isle, LA (23.3°C ± 6.3 and 19.0 ± 7.17 ppt salinity from 2010-2020) (USGS #73802516, *USGS*, 2021). In June 2019, a portion of Sister Lake broodstock was spawned at the Louisiana Sea Grant Oyster Research Laboratory and Mike C. Voisin Oyster hatchery (LSURL). In July 2019, another portion was transported and spawned at Auburn University Shellfish Laboratory hatchery (AUSL) in Dauphin Island, Alabama.

At both hatcheries, diploids used in this study were produced by crossing wild female and male Sister Lake oysters. At LSURL 4 males and 3 females were mated and at AUSL, 33 males and 51 females were mated to produce F_1 diploids. To produce the triploids used in this study, wild

female Sister Lake oysters were crossed with male tetraploid oysters from one of two lines depending on the hatchery; 4DGNL17 line at the LSURL hatchery and 4MC18 line at the AUSL hatchery. The two tetraploid broodstock lines were originally part of the 4MGNL13 line, and each hatchery advanced the lines two generations to produce the two lines of tetraploids used in this study (Bodenstein et al., 2022). Sperm from tetraploid males in the 4DGNL17 and 4MC18 lines were verified by flow cytometry prior to fertilization of Sister Lake female broodstock. At LSURL 2 male tetraploids and 6 female diploids were mated and at AUSL, 8 male tetraploids and 51 female diploids were mated to produce F_1 triploids. At LSURL, corresponding diploid and triploid crosses were not half-siblings, but at AUSL diploid and triploid crosses were half-siblings produced from the same females, using the same batches of eggs. For this study, F_1 oysters produced in LSURL and AUSL will be referred to as LSU and AU cohorts. Diploid and triploid F_1 oysters (crosses) produced in LSURL will be referred to as LSU2N and LSU3N. Diploid and triploid crosses produced at AUSL will be referred to as AU2N and AU3N.

Both hatcheries used standard spawning techniques (temperature shock, Wallace et al., 2008) and each cross were obtained as described previously (Bodenstein et al., 2022). After pediveliger larvae were set on micro-cultch substrate (~ 300 µm in diameter) to produce single-oyster spat, the spat were grown in an upwelling system from July to September at each hatchery until large enough to be deployed in the field (length ~11.0 – 16.4 mm). The AU cohort was transported to LSURL in September 2019 with authorization from the Louisiana Department of Wildlife and Fisheries, and oysters from each cohort were maintained in the longline system at LSURF. Ploidy

verification by flow cytometry (Allen, 1983) was performed on oysters from both cohorts at LSURF in September 2019.

Following a significant increase in interval mortality rates of triploids (but not diploids) of both cohorts at LSURF (2% from mid-March to the end of April to 10% from the end of April to mid-June, Appendix D, Table D.1), oysters of both ploidies and cohorts (~120 of each) were transported in mid-June from the LSURF farm site (30 ± 1°C, 12 ± 4 ppt) to the Animal and Food Sciences Laboratory Building of the Louisiana State University Agricultural Center in Baton Rouge. Oysters were scrubbed to remove any biofouling organisms (e.g., barnacles, algae), and placed in six 400-L tanks filled with aerated artificial seawater adjusted to a salinity of 15 ± 1 ppt and water temperatures of 28 ± 1°C and equipped with biofilters (Crystal Sea Marinemix, Marine Enterprises International, Baltimore, Maryland, USA). Each tank contained approximately 35 oysters from each cohort and ploidy (140 oysters per tank). Oysters were fed ~5% of their dry meat weight with Shellfish Diet 1800® once per day (Reed Mariculture, Campbell, CA).

After 10 d of acclimation to laboratory conditions, clearance rate (CR), valve opening, absorption efficiency (AE), and ammonia excretion rate (NR) were measured with a subset of oysters labeled for identification (Subset A, Table 5.1). At the same time, routine oxygen consumption rates (OCR) were measured with a different subset of oysters (Subset B). After routine OCR measurements were complete, basal OCR were measured on a third subset of oysters (Subset C). Measurements were collected from individual oysters for all physiological rates except ammonia excretion, which was measured using four oysters in the same container. At the end of the study,

oysters in Subset A were processed to determine shell height, gill area, dry meat weight, condition index as described below. Oysters in Subsets B and C were processed to determine dry meat weight. Finally, remaining oysters were used to determine *Perkinsus marinus* (dermo) infection intensity, and gametogenic stage (Subset D). Mortality of all oysters was tracked every other day for the 6-week duration of the study and the percent cumulative mortality of oysters that died during the 6-week period was calculated (Ragone-Calvo et al., 2003).

Table 5.1 . The number of oysters per cross (AU2N, AU3N, LSU2N, and LSU3N) and which subset of oysters that were sampled for each physiological measurement. The same oysters within a subset were used for all measurements listed within that subset. If "All" is listed, the same number of oysters per cross were sampled for that measurement.

Subset	Physiological Measurement	Cross	Number of Oysters per Cross
A	Shell Height	All	24
	Gill Area	All	24
	Dry Meat Weight	All	24
	Clearance Rate	All	22
	Condition Index	All	23
	Clearance Rate	2N AU	18
		3N AU	21
		2N LSU	19
		3N LSU	21
	Absorption Efficiency	2N AU	30
		3N AU	28
		2N LSU	22
		3N LSU	27
	Ammonia Excretion	All	18
	% Valve Open	All	6
B	Routine OCR & Dry Meat Weight	2N AU	23
		3N AU	24
		2N LSU	24
		3N LSU	24
C	Basal OCR & Dry Meat Weight	2N AU	18
		3N AU	21
		2N LSU	20
		3N LSU	19
D	*P. marinus* infection level	All	15
	Gonad Stage	All	8

Clearance Rates

Clearance rate (CR), defined as the volume of water cleared of suspended particles by an animal in a given amount of time, was measured using a static system (Casas et al., 2018b; Riisgard 1988). Oysters were individually placed in 2-L beakers filled with gently aerated 0.5-μm filtered seawater with a salinity of 15 ppt. Oysters were left to acclimate for 1 h after which Shellfish Diet 1800® was added to each beaker to bring the initial suspended particle concentration to 3×10^4 cells mL^{-1}. The per unit volume of algal particles ≥ than 5 μm was measured every 30 sec until particle

counts declined below 50% of original values by use of a particle counter (PAMAS Model S4031 GO, PAMAS Partikelmess-und Analysesysteme, GMBH, Rutesheim, Germany). Beakers containing empty shells, but with the same concentration of algal particles, were used as controls. Only beakers containing oysters with open valves were measured. The same oysters were used to measure clearance rate, absorption efficiency, and ammonia excretion rate.

Individual clearance rate (CR_i) was calculated using the following equation:

$$CR_i\ (L\ h^{-1}) = [(b\text{-}b') \times vol\ (L) \times 60\ min\ h^{-1}$$

where b is the slope of the linear regression between the natural logarithm of cell concentration (cells mL^{-1}) and time (min) for the beaker with oyster, and b' is the slope for the control beaker, vol is the volume of seawater in the beaker (2 L) and 60 (min h^{-1}) is used to convert the time units. Clearance rates were standardized by shell height, specifically to a standard of 80 mm (i.e., average shell height for diploid and triploids, Casas et al., 2018a) using the equation:

$$CR_h=(H_{std}/H_{exp})^b \times CR_i$$

where CR_h is the clearance rate standardized by shell height, CR_i is the individual oyster clearance rate, H_{std} is the shell height of the standard oyster (80 mm), H_{exp} is the shell height of the experimental oyster, and b is the allometric exponent, 1.78 (Cranford et al. 2011). Clearance rates were also expressed relative to gill area (CRa, L h^{-1} cm^{-2}) to express the direct relationship between gill area and clearance rate (Meyhöfer 1985; Riisgård 1988) and to avoid standardization errors related to the different condition indices of the animals compared (Filgueira et al. 2008; Cranford et al. 2011). Finally, clearance rates were weight standardized to 1 g dry meat weight for the calculation of scope for growth according to the equation:

$$CR_w=(W_{std}/W_{exp})^b \times CR_i$$

where CR_w is the clearance rate standardized by dry meat weight, CR_i is the rate of the experimental animal, W_{std} is the standardization meat dry weight (1 g), W_{exp} is the meat dry weight of the experimental oyster, and b is the allometric exponent, 0.58 (Cranford et al. 2011).

Valve Opening

A non-invasive valvometry system (Casas et al., 2018a; Comeau et al., 2018), was used with 24 oysters (six oysters per cross) to estimate the percentage of time valves remained open during a 6-d period. A small magnet (5 × 3mm) was glued (using cyanoacrylate glue) to the left valve and a Hall element sensor (HW-300a, Asahi Kasei, Japan) coated in epoxy was glued directly across from the magnet on the right valve. The magnetic field in the form of output voltage (μV) was recorded at 1-min intervals by a dynamic strain recording device (DC 204R, Tokyo Sokki Kenkyujo Co., Shinagawa-ku, Tokyo, Japan). Oysters were continuously fed for one week at the supplier-recommended feeding rate of 0.05 ml of shellfish diet 1800® (www.reedmariculture.com) per g of wet meat weight per day. Algae were added to a reservoir tank containing refrigerated water at salinity of 15 ppt, and food was dispensed continuously with a peristaltic pump to a 40-L tank holding the oysters. To allow for acclimation following the installation of sensors and magnets, the first day of valve monitoring was discarded from the dataset, and data analyses were restricted to days 2 to 7. Raw voltage data (voltage sec^{-1}) were converted to display the average voltage each min. A regression equation was generated using the baseline (closed) voltage over the study period, to account for possible sensor drift over time. For each oyster, voltage was distributed in a 0 to 100% range, and oysters were considered closed when voltage was in the 0

to 10% range (Comeau et al. 2018). The number of "open" voltage data points was compared to the total number of voltage data points to calculate the percentage of time oyster valves remained open.

Absorption Efficiency

Absorption efficiency (AE) was measured following the direct method in a static system (Smaal & Widdows, 1994). Oysters from each cross were individually placed in containers with 5 L of aerated 0.5-μm filtered water at salinity of 15 ppt. Containers held water but no oysters to serve as controls. Oysters were not fed the day before the assay and were cleaned (by scrubbing their shells) before placing them in the containers. Oysters were fed with 3×10^4 cells mL^{-1} (a concentration at which no pseudofeces are produced) every 90 min for a total of 12 h and left undisturbed for 12 h before collection of feces. For each oyster, all feces produced during the 24-h experimental period were collected, as well as 750 ml of water at time 0 (TPM_0) and 24 h (TPM_{24}). Feces and water samples were filtered through pre-weighed filters (Whatman GF/C), rinsed with 0.5 M ammonium formate to eliminate salts, and dried for 24 h at 70 °C to estimate feces dry weight (F) and food ingested dry weight (I), $I=TPM_0-TPM_{24}$. Absorption efficiency was calculated as:

$$AE = ((I-F)/I) \times 100$$

Oxygen Consumption Rates

Oxygen consumption rates (OCR) of oysters fed daily (routine OCR) and oysters not fed for at least one week (basal OCR) were measured in static 915-mL acrylic chambers filled with 0.5-μm filtered seawater with a salinity of 15 ppt (Casas et al 2018a). Chambers were sealed with clamps except for an opening into which a fitted self-stirring probe with optical dissolved oxygen sensors (ProOBOD, YSI Incorporated, Yellow Springs, OH, USA) was inserted. Dissolved oxygen readings began after an oyster opened its valves and continued until the dissolved oxygen level fell to 70% of the starting level (Casas et al., 2018a). Chambers containing empty shells were used as controls. Individual oyster oxygen consumption rates (OCR_i), basal and routine, were calculated using the equation:

$$OCR_i = ([(c-c') \times Vol] \times 60 \text{ min})$$

where c is the slope of the linear regression of oxygen concentration (mg L^{-1}) in the chamber vs time, c' is the slope for the control, Vol is the volume of the chamber minus oyster volume. Oxygen consumption rates were standardized to 1 g dry meat weight following the formula structure described above for CRw and a weight exponent, b, of 0.58 (Casas et al., 2018a).

$$OCR_w=(W_{std}/W_{exp})^b \times OCR_i$$

Ammonia Excretion Rate

The rate of ammonia excretion (NR) was determined by placing three oysters of each cross in 1-L beakers filled with 1 L of 0.5-μm filtered seawater with a salinity of 15 ppt (Kinsella, 2019; Widdows & Johnson, 1988). A beaker containing 1 L of seawater was used as a control. After 4 h, samples of water were collected to measure ammonia levels using an ammonia ion electrode

(Model Truline, YSI Incorporated, Yellow Springs, OH, USA). Ammonia excretion rates were measured six times for each cross (AU2N, AU3N, LSU2N, LSU3N) using different oysters each time. Six controls using empty shells were also measured for a total of 30 measurements.

Ammonia excretion rate was expressed relative to meat dry weight ($\mu g\ h^{-1}\ g^{-1}$). The NR ($\mu g\ h^{-1}\ g^{-1}$) was calculated using the equation:

$$NR = ([(b-b') \times Vol]/time)/g$$

where b is the ammonia level for the beaker with the oysters (mg/L), b' is the ammonia level for the control beaker (mg/L), Vol is the volume of the beaker (1 L), h is the amount of incubation time (4 h), and g is the combined dry weight of the oysters in the beaker in grams.

Scope for Growth

Scope for growth (SFG) is an index of energy available for growth and reproduction. Scope for growth is estimated from the difference between energy absorbed from the food and the energy expended via respiration and excretion (Smaal & Widdows, 1994; Widdows, 1985). The SFG was calculated by using the equation:

$$SFG = A - (R + U)$$

where A is the energy absorbed, R is the energy respired and U is the energy excreted (Domínguez et al., 2020; Montagnac et al., 2020; Widdows & Johnson, 1988).

To obtain A, the energy consumed (C, J/h) was first calculated using the equation:

$$C = CRw \times open\% \times POM \times 23.5\ J\ mg^{-1}\ POM$$

where CRw are the clearance rate values (L h^{-1} g^{-1}) for oysters (Subset A) in each cross, open% is the percent time valves were open, POM is the particulate organic matter (mg L^{-1}), and 23.5 J mg^{-1} POM is the Joules in 1 mg of POM (Bayne & Newell, 1983). Energy consumed values were calculated for individual oysters in each cross. Individual energy consumed values were multiplied by the average absorption efficiency (AE) for each cross to obtain A values for oysters in each cross. The energy absorbed (A) values were averaged to obtain one value per cross.

The routine OCR values for oysters (Subset B) in each cross (mg O_2 h^{-1} g^{-1}) were multiplied by the number of Joules needed to respire 1 mg of O_2 (14.06 J mg^{-1} O_2) (Gnaiger, 1983) to calculate the energy respired, R. The energy respired values for individual oysters in each cross were then averaged to obtain one value per cross.

Finally, the average energy excreted for each cross, U, was calculated by multiplying the average ammonia excretion rate for each cross (mg NH_3 g^{-1} h^{-1}) by the number of Joules needed to excrete 1 mg of NH_4 (25.1 J mg^{-1} NH_4) (Gnaiger, 1983; Bayne, 2017). Therefore, one energy excreted value was calculated for each cross. The average energy absorbed, respired, and excreted values for each cross were used in the SFG equation to calculate a single average SFG for each cross (AU2N, LSU2N, AU3N, and LSU3N).

Condition Index, P. marinus infection Intensity, and Gametogenic Stage

After completing all physiological measurements, oyster shell height, and whole oyster weight and volume were determined. Oysters were opened and an image of the gill was captured by

digital camera (Nikon Coolpix S9600, Tokyo, Japan) (Casas et al. 2018b). Gill area (cm^2) of all 8 lamella, as seen from a top-down view, was estimated using image analysis software (ImageJ, version 1.53a, National Institutes of Health, USA) that was used to calculate clearance rate by gill area. Additionally, a linear regression analysis was performed to examine the effect of ploidy and shell height on gill area.

Oyster meat from oysters in Subsets A, B, and C were dried at 70° C for 48 h to determine the meat dry weight used to standardize the physiological rates (CR, routine OCR, basal OCR, and NR). Dry meat weights from oysters in Subset A were also used to calculate condition index (CI). The ratio of body mass to cavity shell volume (CI) was calculated using the equation:

CI = (meat dry wt / (whole oyster wt - wet shell wt)) × 100,

(Abbe and Albright, 2003).

Additionally, at the end of the study, 15 oysters from each cross were collected and processed to analyze *P. marinus* infection intensity using the whole-oyster procedure (La Peyre et al., 2018). Eight oysters from each cross were cross-sectioned and processed by standard histological technique (Howard et al., 2004) to determine sex and gametogenic stage. The stages of gametogenic development were determined using established methods (Matt and Allen, 2021). These stages were inactive (≤ 5% follicle coverage of the incipient gonad area), very early active (10–30% follicle coverage), early active (follicles with lumina and 10–40% follicle coverage), active (50–70% follicle coverage), late active (pronounced follicle canals with 75–90% follicle coverage), ripe (follicles filled with oocytes or spermatozoa and ≥ 80% follicle coverage), spawning (slight

follicle contraction with 60–90% follicle coverage), advanced spawning (greater follicle contraction with 30–70% follicle coverage), and spawned out (collapsed follicles with ≤ 50% follicle coverage) (Matt and Allen, 2021).

Data Analysis

All statistical analyses were performed in RStudio (version 4.0.3, R Core Team, 2020). Clearance rate, gill area, condition index, percentage of time valves were opened, absorption efficiency, oxygen consumption rate, and ammonia excretion rate were examined for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett test), transformed as required and analyzed with a two-factor (i.e., cohort and ploidy) analysis of variance (ANOVA) followed by post-hoc Tukey-Kramer pair-wise comparisons when significant differences were found ($p < 0.05$). A linear regression model was used to analyze the effect of shell height and ploidy on gill area. A linear regression model was used to analyze the effect of ploidy and cohort on infection intensity. A linear mixed effect model (R package *nlme*) was used to analyze the effects of cohort and ploidy (fixed effects), and tank (random effect) on cumulative mortality.

Results

Morphology, Clearance Rates, and Valve Opening

Triploids were longer than diploids ($P < 0.001$, 3N: 83.4 $\pm$ 9.9 mm, 2N: 74.6 $\pm$ 7.3 mm), and no differences in shell height were found between cohorts ($P = 0.19$, LSU: 77.8mm, AU: 80.3 mm). Triploids also had larger gill areas (24.62 $\pm$ 6.56 cm^2) than diploids (16.22 $\pm$ 5.37 cm^2) across cohort ($P < 0.001$) (Table 5.2). When the relationship between shell height and gill area was

compared between ploidies, triploids with the same shell heights as diploids had larger gill areas, $P \leq 0.001$ (Figure 5.1).

The larger triploids had greater individual clearance rates, 5.15 ± 3.46 L h^{-1}, than diploids, 2.78 ± 2.11 L h^{-1} ($P < 0.001$). Furthermore, when clearance rates standardized by shell height (CRh) were compared, triploids had greater ($P = 0.01$) clearance rates (5.01 ± 3.23 L h^{-1} 80 mm^{-1}) than diploids (3.24 ± 2.53 L h^{-1} 80 mm^{-1}), with no differences found between cohorts (Appendix D, Table D.3, LSU: 4.14, AU: 4.22). When clearance rates standardized by gill area (CRa) were compared, oysters from both cohorts and ploidies had similar clearance rates, 0.21 ± 0.16 L h^{-1} cm^{-1} ($P \geq 0.23$, for all comparisons) (Table 5.2). Finally, oyster valve movement across cohort and ploidy was not different ($P \geq 0.25$, for all cases). Oysters opened their valves 59% $\pm$ 5 of the time on average.

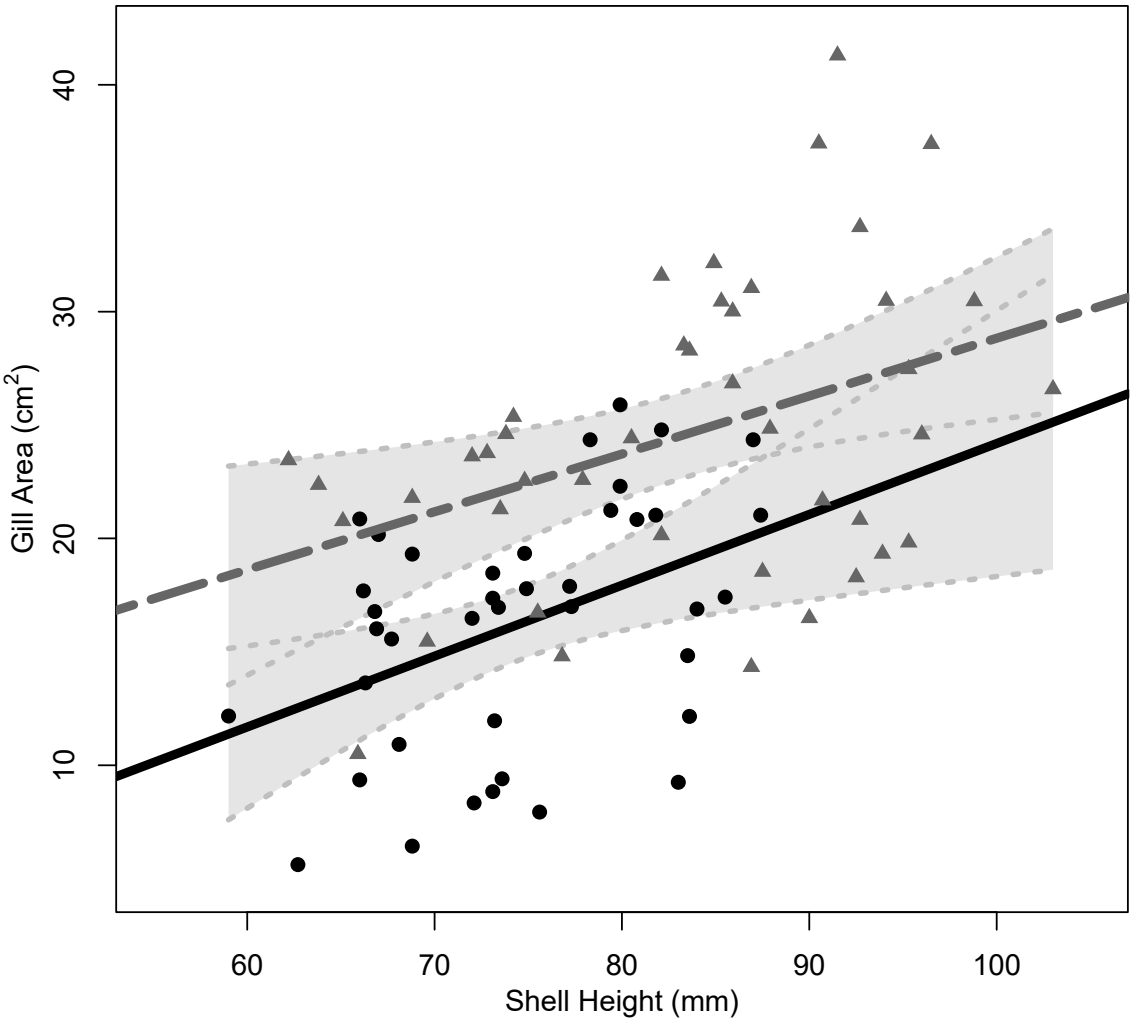

Figure 5.1. Linear regression lines for the relationship of shell height (mm) and gill area (cm^2) in diploids (solid, black line) and triploids (dashed, gray line). Circles represent shell heights and gill areas of individual diploids and gray triangles represent shell heights and gill areas of individual triploids. Light gray, shaded areas behind regression lines represent 95% confidence intervals, with light gray dotted lines indicating upper and lower 95% confidence limits.

Table 5.1. Mean ± standard deviations for shell height, gill area, clearance rate standardized by height (CRh), clearance rate standardized by gill area (CRa), clearance rate standardized by weight (CRw), percent of open valve time openness, and percent absorption efficiency (AE) for each oyster cross (AU2N, LSU2N, AU3N, LSU3N).

Ploidy	Cohort	Shell Height (mm)	Gill Area (cm^2)	CRi ($L\ h^{-1}$)	CRh ($L\ h^{-1}\ 80\ mm^{-1}$)	CRa ($L\ h^{-1}\ cm^{-2}$)	CRw ($L\ h^{-1}\ g^{-1}$)	% Valve Open	AE (%)
2N	AU	75.6 ± 7.48	17.42 ± 5.91	3.23 ± 2.10	3.75 ± 2.64	0.19 ± 0.13	4.60 ± 2.70	57.52 ± 8.28	71.8 ± 19.3
2N	LSU	73.7 ± 7.06	15.12 ± 4.72	2.35 ± 2.07	2.76 ± 2.38	0.18 ± 0.19	3.86 ± 3.89	53.72 ± 12.82	74.9 ± 17.3
3N	AU	84.1 ± 10.9	25.45 ± 6.79	4.87 ± 3.12	4.62 ± 2.88	0.22 ± 0.16	4.62 ± 3.16	64.53 ± 9.85	67.1 ± 22.9
3N	LSU	81.5 ± 9.45	23.82 ± 6.37	5.43 ± 3.83	5.39 ± 3.57	0.24 ± 0.15	5.00 ± 3.32	62.10 ± 20.08	72.6 ± 12.4

Table 5.2. Mean ± standard deviations for basal and routine oxygen consumption rates (basal and routine OCRi), and ammonia excretion rate (NR) for each oyster cross (AU2N, LSU2N, AU3N, LSU3N).

Ploidy	Cohort	basal OCR_w ($mg\ O2\ h^{-1}\ g^{-1}$)	routine OCR_w ($mg\ O2\ h^{-1}\ g^{-1}$)	NR ($\mu g\ h^{-1}\ g^{-1}$)
2N	AU	1.49 ± 0.43	2.49 ± 0.90	21.9 ± 20.7
2N	LSU	1.39 ± 0.56	2.05 ± 0.50	8.39 ± 15.1
3N	AU	1.77 ± 0.49	2.15 ± 0.46	12.2 ± 21.2
3N	LSU	1.25 ± 0.37	2.06 ± 0.62	8.13 ± 9.88

Condition Index

Triploids had higher condition indices (6.0 $\pm$ 1.64) than diploids (3.1 $\pm$ 0.9) ($P \leq 0.01$, for all comparisons).

Absorption Efficiency

The absorption efficiencies (72% $\pm$ 18 on average) were not different between ploidies or between cohorts, ($P \geq 0.31$, for all comparisons).

Oxygen Consumption Rates

The average basal OCR_w of all crosses (1.48 $\pm$ 0.50 mg O_2 h^{-1} g^{-1}) were reduced by 32% compared with routine OCR_w (2.19 $\pm$ 0.66 mg O_2 h^{-1} g^{-1}). Oysters across cohort and ploidy had similar routine OCR_w ($P \geq 0.05$, for all cases) (Table 5.3). However, basal OCR_w were affected by cohort ($P \leq 0.001$). Oysters of the LSU cohort had lower basal OCR_w (1.32 $\pm$ 0.48 mg O_2 h^{-1} g^{-1}) than oysters of the AU cohort, 1.65 $\pm$ 0.48 mg O_2 h^{-1} g^{-1}. Differences in basal OCR_w between triploid (1.52 $\pm$ 0.51 mg O_2 h^{-1} g^{-1}) and diploid (1.44 $\pm$ 0.50 mg O_2 h^{-1} g^{-1}) oysters were not observed ($P = 0.56$).

Ammonia Excretion Rate

The average ammonia excretion rate (NR) for all crosses was 12.65 $\pm$ 17.17 μg NH_3 h^{-1} g^{-1}. Ammonia excretion rate was only affected by cohort ($P = 0.04$). Oysters in the LSU cohort had lower NR (7.11 $\pm$ 8.58 μg NH_3 h^{-1} g^{-1}) than did oysters in the AU cohort (25.35 $\pm$ 20.80 μg NH_3 h^{-1} g^{-1}). No differences in NR between triploid (15.16 $\pm$ 17.71) and diploid (17.29 $\pm$ 19.50) oysters were found.

Scope for Growth

Diploid oysters in the LSU and AU cohorts had the lowest average SFG values (Table 5.4). Triploid oysters (of both cohorts) had higher average SFG values than either diploid cohort, and triploids in the LSU cohort had the highest average SFG value (Table 5.4). Triploid oysters also had higher energy absorption values (21% higher on average) and lower energy excreted (39% lower on average) than diploids (Table 5.4).

Table 5.3. Average values for *P. marinus* infection level, condition index, cumulative mortality, energy absorbed (A, J h^{-1}), energy respired (R, J h^{-1}), energy excreted (U, J mg^{-1}), and Scope for Growth (SFG, J h^{-1} g^{-1}) for each oyster cross (AU2N, LSU2N, AU3N, LSU3N). Only one value was calculated per cross for A, R, U, and SFG, therefore no standard deviation values are provided.

Ploidy	Cohort	*P. marinus* level (log_{10} cell g^{-1})	Condition Index	Cumulative Mortality (%)	A (J h^{-1})	R (J h^{-1})	U (J mg^{-1})	SFG (J h^{-1} g^{-1})
2N	AU	3.04 ± 1.35	3.13 ± 1.13	2.48 ± 2.93	40.19	35.01	0.55	4.63
2N	LSU	4.40 ± 1.58	3.13 ± 0.54	2.94 ± 4.56	32.84	28.82	0.21	3.81
3N	AU	4.00 ± 1.67	5.77 ± 1.50	8.89 ± 8.45	42.30	30.23	0.31	11.77
3N	LSU	3.62 ± 1.60	6.19 ± 1.80	12.10 ± 5.91	47.65	28.96	0.20	18.49

Perkinsus marinus Infection Intensity

Triploids had slightly higher infection intensities (3.79 $\pm$ 1.72 $\log_{10}$ cell/g), on average than diploids (3.69 $\pm$ 1.62 $\log_{10}$ cell/g) (general linear regression, t = 2.30, P = 0.03). The majority of oysters sampled (63%) had light infection intensities (< 10^4 parasites g^{-1} wet tissue), while 20% had moderate (10^4 - 5 × 10^5 parasite g^{-1} wet tissues) and 17% had heavy (> 10^5 parasite g^{-1} wet tissues) infection intensities (Casas et al., 2017). No difference was found between the infection intensities of oysters in the LSU cohort (3.82 $\pm$ 1.62 $\log_{10}$ cell/g) and oysters in the AU cohort (3.66 $\pm$ 1.71 $\log_{10}$ cell/g, P = 0.06).

Gametogenic Stage

Of all oysters sampled at the end of the study that had an active or spawning gonadal stage, 64% were males and 36% were female. Most of the triploids were in the inactive gonadal stage (≤ 5% follicle coverage, LSU: 63%, AU: 88%) or in post-spawning stage with collapsed follicles, hemocytes invading the gonad, and few residual gametes (LSU: 25%, AU: 12%). Only one triploid oyster was observed in advanced spawning stage (LSU: 12%, AU: 0%). Among diploids, most were in the inactive gonadal stage (LSU: 25%, AU: 25%) or spawned out (LSU: 62%, AU: 38%), but there were oysters in spawning stage in the Auburn cohort (25%) and advanced spawning stage in both cohorts (LSU:12%, AU:12%).

Cumulative Mortality

In the laboratory, triploids experienced higher percent mortality (8.9 – 12.1% range) than diploids (2.5 – 2.9% range) over the experiment (linear mixed effects model, t = 3.33, P < 0.001). However,

the percent mortality of the LSU cohort (8 $\pm$ 7% average) was not different from that of the AU cohort (6 $\pm$ 7% average) (linear mixed effects model, t =0.74, *P* = 0.47).

Discussion

The goal of this study was to evaluate if differences in the energetic physiology of triploids and diploids could explain an observed late spring "triploid mortality" event. Six main physiological parameters were measured to determine feeding behavior and metabolism: clearance rate (CR), valve movement, absorption efficiency (AE), basal and routine oxygen consumption rate (OCR), and ammonia excretion rate (NR). These parameters are related, as when oysters open their valves they feed, perform aerobic respiration, and excrete waste (Bayne, 2017; Casas et al., 2018b). The physiological parameters were used to calculate scope for growth (SFG), a function of energy absorbed minus the sum of energy respired and energy excreted. We hypothesized that triploid mortality observed in the field would be explained by increased metabolic demands, decreased feeding behavior, and lower SFG values.

Contrary to the hypothesis of this study, higher triploid mortality could not be explained by the measured physiological parameters. All SFG values fell within the range of SFG values reported for temperate and tropical bivalves (-10.1 – 89.4 J h^{-1} g^{-1}, Domínguez et al., 2020; 3.7 – 72.6 J h^{-1} g^{-1} of dry weight, Kesarcodi-Watson et al. 2001). The LSU triploids had SFG values classified as having high growth potential (> 15 J g^{-1} h^{-1}, Widdows et al., 2002). Triploid Auburn oysters had SFG values that fell into the moderate growth potential category (5 – 15 J g^{-1} h^{-1}, Widdows et al., 2002). Auburn and LSU diploids had SFG values classified as low growth potential (< 5 J g^{-1} h^{-1},

Widdows et al., 2002). A low SFG would indicate that energy expended was similar to energy absorbed and that the animal was in stressful conditions. No associations between low SFG values and high mortality were found in this study. Moreover, despite higher mortality, triploid oysters not only had higher SFG but the condition index values, which indicated how much of the shell cavity was occupied by tissue and a reflection of potential energy reserve, were also greater than diploids.

The higher SFG of triploid oysters was in line with the expectation of greater growth rate of triploid oysters observed in the field (Allen Jr. & Downing, 1986; Dégremont et al., 2012). Scope for growth values represent the net energy that should be available for processes such as somatic growth and meeting metabolic demands (Bayne & Newell, 1983). In accordance with their higher SFG values, triploids had faster growth rates in the field compared to diploids (Bodenstein et al., 2023). Triploids had higher growth rates than diploids because triploids had higher clearance rates when compared at individual basis (CRi) or when standardized by shell height (CRh). This was likely due to the larger average gill area per mm of shell height of triploids (Figure 5.1). As gill area of a bivalve increases so does the capacity to filter suspended particles from the water column, i.e., pumping, filtration, or clearance rates (Jones et al., 1992; Meyhöfer, 1985; Riisgård, 1988). Therefore, triploids consumed a greater amount of energy which in turn led to greater energy absorbed and finally greater SFG values.

It was predicted that triploid oysters, with their higher growth rates, would have higher oxygen consumption rates (metabolic demands) than diploid oysters. Higher oxygen consumption rates

can lead to an imbalance between production and elimination of reactive oxygen species (ROS), resulting in a lethal oxidative stress (Buttemer et al., 2010). Triploids have been observed to have higher rates of digestive enzyme activity and increased ROS production, when compared to diploids (*C. gigas*, Haberkorn et al., 2010). These observations led to the hypothesis that triploids have higher metabolic activity than diploids (Haberkorn et al., 2010) and these higher metabolic demands could be the cause of increased triploid mortality. Moreover, developing and maintaining gonadal tissues over an extended period at high temperatures, without spawning in triploids could elevate metabolism and contribute to oxidative stress and eventually death (Lesser, 2006).

Higher respiration rates have been observed in oysters with higher investment in gametogenesis (Bayne and Widdows, 1978; Casas et al., 2018). In Pacific oysters (*C. gigas*), higher respiration rates and ROS levels were reported before mortality events (Samain, 2011). Higher energy expenditure has long been observed to be predictive of natural mortality with the oxidative stress theory being the most generally accepted explanation (Speakman et al., 2002; Hulbert et al., 2007). Our results, however, showed that this was not the case because no difference could be shown between the oxygen consumption rates of triploid and diploid oysters. Finally, the other parameters (valve movement, clearance rate standardized by gill area, and absorption efficiency) were all similar between the ploidies meaning that feeding behavior, gill function, and digestive efficiency did not explain triploid mortality.

The physiological parameters analyzed in this study attempted to measure energy balance at the organismal level to explain differential die-offs in late spring and early summer between diploid and triploid oysters. Energy balance could have been disrupted on a smaller scale, such as at the cellular or chromosomal levels (Newman & Gregory, 2019; Sokolova et al., 2012). Looking first at the cellular level, triploid cells have larger cell volumes than diploid cells due to increased genomic content (Guo & Allen, 1994). This would result in increased intracellular distances and reduced surface-area-to-volume ratios (Guo & Allen, 1994; Hinegardner, 1974; Miettinen et al., 2017). Transporting molecules over greater distances within a cell can reduce the rate of biochemical reactions. Limiting the surface area of a cell in relation to volume slows down nutrient and oxygen uptake as well as waste disposal on the cellular level. These factors can lead to localized metabolic inefficiency and cell death which may not be detected at the organismal level, but that may eventually lead to organismal death (Miettinen et al., 2017).

Looking at another scale, cells within triploid animals could have had chromosomal abnormalities such as aneuploidy, a condition in which the chromosome number is not an exact multiple of the haploid number (Wachtel & Tiersch, 1993). Triploid oysters have been observed to contain a higher proportion of aneuploid cells than diploids (*C. virginica,* de Sousa et al., 2016; *G. gigas*, Wang et al., 1999) and negative correlations between individual level of aneuploidy, growth, and survival have been observed in bivalves (*Pinctada fucata*, Komaru & Wada, 1994; *C. gigas*, Leitão et al., 2001). Moreover, chromosome alterations (loss or addition) can be extensive during cell division in triploid oysters (Wang et al. 1999, de Sousa et al. 2016), altering the relative expression of hundreds of genes, compromising cellular functions, and causing cell death (Sheltzer & Amon

2011, Vitale et al. 2011, Storchova 2012, Rutledge & Cimini 2016). These events would be expected to be most pronounced at a time when cells are actively dividing and differentiating, such as during larval development. Anecdotally, higher mortality of triploids compared to diploids are observed in hatcheries during larval development stages (Dr. Brian Callam, Mike Voisin Oyster Hatchery, LSU, pers. comm.). Future studies can operate at multiple levels and evaluate physiological parameters (organismal level), cell size and intracellular metabolite gradients (cellular level), and karyotype (chromosomal level) to get a more complete view of how these levels interact and influence oyster bioenergetics.

Finally, triploids and diploids used in this study comprised animals from two cohorts produced at two hatcheries, adding another layer of complexity to analyzing the causes of triploid mortality. Cohort differences may have been caused by hatcheries personnel using different numbers of parents during spawning. The Auburn University hatchery used a greater number of parents (both male and female broodstock) than were used at LSURL when producing F_1 triploids and diploids (Table 1 in Bodenstein et al., 2023). Therefore, Auburn oysters could have had higher heterozygosity (Aho et al., 2006; Hughes et al., 2019) which has been associated with increased survival in bivalves (*Ruditapes decussatus,* Borsa et al., 1992; *C. virginica*, Zouros & Foltz, 1983). Another factor that has been associated with heterozygosity is inbreeding. As proposed in Bodenstein et al. (2022), inbreeding of tetraploid lines could have occurred because of their high genetic load (Plough, 2016). This could have reduced the heterozygosity of triploid offspring and caused differences in the physiological measurements between triploids of each cohort.

Conclusions

Triploid mortality could not be explained with the energetic physiology measurements collected in this study. In contrast to predictions, triploids did not have higher metabolic demands than diploids, and triploids had higher SFG values than diploids. In future experiments, it will be important to account for the possible effects of triploid cell size to determine if metabolic processes at the cellular level, rather than organismal, cause mortality. These studies could include comparing cell size and mitochondrial activity between diploids and triploids. Finally, in the field, environmental conditions are constantly changing, unlike the static conditions within the laboratory. Fluctuating conditions could have driven higher triploid mortality in the field but these conditions were not represented in the laboratory, possibly resulting in similar measurements for triploid and diploid feeding behavior and metabolism. In future experiments, *in situ* field measurements of physiological parameters such as clearance rate could be recorded to more accurately define the effect of environmental conditions on oyster physiology and mortality (Gray & Langdon, 2018).

Chapter 6. Tools for Repository Development in Oyster Aquaculture: Database Management and Modeling Repository Systems

Introduction

Research attempting to identify the causes of oyster mortality and increase the production capacity of oyster aquaculture has been ongoing for decades (Andrews, 1964; Cheney et al., 2000; Matt et al., 2020). Farmers often experience high oyster mortality from numerous causes such as environmental stress (low salinity and high temperature), pathogen infection (*Perkinsus marinus*), stress from gametogenesis, or other unexplained causes (S. Casas et al., 2017; Matt et al., 2020; Rybovich et al., 2016). These stressors may affect oysters differently depending on ploidy level (diploid vs. triploid), broodstock parentage, environmental conditions, or even the hatchery at which oysters were produced (Dégremont et al., 2015; Leonhardt et al., 2017; Meyers et al., 1991). These potential interactions make identifying causes of oyster mortality a complex task. Furthermore, comparing research is often difficult because results from different studies are not collected in a centralized location and the genetics of the animals used in research are often lost after studies are completed. To consolidate research results and preserve genetic resources, data and genetic material from oysters can be preserved in germplasm repositories.

A germplasm repository is a collection of cryopreserved genetic material, such as sperm, stored alongside associated data (e.g., phenotypic, physiological, pedigrees, and genetic) (Torres et al., 2016). Cryopreservation and repository storage have facilitated advanced selective breeding programs and dramatic increases in production for industries such as beef and dairy (Blackburn, 2018). The ability to easily transfer genetic resources (in the form of cryopreserved sperm

samples) is only part of the explanation for the rapid genetic improvement seen in beef and dairy cattle. The comprehensive information stored alongside samples makes them valuable to agricultural industries (Purdy et al., 2016; Varga, 2011). Oyster aquaculture could also benefit from repository storage, particularly because high-throughput, cryopreservation pathways for oysters have been developed (Yang et al., 2012) and the resources required to cryopreserve oysters have been studied (Chapters 2 & 3). Conversely, the resources necessary to manage a database (time and labor) for an oyster germplasm repository have not been evaluated. Furthermore, integrating repository activities, such as data management and quality control policies, into current aquaculture operations without disruption is critical.

The oyster aquaculture facilities most likely to contribute and use repository material are hatcheries. Assessing the needs of oyster hatcheries and their perceptions of cryopreservation is essential to understand how repositories can be built in a manner that best serves the industry. A common technique in industrial engineering called the “Voice of the Customer” analyzes needs of a user group through personal interviews (Griffin & Hauser, 1993). By listening to what hatcheries want and evaluating their needs, a model can be created tracking the exchange of materials and data between the repository and the surrounding community (Damelio, 2011). Such models assist with repository development and with comprehending how the community members (hatcheries, farms, laboratories, etc.) interact to form a repository network. The goal of this study was to provide tools for germplasm repository development by analyzing database management requirements and proposing models for repository communities and networks in oyster aquaculture. The objectives were to: 1) categorize the data needed to build a repository

database and evaluate database management requirements; 2) evaluate the needs of oyster hatchery managers based on personal interviews; 3) create models for repository integration into oyster aquaculture communities, and 4) illustrate the importance of networks in regard to safeguarding repository systems and propose a framework for the oyster repository network.

Methods

Database Management

In previous studies, discrete-event simulation models of a high-throughput oyster cryopreservation protocol (described in Yang et al., 2012) were constructed using the software Simio (2021, v14.230, Simio LLC, Sewickley, PA, Bodenstein et al., 2022). These studies focused on the cryopreservation steps necessary for preserving germplasm and modeled only Steps 4 – 19 (Bodenstein et al., 2022, Figure 2). To evaluate the steps in the cryopreservation pathway necessary to collect and manage data associated with cryopreserved samples, the time study data of Steps 1, 2, 7, 10, and 20 were analyzed. Time study data for these steps was collected during the nine cryopreservation trials (Table 6.1) described in Bodenstein et al. (2022b).

Table 6.1. The date, Number of Oysters processed, number of straws per oyster, and the number of straws processed for each of the nine cryopreservation trials performed between March 2020 and April 2021.

Trial	Date	No. of Oysters	Straws Oyster^{-1}	No. of Straws Processed
1	3/20/20	4	25	96
2	3/24/20	9	25	215
3	3/27/20	6	40	144
4	4/02/20	11	40	515
5	4/17/20	9	40	453
6	6/05/20	20	40	754
7	4/05/21	10	40	385
8	4/09/21	10	45	442
9	4/14/21	10	45	440

Steps 1, 7, 10, and 20 all involved data collection activities. Step 1 (Intake Form) consisted of filling out an Intake Form concerning details of the oysters that were to be cryopreserved. Details such as date, ploidy, age, average size, stock name, collection site location and conditions, broodstock parentage, and the number of oysters to be frozen were recorded. In addition, the cryopreservation protocol to be used was also recorded in the Intake Form including details such as sperm collection method, extender solution, cryoprotectant, container type, equilibrium time, cooling rate, and thawing rate. Steps 7 and 10 were described in detail in previous studies (Bodenstein et al., 2022). In Step 7 (Measure) phenotypic data of individual oysters (weight, height, length, and width) were collected and in Step 10 (Motility) sperm motility data of individual oysters were collected. Step 20 (Post-thaw Motility) contained both data collection and management activities. The data collection activities of Step 20 involved thawing cryopreserved samples and assessing sperm motility, referred to as post-thaw motility.

A subsample of males frozen in a cryopreservation trial were chosen. Two straws from each male were thawed (for 8 s in a 40°C water bath) and post-thaw sperm motility was determined

manually using the Zeiss microscope with a Makler® counting chamber (Sefi Medical Instruments Ltd., Haifa, Israel) at 100× magnification. The time required to complete Steps 1, 7, 10, and 20 were self-timed with a digital stopwatch over the course of the nine cryopreservation trials (Table 6.1). The time data collected for all steps were standardized to time per oyster. The average times to complete data collection activities in Steps 1, 7, 10, and 20 were calculated.

Steps 2 and 20 involved database management activities. Step 2 (Repository Entries) consistent of entering oysters into the National Animal Germplasm (NAGP) Animal GRIN repository (NAGP, 2023). This included registering future samples in the Animal GRIN online database by creating Animal ID, Repository, and Inventory numbers for each male. Under each entry in the database the species name, stock name, phenotype descriptor, germplasm type, date of parturition, provenance (latitude and longitude), and cryopreservation protocol were also listed. The database management activities in Step 20 (Post-thaw Motility), involved entering data collected during and after cryopreservation (initial motility, oyster wet weight, oyster height, oyster width, volume of sperm collected, and post-thaw motility) into Animal GRIN for each male.

Average cumulative mortality and growth rates collected in completed field studies (Bodenstein et al., 2023) as well as additional breeding information (number of sires and dames used) were also entered for each male. The time required to complete Steps 2 and 20 were self-timed with a digital stopwatch over the course of the nine cryopreservation trials. The average times to complete database management activities in Steps 2 and 20 were calculated and standardized to time per oyster. The amount of time required to complete all data collection and database

management steps was calculated based on the Number of Oysters to be processed. The values for the Number of Oysters were based on the oysters processed in the original cryopreservation model (15 oysters from Model A, Bodenstein et al., 2022) and the oysters processed at each production scale in Chapter 3 (50, 1,000, and 10,000 oysters).

Categorizing Repository Data

After the cryopreservation trials ended and all relevant data was entered in Animal GRIN, a list the data collected was compiled. Four categories were formed to group the collected data and data that could be collected in future studies. The groups were Cryopreservation, Sample, Biological, and Genetic Data. Cryopreservation Data consisted of information about the cryopreservation protocol used, such as the gamete collection method, the extender solution, the concentration sperm was diluted to, the cooling rate, and the thawing procedure. Sample Data consisted of measurements from individual males such as wet weight, length, and sperm motility. Biological Data consisted of phenotypic information that applied to the genetic line males belonged to, such as age, provenance, average mortality and growth rates, as well as average values for physiological measurements. Finally, Genetic Data consisted of information pertaining to the genetic makeup of lines, such as pedigrees, genome sequencing, and expected progeny differences (EPD's).

Assessing the Needs of Oyster Hatcheries

To construct models of how cryopreservation could be integrated into oyster hatchery systems, oyster hatchery managers were interviewed. Hatchery managers from private, commercial

hatcheries and from research hatcheries (funded by a university or government agency) were contacted via email. Interviews took place over the phone or via Zoom (Zoom Video Communications, San Jose, CA). A set of discussion questions were developed to first determine the scale and scope of each hatchery, such as the number of seed produced annually, the types of seed or larvae produced (i.e., ploidy, size, genetic line), how many farmers were served, and what challenges the hatchery faced. Managers were asked to discuss their initial perceptions of cryopreservation and why, or why not, they were interested in repository storage. Finally, the potential benefits of cryopreservation and repository storage were discussed, and managers were asked if cryopreservation were to be implemented at their hatchery, would it be preferrable for hatchery personnel to cryopreserve samples or for a cryopreservation service to be contracted. Discussions sometimes deviated from this sequence of questioning to allow for the natural flow of conversation, but all questions were addressed for each interview. In total, 15 managers were interviewed. The discussion questions are listed below:

1. *What does the work at your hatchery entail?*
2. *How many spawns are performed each year?*
3. *What is the average amount of seed produced each year?*
4. *What types of seed or larvae are produced (diploid, triploid, selectively bred, etc.)?*
5. *How many farmers does the hatchery serve?*
6. *What are some of the biggest challenges the hatchery faces?*
7. *Are there currently selective breeding programs run at the hatchery? If not, why? If yes, what are some challenges of running selective breeding programs?*

8. *Are you or anyone at the hatchery interested in cryopreservation? If yes, for what reason? If no, why not?*
9. *Would you be interested in performing cryopreservation yourself or paying for cryopreservation services?*

After the interviews were completed, responses from Questions 6, 8, and 9 were grouped to identify similar viewpoints. This methodology was based on the Critical Incident Technique (CIT), an inductive method where no initial hypotheses are formed so patterns can emerge from the responses (Flanagan, 1954).

Based on identified patterns, researchers can generate theories concerning key ideas of interest (Gremler, 2004). The critical incidents in Critical Incident Technique refer to comments that "make significant contributions … to a [central] activity or phenomenon" (Grove & Fisk, 1997). Using the CIT method, data are collected from the respondent's perspective (in their own words) via interviews and responses are analyzed to identify data categories that summarize and describe the incidents (Grove & Fisk, 1997). In this study, responses were categorized based on the type of hatchery (commercial or research) and the production scale to identify patterns. Commercial hatcheries were defined as private businesses. Research hatcheries were defined as facilities that received some form of government or university funding and were associated with a university. The production scale was defined as the number of seed (juvenile oysters large enough to be sold to farmers, ~6 – 10 mm in length) hatcheries produced annually. The

production scale divisions were < 100 million, 100 – 200 million, and > 200 million seed produced annually.

Creating Models for Repository Development

Based on analysis of the responses, relationship diagrams were developed to outline models for cryopreservation and repository storage integration into commercial and research hatcheries. Relationship diagrams are an industrial engineering tool that visually depicts the interactions among different facilities or community members within a system by illustrating the flow of materials and information (Damelio, 2011; Melnik et al., 2020). These relationship diagrams function at a different level of organizational structure than the cryopreservation process flow diagram developed in Bodenstein et al., 2022 (Figure 2.2 in this document). The cryopreservation diagram was at the Pathway Level of organization, displaying all the steps of a particular activity. The relationship diagrams were developed at the Center Level of organization, one level above the Pathway Level. These diagrams displayed the interactions among hatcheries, repositories, and community members, as well as activities that took place at each facility.

Two separate Center-level diagrams were created to illustrate the relationships surrounding commercial and research hatcheries. To create these diagrams, first, the facilities that would interact with commercial or research hatcheries (i.e., the community members) were established based on interview responses. Next, the relevant activities that would take place at the hatcheries and at each of the community facilities were listed. Hatcheries and community members were placed in the diagram and connected via arrows to represent the flow and

materials and information among the facilities. Finally, according to the type of hatchery represented, a germplasm repository was integrated into the model based on patterns identified using the CIT method.

New interactions (exchanges of materials and information) between the repository and other community members were inserted. Relationship diagrams can also be used to portray interactions at a higher level of organization, the Network Level. Networks expand beyond the direct interactions a of central facility, such as a repository, with community members and encompass interactions community members have among each other, regardless of the central facility's involvement. Network-level diagrams are used to highlight how each member contributes to the repository network and highlights the shared goals of the network. In this study a Network-level diagram was created by identifying all members and placing them together on a diagram with key interactions among members displayed with arrows.

Results

Database Management

A total of 89 males (3,444 French straws) were cryopreserved and entered as samples into the Animal GRIN database (Table 6.1). Germplasm samples (straws) were shipped to the NAGP repository in Fort Collins, CO at the end of April 2021. Data associated with those samples were entered into the NAGP Animal GRIN database. The total average time required to create and manage database entries was 3.5 ± 1.6 min per oyster, which included database management activities in Steps 2 and 20. Updating database entries after cryopreservation and post-thaw

analysis (Step 20, 2.44 ± 1.60 min oyster^{-1}) took 78% longer than creating initial database entries (Step 2, 1.07 ± 0.17 min oyster^{-1}). The average time required to collect data during a cryopreservation trial was 6.8 ± 1.3 min oyster^{-1}, which included data collection activities in Steps 1, 7, 10, and 20. Of these data collection steps, Step 10 (Motility) required the longest amount of time 3.9 ±1.0 min oyster^{-1}, an increase of over 200% from the next longest step (Step 20). On average, data collection took nearly double the amount of time (94% longer) than database management. The time required for data collection and database management based on the Number of Oysters processed was calculated (Table 6.2).

Table 6.2. The time required for database management (Steps 2 and 20) and data collection (Steps 1, 7, 10, and 20) based on the number of oysters processed.

Number of Oysters	Time Required for Data Collection	Time Required for Database Management
15	102.2 ± 18.8 min	52.7 ± 24.1 min
50	5.7 ± 1.0 h	2.9 ± 1.3 h
1,000	14.2 ± 2.6 d	7.3 ± 3.4 d
10,000	142.0 ± 26.0 d	73.2 ± 33.5 d

Categorizing Repository Data

The types of data that were collected for the Cryopreservation, Sample, and Biological Data categories were listed in Table 6.3. Cryopreservation Data were collected during Step 1 (Intake Form) and were entered into the database in Step 2. Sample Data were collected during Steps 7, 10, and 20, while Biological Data were collected either in Step 1 or gathered from other studies on the same genetic line of oysters. Sample and Biological Data were entered into the database in Step 20. No Genetic Data were collected during this study but potential data forms were

suggested based on data found in dairy repository databases, such as USDA Animal GRIN and Bovine Elite (Blackburn, 2009; https://www.bovine-elite.com/; Gadberry et al., 2016).

Table 6.3. Examples of four categories of data (Cryopreservation, Sample, Biological, and Genetic) and forms of data that could be collected in each category, the units and the format for each type of data, and if the data were collected in this study. "Cryo" is used as an abbreviation for cryopreservation, "EDP's" is used as an abbreviation for Expected Progeny Differences.

Category of Data	Data Type	Units	Format	Collected
Cryopreservation	Gamete Collection Method	---	Cryo. Protocol	Y
	Buffering or Extender Solution	---	Solution Protocol	Y
	Concentration of Sperm	cells / mL	Values	Y
	Cryoprotectant and Concentration	---	Solution Protocol	Y
	Cooling rate	°C / min	Values	Y
	Thawing Procedure	°C & min	Values	Y
Sample	Shell Height or Length	mm	Values	Y
	Shell Width	mm	Values	Y
	Whole Wet Weight	g	Values	Y
	Volume of Sperm Collected	mL	Values	Y
	Fresh Sperm Motility (before freezing)	%	Values	Y
	Post-thaw Motility	%	Values	Y
Biological	Latitude and Longitude (of collection or farm site)	D° M' S"	Values	Y
	Collect or Farm Site Conditions (e.g. temperature, salinity)	°C, ppt	Values	Y
	Date of Parturition (spawning date)	date	Values	Y
	No. of Sires and Dames	amount	Values	Y
	Mortality Rate	%	Values	Y
	Growth Rate	mm $month^{-1}$	Values	Y
	Condition Index	---	Values	Y
	Perkinsus marinus Infection intensity and prevalence	cells g^{-1} of tissue, %	Values	Y
	Gonad Development Index	---	Category	Y
	Gill Area	cm^2	Values	Y
	Clearance Rate	L of water filtered $hour^{-1}$	Values	Y
	Absorption Efficiency	%	Values	Y
	Oxygen Consumption Rate	mg O_2 $hour^{-1}$	Values	Y

Cont'd

Category of Data	Data Type	Units	Format	Collected
Biological	Ammonia Excretion Rate	μg ammonia per hour $^{-1}$	Values	Y
Genetic	Pedigree Information	---	Chart	N
	Genotyping or SNPs	---	DNA Sequences	N
	EPD's	---	EPD Chart	N
	Heterozygosity	---	Values	N

Assessing the Needs of Oyster Hatcheries

In total, 14 hatchery managers operating on the Gulf of Mexico and east coast of the United States were interviewed. When categorized by hatchery type, eight were commercial hatcheries and six were research hatcheries. The responses from Question 6 (i.e., perceived hatchery challenges) were classified into the following groups: Access to 4Ns (finding it difficult to obtain tetraploid oyster sperm), Algae Production (difficultly producing sufficient algae to feed oysters), Biosecurity and Diseases (difficulty maintaining biosecurity regulation and disease outbreaks), Funding (insufficient capital to run the hatchery), Harmful Algal Blooms (a source of oyster mortality due to toxic algae), Old Facilities (outdate buildings and equipment), Spawning and Production (difficulties with spawning practices or breeding programs), Staffing (insufficient personnel), and Water Quality (a source of oyster mortality due to water temperature, salinity, pH, pollution, etc.). The greatest challenge (the category with the highest number of responses) for research hatcheries was Water Quality with five responses (Figure 6.1 A). The greatest challenges for commercial hatcheries were Algae Production (six responses) and Water Quality (five responses).

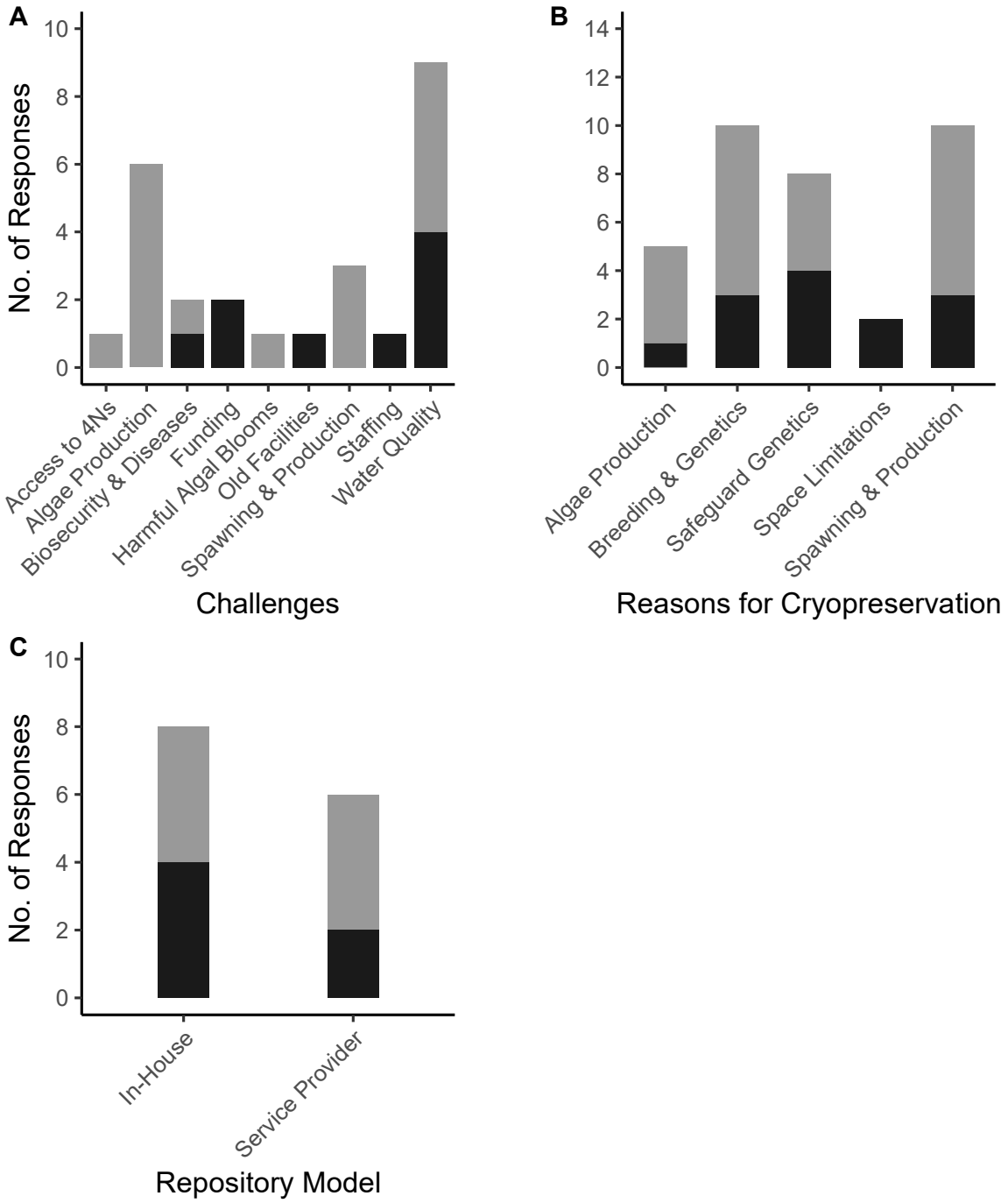

Figure 6.1. Responses of interviewed hatchery managers based on hatchery type to: A) the greatest challenges faced at the hatchery, B) the reasons they were interested in cryopreservation, and C) the repository model that is best suited for the hatchery. Grey bars represent responses from commercial hatcheries (N = 8) and black bars represent responses from research hatcheries (N = 6).

The responses from Question 8 (reasons for cryopreservation) were classified as the following groups: Algae Production (use cryopreserved algae to start cultures and faster recovery from crashes), Breeding and Genetics (use cryopreserved tetraploid sperm to make diploids or cryopreserved diploid sperm for breeding programs), Safeguard Genetics (cryopreserve sperm to protect current genetic lines), Space Limitations (cryopreserve sperm to free up space in the hatchery), and Spawning and Production (use cryopreserve sperm to facilitate spawning or interested in future capabilities to cryopreserve eggs and larvae). In commercial hatcheries, the common reasons for cryopreservation were Spawning and Production as well as Breeding and Genetics, both with seven responses (Figure 6.1 B). In research hatcheries, the most common reason for cryopreservation was to Safeguard Genetics.

The responses from Question 9 (preferred repository model) were classified as the following groups: In-House (hatchery personnel would perform cryopreservation and store samples) and Service Provider (the hatchery would contract an outside repository to cryopreserve and store samples). There were an equal number of commercial hatcheries that chose the In-House and Service Provider models (four responses each), while research hatcheries preferred the In-House model with four responses (Figure 6.1 C).

When hatcheries were categorized by production scale, eleven produced < 100 million seed, two produced between 100 and 200 million seed, and one produced > 200 million seed. The two greatest challenges across all production scales were Algae Production with six responses and Water Quality with nine responses (Figure 6.2 A). All three production scales recognized

Spawning and Production as a reason for cryopreservation (Figure 6.2 B). Finally, the In-House model was the preferred repository model across the production scales with eight responses. For the > 100 million seed scale, the In-House model received six responses while the Service Provider model received five.

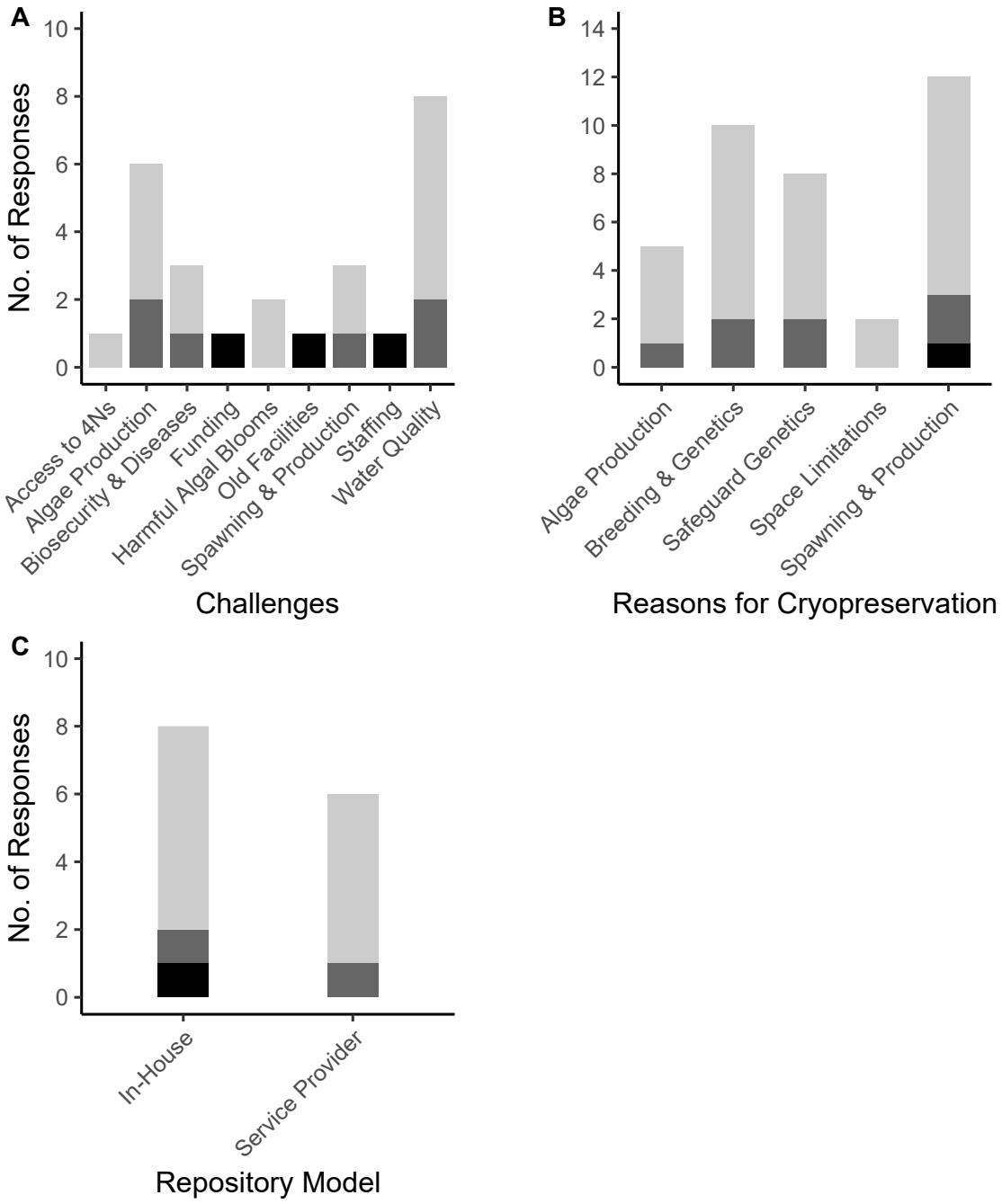

Figure 6.2. Responses of interviewed hatchery managers based on seed production scale to: A) the greatest challenges faced at the hatchery, B) the reasons they were interested in cryopreservation, and C) the repository model that is best suited for the hatchery. Light grey bars represent responses from hatcheries that produced < 100 million seed, dark grey bars represent responses from hatcheries that produced 100 – 200 million seed, and black bars represent responses from hatcheries that produced > 200 million seed. Of the interviewed hatcheries, eleven were at the < 100 million seed, two were at the 100 – 200 million seed scale, and one was at the > 200 million seed scale.

Creating Models for Repository Development

Two Center-level relationship diagrams were created to model repository integration with oyster hatcheries. One diagram was created for commercial hatcheries (Figure 6.3), and one was created for research hatcheries (Figure 6.4). For commercial hatcheries, a central repository served as a service provider that could cryopreserve and store samples, as well as store associated information in a database. For research hatcheries, a repository was integrated into hatchery operations allowing cryopreservation and storage to be done "in-house". Finally, a Network-level relationship diagram was created to model the interactions of among members of an oyster aquaculture systems which included for the Aquatic Germplasm and Genetic Resources Center (AGGRC), research hatcheries, commercial hatcheries, oyster farms, research laboratories, a central repository, and the NAGP (Figure 6.5).

Discussion

Database Management

A database capable of storing information associated with genetic resources, such as germplasm samples, is a necessary part of a comprehensive repository (Blackburn, 2009; Varga, 2011). Data-entry requirements can be extensive, for example at the USDA Animal GRIN database, received samples are not considered completed without entering the date the order was received, who donated the germplasm, the type and quantity of germplasm, breeder information, registration number, date of birth, pedigree, and taxonomy, and inventory location (Irwin et al., 2012). Phenotypic and genotypic descriptors as well as the breed population status can also be recorded in the database (Blackburn, 2009). While creating and maintaining a thorough database is time-

intensive, it is also necessary. In many cases, germplasm samples may not be used for decades, therefore, sufficient data must be stored with physical samples to ensure that future generations understand what the repository holds and how it can be best used (Purdy et al., 2016).

Due to the complex nature of database management, it is not uncommon for a repository facility to employ someone whose main focus is to maintain the database (Irwin et al., 2012; Z. Varga pers. comm.). In the case of the data management requirements calculated in this study, a full-time data manager could be considered at the highest production scale (10,000 oysters per year). At this scale, approximatley 30% of the workday for one operator would be devoted to creating and updating database entries. In addition to these duties, the operator would also be responsible for gathering, verifying, and organizing data collected by other repository personnel. Therefore, as with other large-scale repository facilities, a data management specialist is necessary for a repository that serves oyster hatcheries.

Time requirements for database management comprise only part of the total time needed for a comprehensive repository data system. Collecting data during cryopreservation (e.g. oyster weight, height, sperm motility) takes twice as long as database management (Table 6.2). Furthermore, data collected in the cryopreservation pathway are only a portion of the data that idealy should be stored in the database. It is critical to include phenotypic and genetic information about the oyster lines that are preserved in the repository (Blackburn, 2009; Gadberry et al., 2016; Purdy et al., 2016). Collecting these types of data require extensive resources including trained researchers, equipment, supplies, and time (S. Casas pers. comm.).

For example, the field study to collect growth and mortality data in Bodenstein et al., (2023) required 24 sampling trips (2 per month) made by at least two researchers over a year-long period. Moreover, genetic information could not be collected this study due to time and resource constraints (Table 6.3). A single repository facility would not have to capacity to collect these data for every preserved genetic line. Repositories would have to work with hatcheries, farmers, and research laboratories to collect thorough phenotypic and genetic data. These proposed systems of data exchange are explored further in the *Repository Models* section below.

Phenotypic and genetic data would allow hatchery managers to select genetic traits that would best benefit their breeding programs. For example, if a hatchery was serving oyster farms located in areas of frequent low salinity, germplasm from lines bred for tolerance to low salinity could be utilized to prevent high field mortality (Callam et al., 2016; McCarty et al., 2020). With enough data, breeding values and statistical estimates such as expected progengy differences (EPDs) could be generated for oyster lines (Gutierrez et al., 2018). A combination of pedigree, phenotypic, and genetic information (from sires and progeny) are typically used to calculate breeding values which estimate the genetic merit of an animal for a given trait (Berry et al., 2011). Breeding values are divided in half to calculate expected progeny differences, the predicted amount of change (either positive or negative) for a trait in the offspring (Berry et al., 2011). National Cattle Evaluatons for beef cattle have been published since the 1980s and report breeding values for individual sires and EPDs (Gadberry et al., 2016). Similar to beef and dairy agriculture, this type of breeding information would increase the efficiency of breeding programs and expand production for oysters (Yang et al., 2021b).

Oyster Hatcheries Needs

One of the major challenges identified by research and commercial hatcheries was Water Quality. Although germplasm repositories may not provide direct solutions to water quality problems in a hatchery, it is important to acknowledge the challenge most commonly mentioned by both hatchery types. Water Quality problems arose because all hatcheries interviewed in this study operated primarily by using “flow-through” systems. These systems draw water from a nearby source such as a bay. Water is pumped through a series of filters before flowing through tank systems and out of the hatchery (Dupuy et al., 1977). Only two research hatcheries had the capability to run recirculating systems (where water is drawn from a nearby source, filtered, and recycled after use) for limited periods of time. This left hatcheries vulnerable to poor water quality conditions, such as low salinity, and in some cases was cited as causing high larval mortality. Poor water quality has been named as a major challenge for hatcheries in Oregon and California as well, although ocean acidification (low pH) was the water quality factor cited on the west coast (Green et al., 2023; Ward et al., 2022). As a solution to water quality problems, hatcheries in Oregon and California expressed interest in expanding capabilities to alter the location of water intake when needed, to alter the water chemistry after intake, and to turn off intake pumps during periods of poor water quality (Green et al., 2023; Ward et al., 2022). Implementing these capabilities into hatcheries would be costly, however, they may become necessary if water quality issues persist to the point where no seed can be produced. It is important to note that unintentional selection caused by poor water quality could inadvertently shape the genetics of the seed leaving hatcheries.

Algae Production was another major challenge identified by commercial hatcheries. High mortality in algal cultures (called “crashes”) were a frequent problem and the time needed to restart cultures limited the quantity of oyster larvae that could be grown. Repositories can offer a potential solution to this challenge, if they would house algae samples in addition to oyster germplasm. Having access to high-density, cryopreserved algae would allow hatcheries to reliably start new cultures (Kapoore et al., 2019) and achieve target concentrations more quickly (Gutierrez-Wing pers. comm., more than 5 years of algae cryopreservation experience). This would mitigate the impact of algal crashes and allow hatcheries to more consistently produce seed. Five hatcheries explicitly expressed interest in receiving high-density, cryopreserved algae as a reason for cryopreservation. Implementing algae repository systems into oyster hatcheries would require similar work to that addressed in this dissertation (i.e., time studies and simulation modeling in Chapters 2 and 3, as well as case studies for data collection in Chapters 4 and 5). Incorporation of either type of repository first (algae or oyster) would aid the inclusion of the second type because a repository capability would already be in place, and much of the technology and training would be similar.

Biosecurity and Diseases were the only other major challenges besides Water Quality that research and commercial hatcheries each mentioned. As stated in Chapter 3, cryopreserved sperm could ease the process of navigating state regulations when transferring genetics between states (Tiersch & Jenkins, 2001). During cryopreservation, a sub-sample of collected germplasm (from each male) could be tested, ensuring that germplasm frozen at that time was free of pathogens. If germplasm samples collected from multiple males were pooled together, only a

single pathology report would be required, further expediting the process. This would mean that at any point in time after cryopreservation, all samples could be used immediately upon arrival at a hatchery without requiring additional pathology testing. Easier genetic transfer while maintaining a high level of biosecurity will be important because one of the most commonly cited reasons for cryopreservation was Breeding and Genetics. Managers at research and commercial hatcheries were interested in using cryopreservation to either provide or receive tetraploid and diploid sperm. Research hatcheries often provide broodstock to commercial hatcheries to facilitate breeding programs. Tetraploids are used produce triploids and selected diploids are used in breeding programs to obtain a production trait, such as disease resistance (Guo & Perez, 2019; Yang, 2022). It is often easier to transport frozen straws, rather than live animals, and straws can be used immediately upon arrival without requiring broodstock conditioning.

Two frequently cited reasons for cryopreservation were for Spawning and Production as well as to Safeguard Genetics. Several managers mentioned a desire to have frozen sperm (diploid and tetraploid) available to facilitate spawning. Spawning and Production was another challenge mention by commercial hatcheries. Managers were interested in future capabilities for high-throughput cryopreservation of oyster eggs and larvae (Paniagua-Chavez & Tiersch, 2001; Tervit et al., 2005) to expediate spawning and larval production. This would benefit not only hatcheries but oyster farmers as well because the cost and availability of seed are consistently named as a major problem for farmers (Bosch et al., 2010; Green et al., 2023).

Finally, Safeguarding Genetics was a reason for cryopreservation in commercial and research hatcheries. Developing and maintaining genetic lines takes years of work. For example, Rutgers University has been developing oyster lines that are resistant to the pathogen *Haplosporidium nelsoni* since the 1960s (Yang et al., 2021b). Preserving these valuable genetic resources in germplasm repositories would prevent the unnecessary loss of decades of work and investment. It should be noted that while germplasm repositories offer many potential benefits to oyster aquaculture, there are certain limitations. High-throughput pathways have been developed for sperm cryopreservation, but research is ongoing to obtain similar levels of consistency when freezing oocytes or larvae (e.g., Yang & Huo, 2021). Therefore, genetic transfer occurs only through the male parent. If an entire population were to go extinct, reconstitution efforts would require eggs from females of a different population to be used with cryopreserved sperm. The genetics of the extinct population would not be entirely lost but would also not be fully recovered, a limitation that would remain until oocyte or larvae freezing advances.

While every hatchery interviewed was interested in cryopreservation and repository storage for at least one reason, the specific repository model preferred was different based on the type of hatchery. Research hatcheries preferred an In-House capability meaning that cryopreservation and storage would be performed on-site by hatchery personnel. Commercial hatcheries evenly selected the In-House and Service-Provider models, however, it is important to note that all respondents who preferred the In-House model specified that equipment costs for cryopreservation would have to be affordable. Cryopreservation and repository storage are resource-intensive processes that not every hatchery will have the capacity to incorporate.

Therefore, contracting an outside service to freeze and store samples may be beneficial to smaller-scale hatcheries to avoid large investments in equipment, training, and personnel. Proposed models for repository models are explored further in the following section. Identifying patterns based on production scale was not possible in this study. The majority of hatcheries interviewed fell into the smallest production scale (< 100 million seed produced annually) causing responses to be unbalanced. In future studies, a wider range of hatcheries at all production scales can be intereviewed.

Repository Models

Two separate repository models were created based on responses from commercial and research hatchery managers. While, the same number of commercial hatcheries selected the In-House and Service Provider models, a Center-level diagram was developed to display how a private repository company would interact with commercial hatcheries (Figure 6.3). This model was chosen because of concerns from commercial managers that preserving samples on-site could quickly lead to expensive equipment investments and competition for hatchery resources (e.g., time, space, personnel). In the Service Provider model, a repository company would receive broodstock and associated data from a commercial hatchery. The repository would store both physical germplasm samples and data until the hatchery requested to withdraw their samples. This model represents the centralized approach where samples or animals are transported to a specialized facility that contains high-throughput equipment and trained personnel (Childress et al., 2019). A mobile facility approach could also be incorporated into this model where the repository deploys equipment and personnel in mobile units to commercial hatcheries. There,

repository personnel would preserve samples and return them to the central facility for long-term storage (Childress et al., 2019).

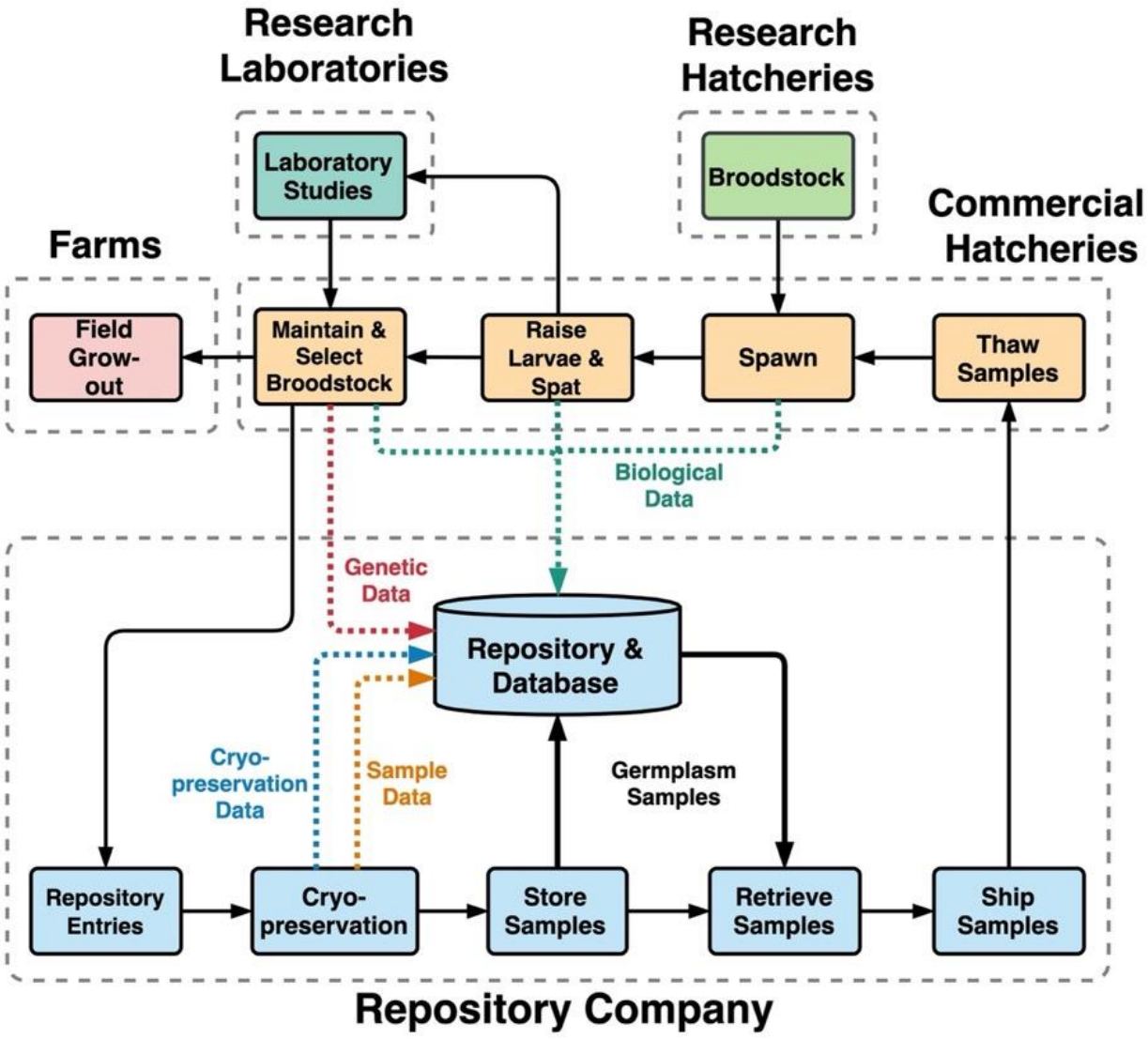

Figure 6.3. Center-level diagram of a central repository facility (in blue) interacting with commercial oyster hatcheries (in yellow), research hatcheries (in green), oyster farms (in red), and research laboratories (in teal). Black arrows indicate the transfer of physical germplasm material and dashed arrow indicate the flow of information (red arrows indicate the transfer of Genetic Data, blue arrows indicate the transfer of Cryopreservation Data, orange arrows indicate the transfer of Sample Data, and green arrows indicate the transfer of Biological Data).

There are currently private repository companies that operate using the centralized and mobile approaches to cryopreserve aquatic species, although they have yet to serve oyster hatcheries (e.g., https://www.cryogenetics.com). Services of this type would allow commercial hatcheries to take advantage of repository storage without costly investments in equipment and training. In this study, some commercial managers expressed interest in trying "in-house" cryopreservation, particularly if contracting repository services proved beneficial. In this case, hatcheries could work with a center that specializes in facilitating repository development, such as the AGGRC. The AGGRC can provide assistance with pathway development (while ensuring repository activities do not disrupt current hatchery operations) and open technology implementation. Open technology would grant hatcheries the ability to cryopreserve with low-cost, open-source devices to can be modified to suit specific needs (Liu et al., 2022).

The majority of research hatcheries preferred the In-House model. Therefore, a Center-level relationship diagram was developed to display how cryopreservation and repository storage could be integrated into current hatchery operations (Figure 6.4). In this model a research hatchery would incorporate repository activities such as cryopreservation, shipping germplasm, and maintaining a database. The hatchery would cryopreserve important genetic lines allowing them to ship germplasm (instead of broodstock) to commercial hatcheries as well as ship seed to farmers. In future work, willingness-to-pay studies could be performed to assess the premiums farmers or commercial hatcheries would pay for oyster seed with certain attributes, such as increased growth or disease resistance. For example, a previous study found that farmers are willing to pay a 14% price premium for fish stocks with a 10% increase in growth rate (Boever et

al., 2006). By calculating willingness-to-pay values, accurate pricing could be generated to sell germplasm samples to commercial hatcheries. These willingness-to-pay values would also help inform acceptable production costs for cryopreserving samples at research hatcheries and what a private repository company could charge commercial hatcheries for cryopreservation services.

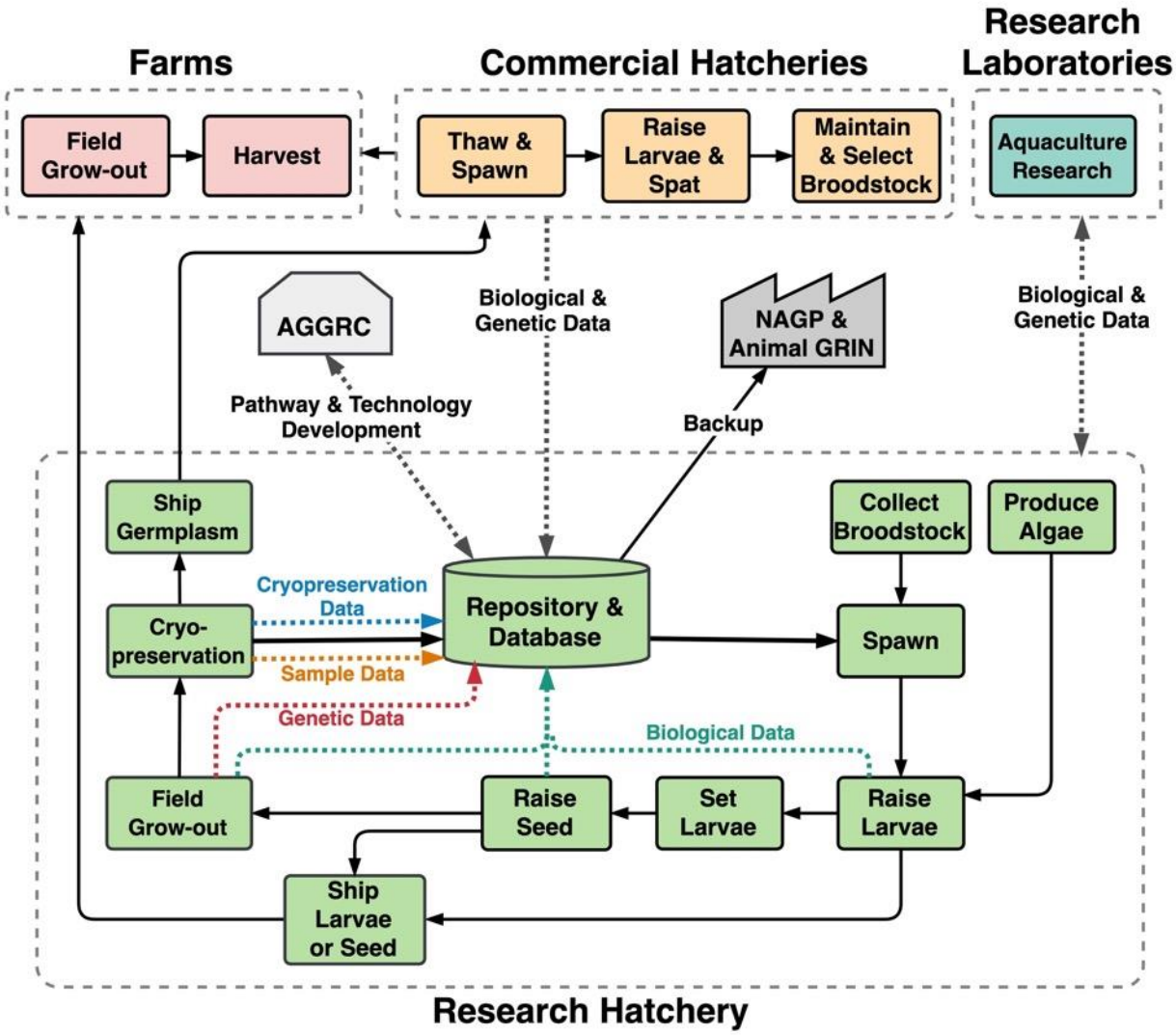

Figure 6.4. Center-level diagram of a research hatchery (in green) acting as a repository and interacting with commercial hatcheries (in yellow), oyster farms (in red) and research laboratories (in teal). Black arrows indicate the transfer of physical germplasm material and dashed arrow indicate the flow of information (red arrows indicate the transfer of Genetic Data, blue arrows indicate the transfer of Cryopreservation Data, orange arrows indicate the transfer of Sample Data, green arrows indicate the transfer of Biological Data, and grey arrows indicate the flow of multiple types of data).

Research hatcheries would collect Biological, Genetic, Sample, and Cryopreservation Data, as well as receive data from cooperating commercial hatcheries and research laboratories.

Germplasm samples and data could be stored as a backup in the USDA NAGP and in Animal GRIN. If intellectual property is a concern, research hatcheries could submit material to the Restricted Collection in which the original owner has control over the release of samples for a limited time based upon an agreement with the USDA Agricultural Research Service (Blackburn, 2009). These types of agreements have allowed the NAGP to store hundreds of thousands of samples from beef and dairy cattle donated by commercial artificial insemination companies and individual breeders (Blackburn, 2009; Purdy et al., 2016). To store germplasm samples and data, originating from the research hatchery and the community, quality management measures must be implemented. Understanding the quality of samples is necessary to ensure the repository can serve the community (Purdy et al., 2016). For example, the USDA NAGP repository receives frozen sperm samples from outside sources and cryopreserves samples in-house. In both cases, sample quality is evaluated by measuring sperm motility with computer-assisted sperm analysis software (CASA), a method that is automated and standardized. With the quality of all samples known the repository can use samples effectively, for example with higher-quality samples used to repopulate breeds, and lower-quality samples used as a source of DNA (Purdy et al., 2016).

Quality management for data collected and stored in the repository would also be necessary. For example, the Zebrafish Information Network (ZFIN) is a database of genetic and genomic data for the biomedical research organism, zebrafish (*Danio rerio*) (www.zfin.org). The ZFIN database stores ontology annotations associated with genes from zebrafish and therefore has quality control policies to ensure all annotations meet the same standards. Detailed documentation of these policies is available online, and meeting the quality control standards is required to submit

this type of data to ZFIN (Ruzicka et al., 2015). Similarly, repositories at research hatcheries must develop and implement quality management policies for data that they generate, or that are received from the community. This will ensure quality, and establish requirements for the forms of data that must be reported, allowing research from multiple institutions to be used cumulatively. To incorporate quality management at the Center Level, members of the repository and community could form a management entity to oversee activities of the repository database. If multiple repositories plan to share data or a database, they must act at the Network Level to develop such policies (discussed further in the next section). Finally, research hatcheries can work with an entity like the AGGRC on development of cryopreservation pathways and open technology. Quality management can be built into cryopreservation pathways and open technology can include sensors that automatically record essential Cryopreservation Data such as equilibration time and cooling rate, to increase quality and standardization.

The Repository Network

To ensure the sustainability of individual repositories, they and their communities should consider formation of a repository network (Hagedorn et al., 2019). Networks can act as a group insurance policy, ensuring repositories remain functional long-term, despite structural or leadership changes in individual members (Gutierrez-Wing, in prep). The formation of networks has been suggested for oyster aquaculture before to allow farmers, managers, policy makers, and scientists to share information, discuss policies, and consider industry needs (Ward et al., 2022). The network proposed in this study (Figure 6.5) includes multiple repositories housed in different facilities and the community members associated with those repositories. Many of the

same relationships illustrated in the two Center-level diagrams (Figures 6.3 & 6.4) can be seen in the Network-level diagram.

Forming a network gives the members opportunities to identify common challenges and propose solutions, in addition to granting members greater influence over policy (Green et al., 2023; Ward et al., 2022). An example of a repository network is the developing National Institutes of Health Aquatic Biomedical Germplasm Repository Network (Voss et al., 2021). This network includes aquatic biomedical stock centers that are each establishing repositories by working collaboratively. Centers meet to share experiences and challenges in creating these repositories which is beneficial to centers earlier in their development. In general, network members can also work together to develop outreach tools, training programs, and quality management guidelines (e.g., for sample and data exchange) to efficiently share samples and incorporate future members.

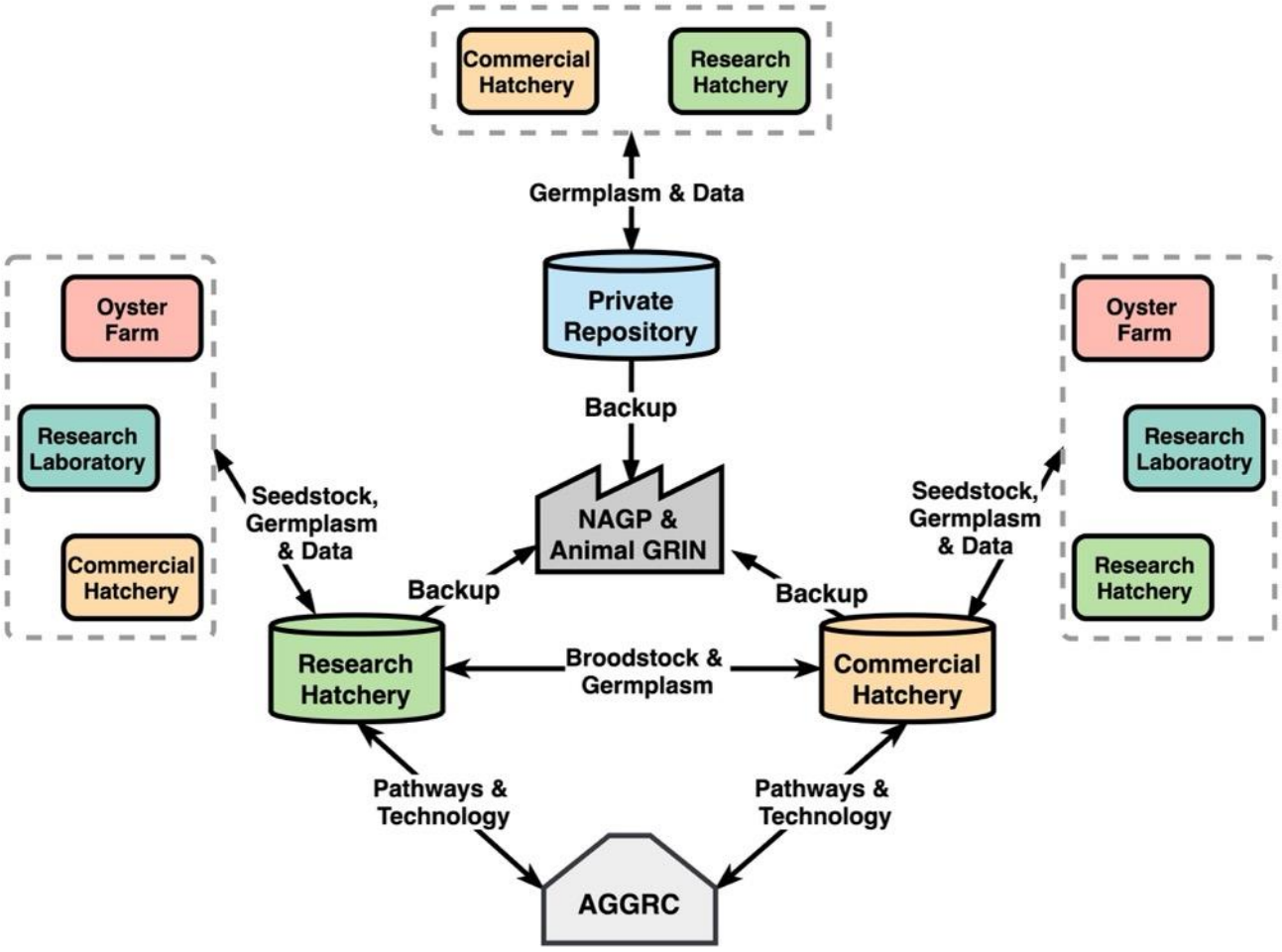

Figure 6.5. Network-level diagram of members interacting with each other. Arrows indicate the flow of materials and information. Facilities inside dashed, gray boxes represent the Center-level communities for each network member.

Similar to how repositories and their communities interact at the Center Level, quality management must be incorporated at the Network Level if multiple repositories plan to share samples and data. Currently, no fully functional repository network for aquaculture species exists and therefore no quality management guidelines have been established. This leaves a future oyster repository network with options for how to develop and incorporate quality management. As mentioned in the previous section, each repository facility could create their own quality

guidelines with their communities. This bottom-up approach would be an effective way to manage sample and data quality for each repository, however, interactions among repositories could be difficult. As the network forms, quality management practices among repositories will not be the same, with different data collected based on different standards. Exchanging data may be difficult and as a network, repositories would have to create new quality management guidelines that all facilities would follow.

Thus, a top-down approach to implementing quality management at the Network Level could also be applied. For example, a governmental organization such as the USDA National Genetic Resources Advisory Council (https://www.ars-grin.gov/Pages/NGRAC) could recommend that USDA establish quality management guidelines that members of a specific network could agree to. This approach would ensure quality management is standardized across the network from the beginning. However, because no oyster repositories currently exist this level of collaborative planning is not currently possible. As repositories are established by early adopters, such as research hatcheries, these facilities can communicate formally or informally to confirm their quality management practices have enough similarities that they can be used as a starting point (either by network members or a government agency) to establish network-level practices in the future.

Conclusions

The goal of this study was to provide repository development tools by analyzing database management requirements and proposing repository and network models for oyster

aquaculture. The specific resources required for database management are typically not discussed in studies that address repositories development for aquatic species. (Liu et al., 2019; Torres et al., 2017). These requirements can be substantial, however, particularly if hatcheries need to develop their own databases and standardized data collection methods. In future work, it will be necessary to create database guidelines for submitting samples to a repository. These would include the minimum data requirements that must be submitted with the sample, such as the cryopreservation protocol used (Cryopreservation Data), quality control metrics (Sample Data), and location of origin for the line (Biological Data) (Liu et al., 2019). Increasing efforts to collect Genetic Data is also important to accelerate selective breeding work. In addition, to further facilitate genetic transfer, future work must focus on integrating biosecurity protocols into high-throughput sperm cryopreservation. Collecting and maintaining all this data is a network-wide effort. Members can each contribute different data types and collectively decide on data guidelines and standards. Similar to actions taken by the aquatic biomedical community through the AGGRC acting as a Hub (www.aggrc.com), network development workshops could be arranged. Interested industry professionals (e.g., hatchery managers, farmers, researchers, government officials) could gather to discuss shared challenges, common goals, and preliminary relationships among members. This would be a first step in developing a network and building the capabilities for future repository and database systems.

Chapter 7. Summary and Conclusions

The goal of this dissertation was to advance germplasm repository development in commercial aquaculture species by integrating repository activities into oyster aquaculture systems as a model at the Pathway, Center, and Network Levels. The tools developed in this dissertation are meant to assist oyster aquaculture professionals with establishing repository capabilities. These tools are also meant to be generalizable and useful to other aquaculture industries, and aquatic species in general. The research performed in Chapters 2 and 3 operated at the Pathway Level and provided process maps and simulation models of applied cryopreservation – a pathway. In addition to publishing this work, process maps, simulation models, and an introduction to their utility were made available online on the AGGRC website (aggrc.com). Research performed in Chapters 4 and 5 operated at the Center Level (e.g., hatchery or genetic stock center) and provided an overview of the forms of data that can be collected about genetic lines stored in a repository. In these chapters, the methodology and data interpretation of the studies were discussed. This can provide direction for future aquaculture research and assist with establishing minimum data requirements necessary to submit samples to repositories. Finally, research performed in Chapter 6 operated at the Center and Network Levels. This chapter provided tools to assist with database management and outlined the exchange of materials and information between a repository and its surrounding community. Initial steps were taken to assess the needs of the oyster aquaculture community, which utilized interviews with individual hatchery managers.

The research performed in this dissertation offers a strong starting point for repository development, but this work must be continued to realize the goal of protecting the genetic resources of oyster aquaculture, and aquatic species in general. Therefore, this chapter will recommend steps for the next phases of germplasm repository development into oyster aquaculture systems at the Pathway, Center, and Network Levels (Figure 7.1). The overall goal of this will be to establish an oyster repository network and to begin the preservation, storage, exchange, and ultimately commercialization of germplasm samples and data. To accomplish this, work should begin at the Network Level. While the work in this dissertation began at the Pathway Level (the organization level operating at the smallest scale) and ended at the Network Level, the next phases of repository development could begin by identifying network members and establishing communication. This approach was implemented by the AGGRC in coordination with National Institutes of Health Aquatic Biomedical Germplasm Repository Network. Starting at the Network Level allowed members to develop repositories in relation to one another. This provided opportunity for network members to offer mutual support to deal with the shared challenges of building a repository. For example, members of the Aquatic Biomedical Germplasm Repository Network all face resource limitations and have begun looking into 3-D printed device options to begin cryopreservation efforts. Network members can also work together to secure resources through grant applications or negotiations with government agencies. Most importantly, starting at the Network Level allows members to create shared quality management policies for sample and data collection and storage. This ensures consistent quality as well as facilitates sample and data exchange from the outset of the network (Torres & Tiersch, 2018).

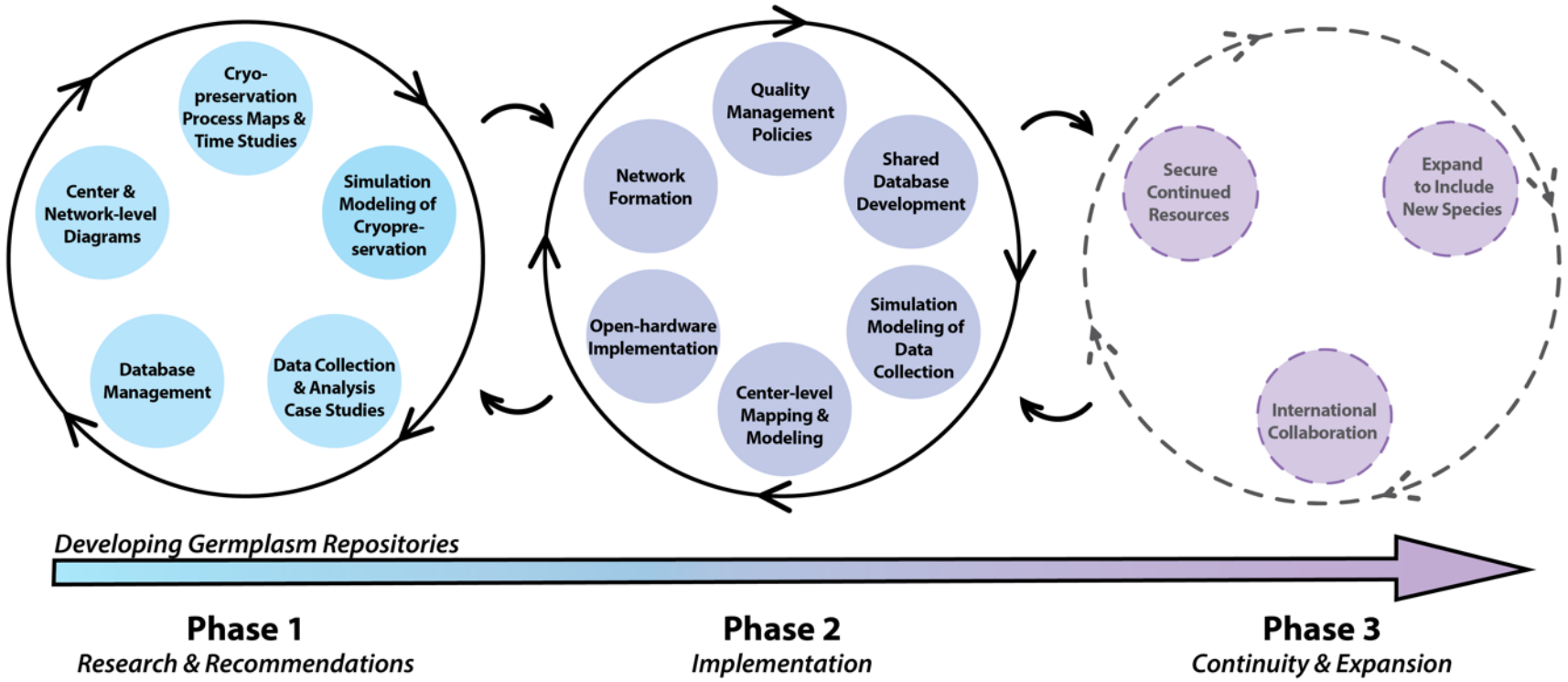

Figure 7.1. Three proposed phases for development of germplasm repositories and repository networks. Phase 1 encompasses research performed in Chapters 2 – 6 of this dissertation [e.g., process mapping and simulation modeling of cryopreservation (Chapters 2 & 3), case studies of data collection (Chapters 4 & 5), and database management and network-level diagrams (Chapter 6)]. Phase 2 would involve continued research efforts and implementation of research from Phase 1 to build a repository network (discussed in detail in Chapter 7). Phase 3 would include work to ensure the sustainability of the repository network and allow for expansion (to include additional species or new repositories).

In general, forming a repository network can be approached in two ways. The top-down approach for example, could involve a government agency providing resources and gathering principal community members for the purpose of creating repositories and forming a network. This was used to create the National Institutes of Health Aquatic Biomedical Germplasm Repository Network (Voss et al., 2021). The bottom-up approach involves identifying "early adopters" to begin the process of preserving and storing genetic resources. These early adopters can also encourage other community members to engage in repository activities and the network. Oyster research hatcheries, for example, are well positioned to serve as early adopters based on interview results from Chapter 6.

These hatcheries are already trusted members of the oyster aquaculture community, and they support commercial hatcheries and farmers by providing broodstock and oyster seed. Incorporation of repository activities would be a natural extension of how research hatcheries can protect their genetic resources and bring cryopreservation advances to oyster aquaculture. The bottom-up approach would gradually encourage more hatcheries to use repository samples, develop their own cryopreservation capabilities, and eventually join the network. If industry support was great enough, private repository companies could be established to serve the community. Having private and public repository options would provide greater flexibility and help ensure the sustainability of oyster germplasm repositories. Regardless of which approaches are taken, focusing on the formation of a network first would help ensure efficient organization and longevity of repositories at individual facilities.

Along with formation of a repository network, members should focus on creating quality management policies to maintain sample and data quality across different facilities. Methods of sample and data collection must be reproducible across different facilities despite use of different methods and devices (i.e., harmonization) and data must be standardized to the same formats within databases. One way to ensure standardization is to have a shared database for network members and require certain data types to be submitted with samples. For example, certain Cryopreservation, Sample, and Biological Data types should be collected (Table 6.3, Chapter 6). Recommendations for required Cryopreservation Data information could include: extender solution, final sperm concentration, cryoprotectant (type and final concentration), cooling curve (from which rate could be calculated), and thawing conditions (from which rate could be calculated). Recommendations for Sample Data include: shell height, wet weight, and sperm motility (pre-freezing and post-thaw) which is a basic quality control metric for samples. Recommendations for Biological Data include: collection site coordinates and conditions (e.g. salinity and temperature), spawning date, mortality rate, and growth rate. Although Genetic Data were not collected in this dissertation, a minimum would be to provide pedigree information to the repository database. It is important to note that these data types would be necessary for samples collected for oyster aquaculture, however, different data would need to be collected for samples intended for conservation or fisheries management, such as the history of stocking efforts in an area.

The data collection necessary to populate a repository database can be time-consuming and resource-intensive, requiring trained personnel, specialized equipment, and supplies. The

decision of what data types must be submitted to the repository must consider the time and resources necessary for data collection. Therefore, members of the repository network could perform time studies (a Pathway-Level activity) on the various data collection steps discussed in Chapters 4 and 5. This would allow simulation models to be created for processes such as collection of field mortality data or oxygen consumption rates. With these models, the time and costs associated with data collection could be calculated, data requirement guidelines could be created, and researchers could evaluate the feasibility of collecting multiple data types. While no formal time studies of the data collection processes described in Chapters 4 and 5 were performed in this dissertation, a general timeline to collect the field and physiology data and the Cryopreservation Data was constructed (Figure 7.2). This timeline demonstrates that the data collection in Chapters 4 and 5 was resource intensive and operated on different time scales than the cryopreservation data collected in Chapters 2 and 3. Field data took more than a year to collect, physiology data took months, and Cryopreservation Data took days. Providing this expanded Pathway-level research (including simulation analyses) would assist networks in determining essential data. A single research facility would likely not have the capacity to collect all such data types, reinforcing the value of an interconnected repository network.

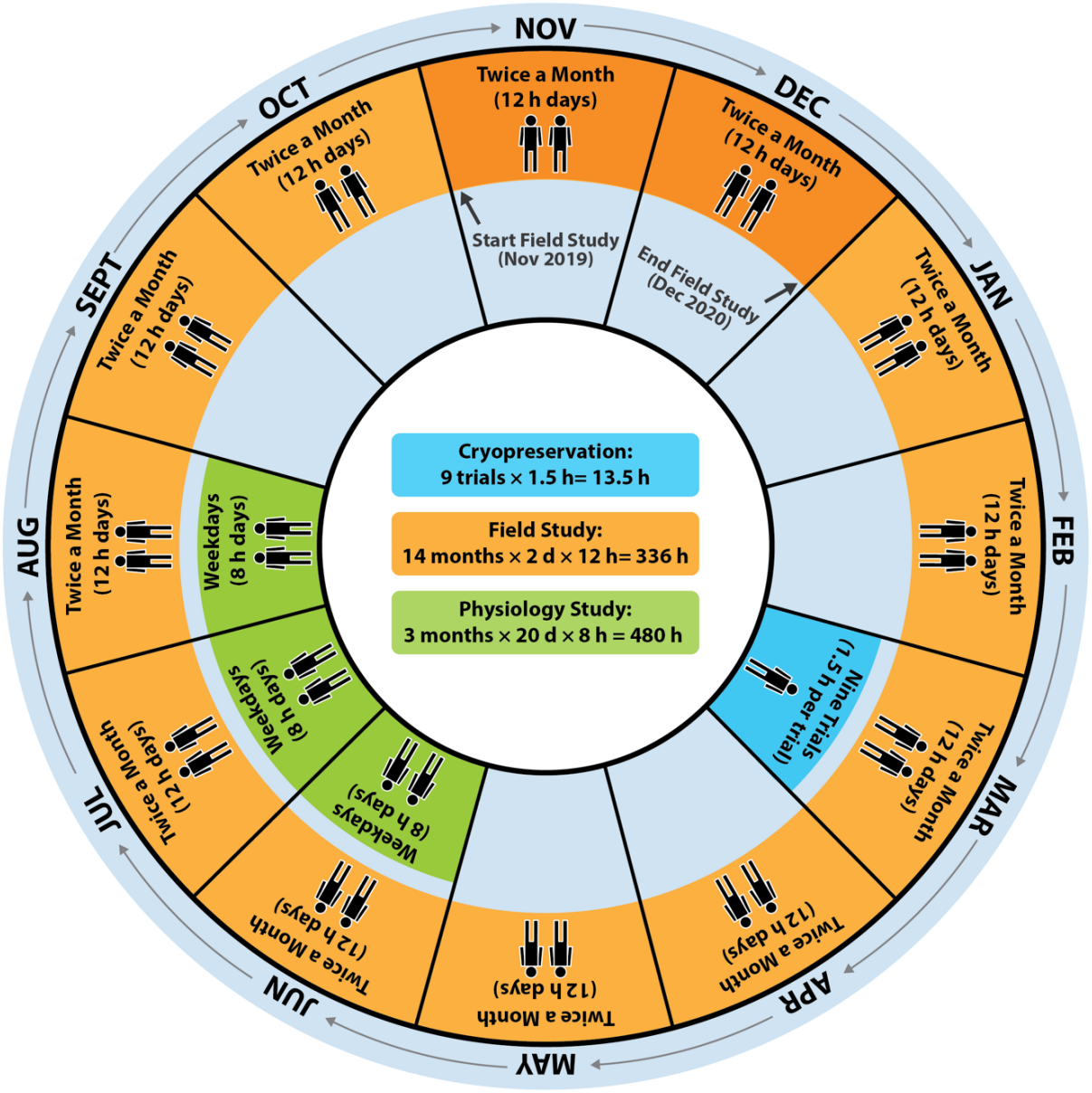

Figure 7.2. Generalized timeline to collect the field and physiology data described in Chapters 4 and 5, as well as the Cryopreservation Data collected in Chapters 2 and 3. Cryopreservation Data were collected with one researcher during 9 cryopreservation trials. Each trial required 1.5 h of work for each trial, for a total of 13.5 h. Field studies began in November, 2019, and continued for 14 months until December, 2020. Field work was performed during two work trips each month. Trips required two researchers working for 12 h, for a total of 336h. Physiology work was performed over a 3-month period, requiring two researchers to work each weekday for 8 h, for a total of 480 h.

In the next phase of repository development, members of a repository network would need the resources to develop a shared database designed for aquaculture species. In this dissertation, data (and samples) from Chapters 2 – 5 were stored in the USDA NAGP Animal GRIN database (aggrc.com), which was designed primarily with livestock species in mind. There are fields for users to enter data about individual animals and mechanisms to group individuals together based on when they were submitted to the repository.

In aquaculture species, such as oysters, the majority of Biological Data is collected at the population level. For example, to calculate the growth rate of a group of oysters, shell heights of a subsample are measured and averaged. Most oyster breeding programs are also based on the traits measured at the population level. A database where information that applies to multiple individuals (all belonging to the same genetic line or population) could be entered once would make the data entry process more efficient. An oyster repository network could advocate for this capability to be added to the USDA Animal GRIN database, the only major repository currently storing oyster samples. Additionally, specific fields could be added that apply to aquaculture species such as farm site conditions, mortality rate, and scope for growth.

In addition to data and germplasm samples, the network could decide to store other sample types in oyster repositories to be used for genetic analysis. While germplasm repositories were initially conceived to conserve genetic diversity and re-establish breeds, other uses have become apparent, such as genetic studies and reproductive physiology experiments (Blackburn, 2018). Mapping the genetic structure of oyster populations would be useful to direct conservation and

breeding efforts (Blackburn, 2006), and would be directly supported by well-organized germplasm repositories. In addition, having genetic records of tetraploid oysters, for example, would be useful to compare inbreeding levels through generations or to perform genetic distance analyses. Repositories can also be used to facilitate whole-genome sequencing studies. Oyster repositories can also offer specific benefits. For example, oysters are protandrous hermaphrodites, generally beginning life as males and changing into females as they age (Galtsoff, 1964). With the development of non-lethal sperm collection methods, sperm can be extracted from males and be cryopreserved (Yang et al., 2015). Those individuals can be maintained until they reconstitute as females, whereupon eggs can be extracted and fertilized with their own cryopreserved sperm. This has allowed for the creation of self-fertilized inbred lines that can be used as reference populations for genetic mapping (Yang et al., 2015).

During the next phase of repository development, members of a repository network would also have to engage in Center-level research, such as creating relationship diagrams to map the activities of each repository facility and their interactions with the local community. This work would outline how each repository could best serve its community and indicate at what production scale the repository will need to operate. Efficient operation within repository facilities could be planned, implemented, and improved with simulation analyses. The process mapping and simulation modeling in this dissertation focused on the cryopreservation pathway, however, many other activities also take place at a repository facility. These activities would share resources such as space, personnel, and equipment. To avoid resource competition, multiple pathways representing all the activities that take place at a repository could be mapped and

simulated. This would also be a useful tool to evaluate how cryopreservation would interact with other activities at an established facility to ensure the smooth integration of repository storage. This research would involve using the Center-level diagrams of each facility and Pathway-level maps for each activity. Simulation models for the activities would be generated and linked to one another. With interlinking models, schedules for operators and pieces of equipment required by multiple pathways could be made to ensure efficient resource sharing.

Future studies with simulation models could also include a wider range of device options. Modeling time and resource requirements for cryopreservation with 3-D printed devices would allow for improved recommendations at facilities with different production demands. With information from these models, newly formed repository facilities could assess production demands in relation to open hardware. Commercial-scale cryopreservation devices are often expensive, and using open-source devices may be the only way a facility could begin building a repository (Figure 7.3). Likewise, if the community surrounding the repository wants to begin cryopreserving their own samples, open hardware is a viable option that does not require high initial equipment costs.

Furthermore, at the Network-level, incorporating open hardware ensures a level of harmonization (and possibly standardization) where users can collect samples and data using multiple device options but maintain similar levels of quality. Open hardware can incorporate electronic sensors that automatically collect important data such as cooling rates (Shamkhalichenar et al., 2019). The strength of this proposed phase of repository development

is the opportunity for interdisciplinary collaboration. Availability of multiple tools, such as open hardware and simulation modeling, can provide greater access to networks wishing to advance repository capabilities and a greater understanding of to how build and maintain repository systems at multiple levels of organization. Applying Pathway-, Center-, and Network-level research can provide sustainable germplasm repositories that offer oyster aquaculture the ability to protect and more effectively utilize its genetic resources. With an interdisciplinary approach, repositories can be integrated into aquaculture systems and sustained for generations to come.

Figure 7.3. Examples of innovative technologies are developed at the AGGRC in the form of open hardware that can be shared as computer files with user communities to support germplasm repositories. These devices address steps in sample processing, freezing and thawing, and are custom designed for fabrication by use of consumer-level 3D printers, electronics and microcontrollers (source: https://www.lsuagcenter.com/articles/page1655756705673).

Appendix A. Supplementary Material from Chapter 2

Table A.1. Time distributions from data collected in Trials 7-9.

Step	Distribution (s/oyster)
Shuck	Lognormal(3.864, 0.573)
Verify	Lognormal(3.830, 0.334)
Measure	Lognormal(4.021, 0.346)
Strip	Lognormal(5.089, 0.136)
Motility	Lognormal(5.468, 0.117)
Filter	Weibull(2.723, 64.614)
Prep CPA	Lognormal(3.999, 0.232) (no change)
Combine CPA	Lognormal(2.403, 0.448) (no change)
Fill	20
Freeze	8
Gather	Normal(0.375, 0.106) # straws batch size
Sort	Normal(3.175, 0.479) # straws batch size
Store	Lognormal(2.932, 0.577) batch size
Clean	Lognormal(5.157, 0.349) batch size

Appendix B. Supplementary Material from Chapter 3

Table B.1. Linear regression results for the Baseline and Enhanced Models analyzing the effect of three parameters (Straws per Oyster, Batch Size, and Number of Operators) on three output statistics (Throughput, Time in System (TIS), and Cost per Oyster). Bolded P values are significant.

Model	Output Statistic	Parameter	Estimate	t value	*P* value	R^2 value
A	Throughput	Straws per Oyster	-0.03	-11.36	**<0.01**	0.88
		Batch Size	1.30	4.12	**0.01**	0.77
		No. Operators	15.32	13.17	**<0.01**	0.98
	TIS (h)	Straws per Oyster	0.01	9.67	**<0.01**	0.85
		Batch Size	0.41	4.52	**<0.01**	0.80
		No. Operators	-0.05	-1.10	0.33	0.23
	Operating Cost ($)	Straws per Oyster	2.14	26.32	**<0.01**	0.98
		Batch Size	11.37	4.71	**0.01**	0.82
		No. Operators	515.82	25.47	**<0.01**	0.99
	Cost Per Oyster ($)	Straws per Oyster	0.24	1596.30	**<0.01**	1
		Batch Size	-2.47	-3.88	**0.01**	0.75
		No. Operators	0.11	3.60	**0.02**	0.76
E	Throughput	Straws per Oyster	-0.018	-10.38	**<0.01**	0.86
		Batch Size	2.12	4.13	**0.01**	0.77
		No. Operators	20.39	14.75	**<0.01**	0.98
	TIS (h)	Straws per Oyster	0.00	0.64	0.53	0.02
		Batch Size	0.33	5.29	**<0.01**	0.85
		No. Operators	-0.12	-1.67	0.17	0.41
	Operating Cost ($)	Straws per Oyster	4.73	54.90	**<0.01**	0.99
		Batch Size	27.18	6.20	**<0.01**	0.88
		No. Operators	626.34	24.68	**<0.01**	0.99
	Cost Per Oyster ($)	Straws per Oyster	0.23	2317.20	**<0.01**	1
		Batch Size	-2.48	-3.71	**0.01**	0.73
		No. Operators	0.07	2.41	0.07	0.59

Appendix C. Supplementary Material from Chapter 4

Table C.1. The condition index, prevalence (percent of oysters with > 0 parasites g^{-1} wet tissue), and *P. marinus* infection intensity (mean ± standard deviation, count g^{-1} x 10^2) for the sampling dates of June and September for oysters from both sites (LSURF and LUMON), both cohorts (AU and LSU), both ploidies, (2N and 3N), and all stocks (CL, SL, and VB). Crosses with "NA" listed under *P. marinus* infection had no infected individuals. Crosses with "NA" listed for standard deviation only had one infected oyster.

Month	Site	Cohort	Ploidy	Stock	Condition Index	Prevalence (%)	*P. marinus* Infection (count g^{-1})
June	LSURF	AU	2N	CL	15.8 ± 3.27	20	0.38 ± 0.53
				SL	15.4 ± 3.1	65	0.38 ± 0.32
				VB	14.3 ± 3.2	45	221.71 ± 661.64
			3N	CL	16 ± 1.95	35	0.87 ± 1.93
				SL	16.4 ± 1.47	95	5.75 ± 23.6
				VB	14.3 ± 2.58	60	11.81 ± 29.95
		LSU	2N	CL	12.1 ± 1.56	45	0.12 ± 0.06
				SL	8.5 ± 3.64	45	0.51 ± 1.13
				VB	9.71 ± 9.54	45	0.28 ± 0.58
			3N	CL	15.5 ± 2.37	30	31.57 ± 67.99
				SL	15.4 ± 9.15	40	15.77 ± 44.24
				VB	15.7 ± 9.62	35	2.46 ± 4.89
	LUMCON	AU	2N	CL	15.5 ± 4.31	15	0.17 ± 0.03
				SL	12.5 ± 4.36	15	0.21 ± 0.09
				VB	12.6 ± 3.13	25	0.15 ± 0.03
			3N	CL	16.9 ± 2.41	15	0.15 ± 0.04
				SL	14.5 ± 2.5	15	0.13 ± 0.02
				VB	14.7 ± 3.53	10	0.08 ± 0.02
		LSU	2N	CL	12 ± 3.93	35	0.18 ± 0.14
				SL	10.6 ± 1.47	30	0.14 ± 0.11
				VB	11.2 ± 3.07	25	0.21 ± 0.12
			3N	CL	15.7 ± 7.66	30	0.26 ± 0.31
				SL	14.1 ± 4.01	25	0.09 ± 0.05
				VB	12.4 ± 2.77	25	0.11 ± 0.06
Sept	LSURF	AU	2N	CL	6.35 ± 1.91	0	NA
				SL	5.94 ± 2.37	10	0.21 ± 0.01
				VB	6.7 ± 1.41	15	0.08 ± 0.03

Cont'd

Month	Site	Cohort	Ploidy	Stock	Condition Index	Prevalence (%)	*P. marinus* Infection (count g^{-1})
Sept	LSURF	AU	3N	CL	8.39 ± 1.75	5	31.97 ± NA
				SL	7.27 ± 2.11	0	NA
				VB	8.29 ± 1.86	20	2.98 ± 5.05
		LSU	2N	CL	5.19 ± 3.86	100	111.53 ± 241.0 ($x10^3$)
				SL	5.69 ± 2.06	100	260.55 ± 442.95 ($x10^3$)
				VB	6.49 ± 3.61	100	7700.8 ± 1436.8 ($x10^3$)
			3N	CL	9.63 ± 2.74	100	1,633.43 ± 3,361.68 ($x10^3$)
				SL	9.19 ± 1.68	95	410.78 ± 768.33 ($x10^3$)
				VB	9.35 ± 9.62	100	937.31 ± 1402.55 ($x10^3$)
	LUMCON	AU	2N	CL	7.6 ± 2.57	5	0.07 ± NA
				SL	7.43 ± 2.62	5	0.1 ± NA
				VB	6.36 ± 1.97	0	NA
			3N	CL	10.1 ± 2.59	0	NA
				SL	10.7 ± 2.01	5	0.13 ± NA
				VB	8.84 ± 2.13	15	0.12 ± 0.03
		LSU	2N	CL	6.6 ± 2.14	90	127.1 ± 520.62
				SL	10.9 ± 10.6	90	606.19 ± 958.02
				VB	7.52 ± 1.33	100	491.85 ± 1582.30 ($x10^3$)
			3N	CL	8.89 ± 3.64	95	643.04 ± 1373.78
				SL	11.4 ± 2.58	95	364.3 ± 685.83
				VB	8.22 ± 3.13	95	520.46 ± 1138.70 ($x10^3$)

Appendix D. Supplementary Material from Chapter 5

Table D.1. The average interval mortality per interval and per day, and shell height (± standard deviation) for four sampling intervals at the LSURF field site or in the laboratory for diploid and triploids oysters (2N and 3N) of both cohorts (AU and LSU) spawned in June 2019 using Sister Lake broodstock.

Sampling Interval	Temperature (°C)	Salinity (ppt)	Ploidy	Cohort	Interval Mortality for the Interval (%)	Interval Mortality d^{-1} (%)	Shell Height (mm)
3/13/20-4/20/20 Field	23.3 ± 1.7	10.1 ± 3.7	2N	AU	0.0	0.00	59.5 ± 3.9
			3N	AU	1.0	0.02	68.5 ± 3.1
			2N	LSU	0.3	0.01	63.5 ± 3.3
			3N	LSU	2.0	0.05	71.0 ± 3.0
4/20/20 - 6/18/20 Field	23.8 ± 5.9	12.1 ± 7.6	2N	AU	1.0	0.02	69.7 ± 3.5
			3N	AU	1.0	0.02	78.5 ± 2.0
			2N	LSU	0.6	0.01	71.6 ± 3.5
			3N	LSU	18.7	0.31	78.5 ± 3.7
6/18/20 - 7/14/20 Field	29.5 ± 1.7	11.8 ± 3.8	2N	AU	10.0	0.37	73.9 ± 3.2
			3N	AU	10.7	0.40	83.7 ± 2.4
			2N	LSU	10.5	0.39	76.1 ± 2.8
			3N	LSU	13.2	0.49	81.5 ± 2.8
7/24/20-9/9/20 Laboratory	27 ± 1	15 ± 1	2N	AU	2.5	0.05	75.7 ± 7.5
			3N	AU	8.9	0.19	84.2 ± 10.9
			2N	LSU	2.9	0.06	73.7 ± 7.1
			3N	LSU	12.1	0.26	81.5 ± 9.4

Table D.2. Results of linear regression model for the effects of cohort and ploidy on *P. marinus* infection intensity, and results of linear mixed effects model for the effects of cohort and ploidy on cumulative mortality.

Dependent Variable	Independent Variable	DF	Estimate Std.	Std. Error	t value	P value
Infection Intensity	Intercept	56	2.84	0.43	6.56	**<0.01**
	cohort	56	1.15	0.60	1.91	0.06
	ploidy	56	1.26	0.61	2.06	**0.04**
	cohort:ploidy	56	-1.64	0.87	-1.89	0.06
Mortality	Intercept	39	2.00	2.07	0.96	0.34
	cohort	39	1.22	2.04	0.60	0.55
	ploidy	39	6.09	2.04	2.98	**<0.01**
	cohort:ploidy	39	4.76	2.89	1.65	0.11

Table D.3. Results of two-factor ANOVA analyses used to test the effects of cohort and ploidy on shell height, gill area, clearance rate measurement (CRi, CRh, CRa, CRw), condition index (CI), percentage time open, absorption efficiency (AE), basal and routine oxygen consumption rate (OCRw), and ammonia excretion rate (NR).

Dependent Variable	Independent Variable	Df	Sum Sq	Mean Sq	F value	P value
Shell Height	cohort	1	141.00	141.00	1.75	0.19
	ploidy	1	1488.00	1488.40	18.51	**<0.01**
	cohort:ploidy	1	3.00	2.80	0.04	0.85
Gill Area	cohort	1	110.50	110.50	3.05	0.08
	ploidy	1	1445.50	1445.50	39.89	**<0.01**
	cohort:ploidy	1	3.00	3.00	0.08	0.77
CRi	cohort	1	0.01	0.01	0.03	0.86
	ploidy	1	3.97	3.97	15.54	**<0.01**
	cohort:ploidy	1	0.94	0.94	3.66	0.06
CRh	cohort	1	0.29	0.29	0.33	0.57
	ploidy	1	6.00	6.00	6.85	**0.01**
	cohort:ploidy	1	1.72	1.72	1.96	0.16
CRa	cohort	1	0.02	0.02	0.05	0.82
	ploidy	1	0.55	0.55	1.48	0.23
	cohort:ploidy	1	0.23	0.23	0.63	0.43
CRw	cohort	1	0.12	0.12	0.34	0.56
	ploidy	1	0.27	0.27	0.74	0.39
	cohort:ploidy	1	0.51	0.51	1.42	0.24
CI	cohort	1	0.25	0.25	0.14	0.71
	ploidy	1	129.52	129.52	73.98	**<0.01**
	cohort:ploidy	1	0.68	0.68	0.39	0.54
Time Open	cohort	1	42.19	42.19	0.24	0.63
	ploidy	1	258.41	258.41	1.45	0.25
	cohort:ploidy	1	1.97	1.97	0.01	0.92
AE	cohort	1	187862.00	187862.00	0.15	0.70
	ploidy	1	1320523.00	1320523.00	1.05	0.31
	cohort:ploidy	1	288402.00	288402.00	0.23	0.63
Basal OCRw	cohort	1	2.02	2.02	8.97	**<0.01**
	ploidy	1	0.08	0.08	0.36	0.55
	cohort:ploidy	1	0.84	0.84	3.75	0.06
Routine OCRw	cohort	1	0.14	0.14	3.93	0.05
	ploidy	1	0.05	0.05	1.37	0.24
	cohort:ploidy	1	0.04	0.04	1.15	0.29
NR	cohort	1	1331.70	1331.70	5.41	**0.04**
	ploidy	1	18.20	18.20	0.07	0.79
	cohort:ploidy	1	574.00	574.00	2.33	0.15

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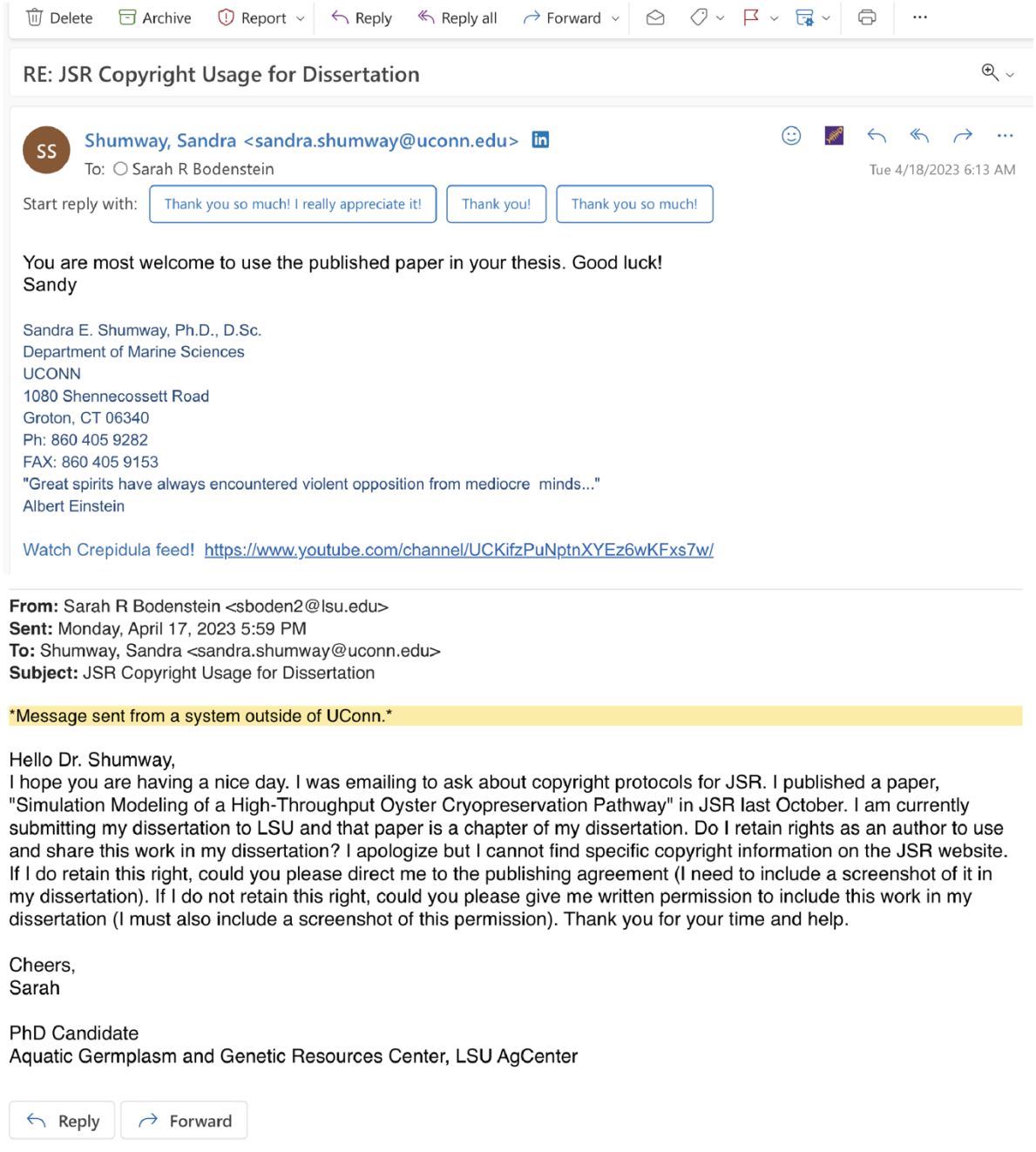

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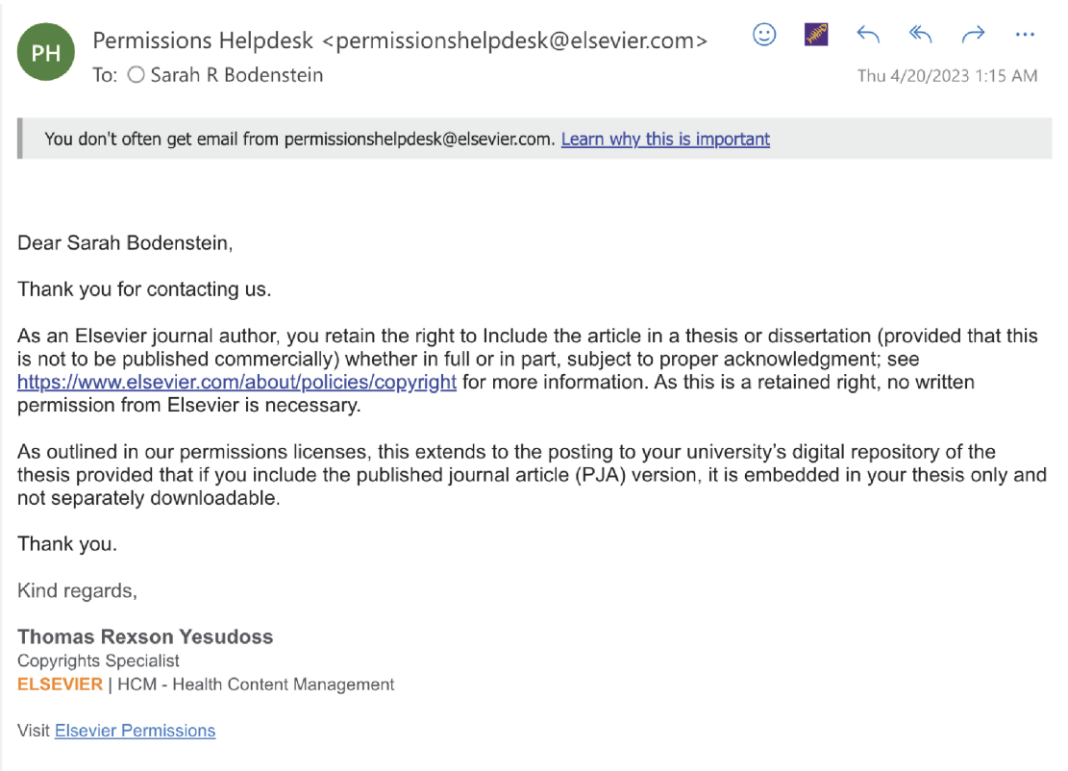

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References

Abbe, G.R. & Albright, B.W. (2003). An improvement to the determination of meat condition index for the eastern oyster, *Crassostrea virginica* (Gmelin 1791). *Journal of Shellfish Research*, *22*(3), 747-752.

Agarwal, A., Gupta, S., Sharma, R. (2016). Semen Analysis Using Hamilton-Thorne Computer Assisted Semen Analyzer (CASA), in: Agarwal, A., Gupta, S., Sharma, R. (Eds.), Andrological Evaluation of Male Infertility: A Laboratory Guide. Springer International Publishing, Cham, pp. 47–58. https://doi.org/10.1007/978-3-319-26797-5_5

Aho, T., Rönn, J., Piironen, J., & Björklund, M. (2006). Impacts of effective population size on genetic diversity in hatchery reared Brown trout (*Salmo trutta* L.) populations. *Aquaculture*, *253*(1), 244–248. https://doi.org/10.1016/j.aquaculture.2005.09.013

Allen, S. K. (1983). Flow cytometry: Assaying experimental polyploid fish and shellfish. *Aquaculture*, *33*(1), 317–328. https://doi.org/10.1016/0044-8486(83)90412-X

Allen Jr., S. K., & Downing, S. L. (1986). Performance of triploid Pacific oysters, *Crassostrea gigas* (Thunberg). I. Survival, growth, glycogen content, and sexual maturation in yearlings. *Journal of Experimental Marine Biology and Ecology*, 102(2), 197–208. https://doi.org/10.1016/0022-0981(86)90176-0

Allen, M., Spencer, A., Gibson, A., Matthews, J., Allwood, A., Prosser, S., Pitt, M. (2015). Right cot, right place, right time: improving the design and organisation of neonatal care networks–a computer simulation study. Heal Serv Deliv Res 3.

Andrews, J. (1964). Oyster mortality studies in Virginia IV. MSX in James River public seed beds. Proceedings of the National Shellfisheries Association, 53, 65–84.

Arunagiri, P., & Gnanavelbabu, A. (2014). Identification of Major Lean Production Waste in Automobile Industries using Weighted Average Method. *Procedia Engineering*, 97, 2167–2175. https://doi.org/10.1016/j.proeng.2014.12.460

Baesler, Jahnsen, & DaCosta. (2003). The use of simulation and design of experiments for estimating maximum capacity in an emergency room. Proceedings of the 2003 Winter Simulation Conference, 2003., 2, 1903–1906 vol.2. https://doi.org/10.1109/WSC.2003.1261651

Baghurst, B.C., Mitchell, J.G. (2002). Sex-specific growth and condition of the Pacific oyster (*Crassostrea gigas* Thunberg). *Aquac. Res*. 33, 1253–1263. https://doi.org/10.1046/j.1365-2109.2002.00788.x

Barbas, J. P., & Mascarenhas, R. D. (2009). Cryopreservation of domestic animal sperm cells. *Cell and Tissue Banking*, 10(1), 49–62. https://doi.org/10.1007/s10561-008-9081-4

Bayne, B. L. (2017). *Biology of Oysters* (1st edition). Academic Press.Berry, D. P., Bermingham,

Bayne, B. L., & Newell, R. C. (1983). 9—Physiological Energetics of Marine Molluscs. In A. S. M. Saleuddin & K. M. Wilbur (Eds.), *The Mollusca* (pp. 407–515). Academic Press. https://doi.org/10.1016/B978-0-12-751404-8.50017-7

Bayne, B.L., & Widdows, J. (1978). The physiological ecology of two populations of *Mytilus edulis* L. *Oecologia* 37: 137–62. https://doi.org/10.1007/BF00344987 https://doi.org/10.1186/2046-0481-64-5

Berry, D. P., Bermingham, M. L., Good, M., & More, S. J. (2011). Genetics of animal health and disease in cattle. *Irish Veterinary Journal*, 64(1), 5. https://doi.org/10.1186/2046-0481-64-5

Blackburn, H. D. (2006). The National Animal Germplasm Program: Challenges and Opportunities for Poultry Genetic Resources. *Poultry Science*, 85(2), 210–215. https://doi.org/10.1093/ps/85.2.210

Blackburn, H. D. (2009). Genebank development for the conservation of livestock genetic resources in the United States of America. *Livestock Science*, 120(3), 196–203. https://doi.org/10.1016/j.livsci.2008.07.004

Blackburn, H. D. (2018). Biobanking Genetic Material for Agricultural Animal Species. Annual *Review of Animal Biosciences,* 6(1), 69–82. https://doi.org/10.1146/annurev-animal-030117-014603

Bodenstein, S., Callam, B. R., Walton, W. C., Rikard, F. S., Tiersch, T. R., & La Peyre, J. F. (2023). Survival and growth of triploid eastern oysters, *Crassostrea virginica*, produced from wild diploids collected from low-salinity areas. *Aquaculture*, 739032. https://doi.org/10.1016/j.aquaculture.2022.739032

Bodenstein, S., Nahmens, I., & Tiersch, T. R. (2022b). Simulation Modeling of a High-Throughput Oyster Cryopreservation Pathway. *Journal of Shellfish Research*, 41(2), 209–221. https://doi.org/10.2983/035.041.0206

Bodenstein, S., Walton, W.C., Steury, T.D. (2021). Effect of farming practices on growth and mortality rates in triploid and diploid eastern oysters *Crassostrea virginica. Aquac. Environ. Interact.* 13, 33–40. https://doi.org/10.3354/aei00387

Boever, B. P., Harrison, R. W., & Tiersch, T. R. (Eds.). (2006). Willingness-to-Pay for Genetic Attributes in Aquaculture Industries. In *Southern Agricultural Economics Association Annual Meetings*. https://doi.org/10.22004/ag.econ.35337

Borsa, P., Jousselin, Y., & Delay, B. (1992). Relationships between allozymic heterozygosity, body size, and survival to natural anoxic stress in the palourde *Ruditapes decussatus* L. (Bivalvia: Veneridae). *Journal of Experimental Marine Biology and Ecology*, *155*(2), 169–181. https://doi.org/10.1016/0022-0981(92)90061-E

Bosch, D., Kuminoff, N., Stephenson, K., Miller, A., Pope, J., & Harris, A. (2010). Evaluation of Policy Options for Expanding Oyster Aquaculture in Virginia. *Aquaculture Economics & Management*, 14(2), 145–163. https://doi.org/10.1080/13657301003776698

Botta, R., Asche, F., Borsum, J. S., & Camp, E. V. (2020). A review of global oyster aquaculture production and consumption. *Marine Policy*, 117, 103952. https://doi.org/10.1016/j.marpol.2020.103952

Bougrier, S., Rabenomanana, L.D. (1986). Cryopreservation of spermatozoa of the Japanese oyster, *Crassostrea gigas. Aquaculture* 58, 277–280. https://doi.org/10.1016/0044-8486(86)90093-1

Buttemer, W. A., Abele, D., & Costantini, D. (2010). From bivalves to birds: Oxidative stress and longevity. *Functional Ecology*, *24*(5), 971–983. https://doi.org/10.1111/j.1365-2435.2010.01740.x

Byrne, P.F., Volk, G.M., Gardner, C., Gore, M.A., Simon, P.W., Smith, S. (2018). Sustaining the Future of Plant Breeding: The Critical Role of the USDA-ARS National Plant Germplasm System. Crop Sci. 58, 451–468. https://doi.org/10.2135/cropsci2017.05.0303

Caffey, R.H., Tiersch, T.R. (2000). Cost Analysis for Integrating Cryopreservation into an Existing Fish Hatchery. *J. World Aquac.* Soc. 31, 51–58. https://doi.org/10.1111/j.1749-7345.2000.tb00697.x

Callam, B. R., Allen, S. K., & Frank-Lawale, A. (2016). Genetic and environmental influence on triploid *Crassostrea virginica* grown in Chesapeake Bay: Growth. *Aquaculture*, 452, 97–106. https://doi.org/10.1016/j.aquaculture.2015.10.027

Carvalho, G. R., Faria, W. R., Nardy, V. P. D. R., & Junior, A. A. B. (2022). Hedonic pricing analysis for semen of dairy bulls in Brazil. *PLOS ONE*, 17(4), e0267109. https://doi.org/10.1371/journal.pone.0267109

Casas, S. M., Filgueira, R., Lavaud, R., Comeau, L. A., La Peyre, M. K., & La Peyre, J. F. (2018b). Combined effects of temperature and salinity on the physiology of two geographically-distant eastern oyster populations. *Journal of Experimental Marine Biology and Ecology*, 506, 82–90. https://doi.org/10.1016/j.jembe.2018.06.001

Casas, S. M., Lavaud, R., La Peyre, M. K., Comeau, L. A., Filgueira, R., & La Peyre, J. F. (2018a). Quantifying salinity and season effects on eastern oyster clearance and oxygen consumption rates. *Marine Biology*, *165*(5), 90. https://doi.org/10.1007/s00227-018-3351-x

Casas, S., Walton, W., Chaplin, G., Rikard, S., Supan, J., & Peyre, J. L. (2017). Performance of oysters selected for dermo resistance compared to wild oysters in northern Gulf of Mexico estuaries. *Aquaculture Environment Interactions*, 9, 169–180. https://doi.org/10.3354/aei00222

Chávez-Villalba, J., Pommier, J., Andriamiseza, J., Pouvreau, S., Barret, J., Cochard, J.-C., & Le Pennec, M. (2002). Broodstock conditioning of the oyster *Crassostrea gigas*: Origin and temperature effect. *Aquaculture*, 214(1–4), 115–130. https://doi.org/10.1016/S0044-8486(01)00898-5

Cheney, D., Macdonald, B., & Elston, R. (2000). Summer mortality of Pacific oysters, *Crassostrea gigas* (Thunberg): Initial finding on multiple environmental stressors in Puget Sound, Washington, 1998. *Journal Of Shellfish Research*, 19(1), 353–359.

Child, A. R., & Watkins, H. P. (1994). A simple method to identify triploid molluscan bivalves by the measurement of cell nucleus diameter. *Aquaculture*, *125*(1), 199–204. https://doi.org/10.1016/0044-8486(94)90296-8

Childress, W. M., Bosworth, B., Chesney, E., Walter, R. B., & Tiersch, T. R. (2019). On-Site Capabilities of a Mobile Laboratory for Aquatic Germplasm Cryopreservation. North American Journal of Aquaculture, 81(4), 349–363. https://doi.org/10.1002/naaq.10099

Childress, W. M., Caffey, R. H., & Tiersch, T. R. (2018). Design and Cost Analysis of a Self-contained Mobile Laboratory for Commercial-scale Aquatic Species Cryopreservation. *Journal of the World Aquaculture Society*, 49(5), 805–826. https://doi.org/10.1111/jwas.12525

Childress, W.M., Liu, Y., Tiersch, T.R. (2021). Design, alpha testing, and beta testing of a 3-D printed open-hardware portable cryopreservation device for aquatic species. *J. Appl. Aquac*. 0, 1–24. https://doi.org/10.1080/10454438.2021.1955805

Chong, K. E., & Ng, K. C. (2016). Relationship between overall equipment effectiveness, throughput and production part cost in semiconductor manufacturing industry. 2016 IEEE International Conference on Industrial Engineering and Engineering Management (IEEM), 75–79. https://doi.org/10.1109/IEEM.2016.7797839

Comai, L. (2005). The advantages and disadvantages of being polyploid. *Nature Reviews Genetics*, *6*(11), 836–846. https://doi.org/10.1038/nrg1711

Comeau, L. A., Arsenault, G., & Davidson, J. (2011). Off bottom oyster (*Crassostrea virginica* Gmelin) culture in Prince Edward Island: An evaluation of seed sources and stocking density. *Can. Tech. Rep. Fish. Aquat. Sci.*, *2976*, 21.

Comeau, L. A., Babarro, J. M. F., Longa, A., & Padin, X. A. (2018). Valve-gaping behavior of raft-cultivated mussels in the Ría de Arousa, Spain. *Aquaculture Reports*, *9*, 68–73. https://doi.org/10.1016/j.aqrep.2017.12.005

Cotter, E., Malham, S. K., O'Keeffe, S., Lynch, S. A., Latchford, J. W., King, J. W., Beaumont, A. R., & Culloty, S. C. (2010). Summer mortality of the Pacific oyster, *Crassostrea gigas*, in the Irish Sea: The influence of growth, biochemistry and gametogenesis. *Aquaculture*, *303*(1), 8–21. https://doi.org/10.1016/j.aquaculture.2010.02.030

Cranford, P. J., Ward, J. E., & Shumway, S. E. (2011). Bivalve Filter Feeding: Variability and Limits of the Aquaculture Biofilter. In S. E. Shumway (Ed.), *Shellfish Aquaculture and the Environment* (pp. 81–124). Wiley-Blackwell. https://doi.org/10.1002/9780470960967.ch4

Damelio, R. (2011). The Basics of Process Mapping, 2nd Edition. CRC Press.

Davis, J. (2013). *Effects of basket arrangement and stocking density when using the adjustable long-line system for oyster grow-out* [Thesis]. https://etd.auburn.edu//handle/10415/3502

Davis, J. P. (1994). *Studies on the influence of ambient temperature and food supply on growth rate, carbohydrate content and reproductive output in diploid and triploid Pacific oysters, Crassostrea gigas (Thunberg)* [Ph.D., University of Washington]. https://www.proquest.com/docview/304128034/abstract/AF0D5ABA42F74045PQ/1

Davis, C. V., & Barber, B. J. (1999). Growth and survival of selected lines of eastern oysters, *Crassostrea virginica* (Gmelin 1791) affected by juvenile oyster disease. *Aquaculture*, 178(3), 253–271. https://doi.org/10.1016/S0044-8486(99)00135-0

de Sousa, J. T., Allen, S. K., Baker, H., & Matt, J. L. (2016). Aneuploid progeny of the American oyster, *Crassostrea virginica*, produced by tetraploid × diploid crosses: Another example of chromosome instability in polyploid oysters. *Genome*, *59*(5), 327–338. https://doi.org/10.1139/gen-2015-0222

Dégremont, L., Garcia, C., & Allen, S. K. (2015). Genetic improvement for disease resistance in oysters: A review. Journal of Invertebrate Pathology, 131, 226–241. https://doi.org/10.1016/j.jip.2015.05.010

Dégremont, L., Garcia, C., Frank-Lawale, A., & Allen, S. K. (2012). Triploid Oysters in the Chesapeake Bay: Comparison of Diploid and Triploid *Crassostrea virginica*. *Journal of Shellfish Research*, *31*(1), 21–31. https://doi.org/10.2983/035.031.0103

Dégremont, L., Soletchnik, P., & Boudry, P. (2010). Summer Mortality of Selected Juvenile Pacific Oyster *Crassostrea gigas* Under Laboratory Conditions and in Comparison with Field Performance. *Journal of Shellfish Research*, *29*(4), 847–856. https://doi.org/10.2983/035.029.0419

Devotta, A.F. (1983). *A survey of performance rating research in work measurement* (Master's Thesis). University Of Madras, India.

Diego Fernando, M. D., & Rivera Cadavid, L. (2007). Lean manufacturing measurement: The relationship between lean activities and lean metrics. *Estudios Gerenciales*, 23(105), 69–83. https://doi.org/10.1016/S0123-5923(07)70026-8

Domínguez, R., Vázquez, E., Woodin, S. A., Wethey, D. S., Peteiro, L. G., Macho, G., & Olabarria, C. (2020). Sublethal responses of four commercially important bivalves to low salinity. *Ecological Indicators*, *111*, 106031. https://doi.org/10.1016/j.ecolind.2019.106031

Dong, Q., Eudeline, B., Huang, C., Allen, S. K., & Tiersch, T. R. (2005). Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. *Cryobiology*, 50(1), 1–16. https://doi.org/10.1016/j.cryobiol.2004.09.003

Dong, Q., Huang, C., Tiersch, T.R. (2007). Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: Evidence from sperm agglutination in oysters. *Cryobiology* 54, 87–98. https://doi.org/10.1016/j.cryobiol.2006.11.007

Duggan, K. J. (1998). Facilities Design for Lean Manufacturing. *IIE SOLUTIONS*. https://www.semanticscholar.org/paper/FACILITIES-DESIGN-FOR-LEAN-MANUFACTURING-Duggan/6bdb7318e3f82c4d96487e50cee4a180a0584315

Dupuy, J. L., Windsor, N. T., & Sutton, C. E. (1977). Manual for Design and Operation of an Oyster Seed Hatchery for the American Oyster, *Crassostrea virginica*. *Applied Marine Science and Ocean Engineering of The Virginia Institute of Marine Science*, 142, 127.

Eastburn, M. C. (2021). *Testing Maternal Contributions in Triploid Eastern Oysters (*Crassostrea virginica*): A Field Evaluation in Coastal Alabama* [Auburn University]. https://etd.auburn.edu//handle/10415/7733

Edwards, D.K. (1996). Practical guidelines for lean manufacturing equipment. *Prod. Inventory Manag*. J. 37, 51.

Ferry Cove Oyster Seed Order Form. (2022). Ferry Cove Shellfish, Inc. https://www.ferrycove.org/order-now/

Gadberry, S., Jennings, J., Ward, H., Beck, P., Kutz, B., & Troxel, T. (2016). Beef cattle production. University of Arkansas, United States Department of Agricultur.

Galtsoff, P. S. (1964). The American Oyster, *Crassostrea virginica* Gmelin. U.S. Government Printing Office.

Gandonou, J.-M. A., Dillon, C. R., Shearer, S. A., & Stombaugh, T. (2006). Precision Agriculture Equipment Ownership versus Custom Hire: A Break-even Land Area Analysis. *Journal of ASFMRA*, 106–116.

Gittins, P., McElwee, G., Tipi, N. (2020). Discrete event simulation in livestock management. *J. Rural Stud*. 78, 387–398. https://doi.org/10.1016/j.jrurstud.2020.06.039

Glazier, D. S. (2021). Genome Size Covaries More Positively with Propagule Size than Adult Size: New Insights into an Old Problem. *Biology*, *10*(4), Article 4. https://doi.org/10.3390/biology10040270

Gnaiger, E. (1983). Heat dissipation and energetic efficiency in animal anoxibiosis: Economy contra power. *Journal of Experimental Zoology*, *228*(3), 471–490. https://doi.org/10.1002/jez.1402280308

Gray, M. W., & Langdon, C. J. (2018). Ecophysiology of the Olympia Oyster, *Ostrea lurida*, and Pacific Oyster, *Crassostrea gigas*. *Estuaries and Coasts*, *41*(2), 521–535. https://doi.org/10.1007/s12237-017-0273-7

Green, K. M., Spalding, A. K., Ward, M., Levine, A., Wolters, E. A., Hamilton, S. L., & Rice, L. (2023). Oregon shellfish farmers: Perceptions of stressors, adaptive strategies, and policy linkages. *Ocean & Coastal Management*, 234, 106475. https://doi.org/10.1016/j.ocecoaman.2022.106475

Gremler, D. D. (2004). The Critical Incident Technique in Service Research. *Journal of Service Research*, 7(1), 65–89. https://doi.org/10.1177/1094670504266138

Griffin, A., & Hauser, J. R. (1993). The Voice of the Customer. *Marketing Science*, 12(1), 1–27. https://doi.org/10.1287/mksc.12.1.1

Grove, S. J., & Fisk, R. P. (1997). The impact of other customers on service experiences: A critical incident examination of "getting along." *Journal of Retailing*, 73(1), 63–85. https://doi.org/10.1016/S0022-4359(97)90015-4

Guévélou, E., Carnegie, R. B., Small, J. M., Hudson, K., Reece, K. S., & Rybovich, M. M. (2019). Tracking Triploid Mortalities of Eastern Oysters *Crassostrea virginica* in the Virginia Portion of the Chesapeake Bay. *Journal of Shellfish Research*, *38*(1), 101–113. https://doi.org/10.2983/035.038.0110

Guo, X., & Allen, S. K., Jr. (1994). Sex determination and polyploid gigantism in the dwarf surfclam (*Mulinia lateralis* Say). *Genetics*, *138*(4), 1199–1206. https://doi.org/10.1093/genetics/138.4.1199

Guo, X., DeBrosse, G. A., & Allen, S. K. (1996). All-triploid Pacific oysters (*Crassostrea gigas* Thunberg) produced by mating tetraploids and diploids. *Aquaculture*, *142*(3), 149–161. https://doi.org/10.1016/0044-8486(95)01243-5

Guo, X., & Perez, D. (2019). Improved Eastern Oysters (p. 1) [Rutgers Office of Technology Commercialization]. Rutgers University. https://hsrl.rutgers.edu/wp-content/uploads/2022/11/Improved_Eastern_Oyster_2018-070_Technology_Summary_2019.pdf

Gupta, M.C., Boyd, L.H. (2008). Theory of constraints: a theory for operations management. *Int. J. Oper. Prod. Manag.* 28, 991–1012. https://doi.org/10.1108/01443570810903122

Gutierrez, A. P., Matika, O., Bean, T. P., & Houston, R. D. (2018). Genomic Selection for Growth Traits in Pacific Oyster (*Crassostrea gigas*): Potential of Low-Density Marker Panels for Breeding Value Prediction. *Frontiers in Genetics*, 9. https://www.frontiersin.org/articles/10.3389/fgene.2018.00391

Haberkorn, H., Lambert, C., Le Goïc, N., Guéguen, M., Moal, J., Palacios, E., Lassus, P., & Soudant, P. (2010). Effects of *Alexandrium minutum* exposure upon physiological and hematological variables of diploid and triploid oysters, *Crassostrea gigas*. *Aquatic Toxicology*, *97*(2), 96–108. https://doi.org/10.1016/j.aquatox.2009.12.006

Hagedorn, M., Varga, Z., Walter, R. B., & Tiersch, T. R. (2019). Workshop report: Cryopreservation of aquatic biomedical models. *Cryobiology*, 86, 120–129. https://doi.org/10.1016/j.cryobiol.2018.10.264

Halachmi, I., Simon, Y., Guetta, R., Hallerman, E.M. (2005). A novel computer simulation model for design and management of re-circulating aquaculture systems. *Aquac. Eng*. 32, 443–464. https://doi.org/10.1016/j.aquaeng.2004.09.010

Hand, R. E., Nell, J. A., & Thompson, P. A. (2004). Studies on triploid oysters in Australia: XIII. Performance of diploid and triploid Sydney rock oyster, *Saccostrea glomerata* (Gould, 1850), progeny from a third generation breeding line. *Aquaculture*, *233*(1), 93–107. https://doi.org/10.1016/j.aquaculture.2003.09.017

Haskin, H. H., & Ford, S. E. (1979). Development of Resistance to *Minchinia nelsoni* (MSX) Mortality in Laboratory-Reared and Native Oyster Stocks in Delaware Bay. *Marine Fisheries Review*, 41, 54–63.

Hedelind, M., Jackson, M. (2011). How to improve the use of industrial robots in lean manufacturing systems. *J. Manuf. Technol. Manag*. 22, 891–905. https://doi.org/10.1108/17410381111160951

Heneman, R.L., Moore, M.L., Wexley, K.N. (1987). Performance-rating accuracy: A critical review. *J. Bus. Res.* 15, 431–448. https://doi.org/10.1016/0148-2963(87)90011-7

Hinegardner, R. (1974). Cellular DNA content of the Mollusca. *Comparative Biochemistry and Physiology Part A: Physiology*, *47*(2), 447–460. https://doi.org/10.1016/0300-9629(74)90008-5

Howard, D. W., Lewis, E., Keller, B., & Smith, C. (2004). Histological Techniques for Marine Bivalve Mollusks. In *NOAA Technical Memorandum NOS NCCOS 5* (p. 218).

Hu, E. (2012). *High-throughput sperm cryopreservation of aquatic species* [PhD, Louisiana State University]. https://digitalcommons.lsu.edu/gradschool_dissertations/1202

Hu, E., Bosworth, B., Baxter, J., Tiersch, T.R. (2014). On-site evaluation of commercial-scale hybrid catfish production using cryopreserved blue catfish sperm. *Aquaculture* 426–427, 88–95. https://doi.org/10.1016/j.aquaculture.2014.01.024

Hu, E., Liao, T.W., Tiersch, T.R. (2013). A quality assurance initiative for commercial-scale production in high-throughput cryopreservation of blue catfish sperm. *Cryobiology* 67, 214–224. https://doi.org/10.1016/j.cryobiol.2013.07.001

Hu, E., Liao, T.W., Tiersch, T.R. (2015). Simulation modelling of high-throughput cryopreservation of aquatic germplasm: a case study of blue catfish sperm processing. *Aquac. Res.* 46, 432–445. https://doi.org/10.1111/are.12192

Hu, E., Yang, H., Tiersch, T.R. (2011). High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): Establishment of an approach for commercial-scale processing. *Cryobiology* 62, 74–82. https://doi.org/10.1016/j.cryobiol.2010.12.006

Hughes, A. R., Hanley, T. C., Byers, J. E., Grabowski, J. H., McCrudden, T., Piehler, M. F., & Kimbro, D. L. (2019). Genetic diversity and phenotypic variation within hatchery-produced oyster cohorts predict size and success in the field. *Ecological Applications*, *29*(6), e01940. https://doi.org/10.1002/eap.1940

Hulbert, A.J., Pamplona, R., Buffenstein, R. & Buttemer, W.A. (2007). Life and death: metabolic rate, membrane composition, and life span of animals. *Physiol. Rev.*, 87, 1175–1213.

Huvet, A., Normand, J., Fleury, E., Quillien, V., Fabioux, C., & Boudry, P. (2010). Reproductive effort of Pacific oysters: A trait associated with susceptibility to summer mortality. *Aquaculture*, *304*(1), 95–99. https://doi.org/10.1016/j.aquaculture.2010.03.022

Irwin, G., Wessel, L., & Blackburn, H. (2012). The Animal Genetic Resource Information Network (AnimalGRIN) Database: A Database Design & Implementation Case. *Journal of Information Systems Education*, 23(1), 19–28.

Johnson, K. M., & Kelly, M. W. (2020). Population epigenetic divergence exceeds genetic divergence in the Eastern oyster *Crassostrea virginica* in the Northern Gulf of Mexico. *Evolutionary Applications*, 13(5), 945–959. https://doi.org/10.1111/eva.12912

Jones, H. D., Richards, O. G., & Southern, T. A. (1992). Gill dimensions, water pumping rate and body size in the mussel *Mytilus edulis* L. *Journal of Experimental Marine Biology and Ecology*, *155*(2), 213–237. https://doi.org/10.1016/0022-0981(92)90064-H

Jones, J.W., Antle, J.M., Basso, B., Boote, K.J., Conant, R.T., Foster, I., Godfray, H.C.J., Herrero, M., Howitt, R.E., Janssen, S., Keating, B.A., Munoz-Carpena, R., Porter, C.H., Rosenzweig, C., Wheeler, T.R. (2017). Brief history of agricultural systems modeling. *Agric. Syst.* 155, 240–254. https://doi.org/10.1016/j.agsy.2016.05.014

Joseph, T.P. (2006). Design a Lean laboratory layout. *Med. Lab. Obs.* 38, 24–31.

Jouaux, A., Blin, J. L., Adeline, B., Heude-Berthelin, C., Sourdaine, P., Mathieu, M., & Kellner, K. (2013). Impact of energy storage strategies on gametogenesis and reproductive effort in diploid and triploid Pacific oysters *Crassostrea gigas*—Involvement of insulin signaling. *Aquaculture*, *388–391*, 173–181. https://doi.org/10.1016/j.aquaculture.2013.01.009

Kapoore, R. V., Huete-Ortega, M., Day, J. G., Okurowska, K., Slocombe, S. P., Stanley, M. S., & Vaidyanathan, S. (2019). Effects of cryopreservation on viability and functional stability of an industrially relevant alga. *Scientific Reports*, 9(1), Article 1. https://doi.org/10.1038/s41598-019-38588-6

Kato, I. (2000). Toyota Production System Basic Handbook. http://artoflean.com/wp -content/uploads/2019/01/Basic_TPS_Handbook.pdf

Kesarcodi-Watson, A., Klumpp, D. W., & Lucas, J. S. (2001a). Comparative feeding and physiological energetics in diploid and triploid Sydney rock oysters (*Saccostrea commercialis*): II. Influences of food concentration and tissue energy distribution. *Aquaculture*, *203*(1), 195–216. https://doi.org/10.1016/S0044-8486(01)00623-8

Kinsella, J. (2019). Environmental Effects on Cultured Oyster Crassostrea virginica: Implications for filtration capacity and production [Master's Thesis]. University of North Carolina Wilmington.

Koganezawa, A. (1974). Present status of studies on the mass mortality of cultured oyster in Japan and its prevention. *Proceedings of the Third U.S.-Japan Meeting on Aquaculture*, 29–34. https://cir.nii.ac.jp/crid/1573668925131893632

Komaru, A., & Wada, K. T. (1994). Meiotic maturation and progeny of oocytes from triploid Japanese pearl oysters (*Pinctada fucata martensii*) fertilized with spermatozoa from diploids. *Aquaculture*, *120*(1), 61–70. https://doi.org/10.1016/0044-8486(94)90222-4

Kumar, S. (2014). Lean Manufacturing and its Implementation. *International Journal of Advanced Mechanical Engineering*, 4(2), 231–238.

La Peyre, J.F., Casas, S.M., Richards, M., Xu, W., Xue, Q. (2019). Testing plasma subtilisin inhibitory activity as a selective marker for dermo resistance in eastern oysters. *Diseases of Aquatic Organisms,* 133, 127–139. https://doi.org/10.3354/dao03344

La Peyre, J. F., Casas, S. M., & Supan, J. E. (2018). Effects of controlled air exposure on the survival, growth, condition, pathogen loads and refrigerated shelf life of eastern oysters. *Aquaculture Research*, *49*(1), 19–29. https://doi.org/10.1111/are.13427

La Peyre, J. F., Chu, F.-L. E., & Meyers, J. M. (1995). Haemocytic and humoral activities of eastern and Pacific oysters following challenge by the protozoan *Perkinsus marinus*. *Fish & Shellfish Immunology*, *5*(3), 179–190. https://doi.org/10.1016/S1050-4648(05)80012-9

La Peyre, M. K., Eberline, B. S., Soniat, T. M., & La Peyre, J. F. (2013). Differences in extreme low salinity timing and duration differentially affect eastern oyster (*Crassostrea virginica*) size class growth and mortality in Breton Sound, LA. *Estuarine, Coastal and Shelf Science*, *135*, 146–157. https://doi.org/10.1016/j.ecss.2013.10.001

La Peyre, M. K., Gossman, B., & La Peyre, J. F. (2009). Defining Optimal Freshwater Flow for Oyster Production: Effects of Freshet Rate and Magnitude of Change and Duration on Eastern Oysters and *Perkinsus marinus* Infection. *Estuaries and Coasts*, *32*(3), 522–534. https://doi.org/10.1007/s12237-009-9149-9

Lannig, G., Eilers, S., Pörtner, H. O., Sokolova, I. M., & Bock, C. (2010). Impact of Ocean Acidification on Energy Metabolism of Oyster, *Crassostrea gigas*—Changes in Metabolic Pathways and Thermal Response. *Marine Drugs*, *8*(8), Article 8. https://doi.org/10.3390/md8082318

Leitão, A., Boudry, P., & Thiriot-Quiévreux, C. (2001). Negative correlation between aneuploidy and growth in the Pacific oyster, *Crassostrea gigas*: Ten years of evidence. *Aquaculture*, *193*(1), 39–48. https://doi.org/10.1016/S0044-8486(00)00488-9

Leonhardt, J. M., Casas, S., Supan, J. E., & La Peyre, J. F. (2017). Stock assessment for eastern oyster seed production and field grow-out in Louisiana. *Aquaculture*, 466, 9–19. https://doi.org/10.1016/j.aquaculture.2016.09.034

Lesser, M. P. (2006). Oxidative stress in marine environments: Biochemistry and physiological ecology. *Annual Review of Physiology*, *68*(1), 253–278.

Lowe, M. R., Sehlinger, T., Soniat, T. M., & Peyre, M. K. L. (2017). Interactive Effects of Water Temperature and Salinity on Growth and Mortality of Eastern Oysters, *Crassostrea virginica*: A Meta-Analysis Using 40 Years of Monitoring Data. *Journal of Shellfish Research*, 36(3), 683–697. https://doi.org/10.2983/035.036.0318

Lind, C. E., Evans, B. S., Taylor, J. J. U., & Jerry, D. R. (2010). The consequences of differential family survival rates and equalizing maternal contributions on the effective population size (Ne) of cultured silver-lipped pearl oysters, *Pinctada maxima*. *Aquaculture Research*, *41*(8), 1229–1242. https://doi.org/10.1111/j.1365-2109.2009.02410.x

Liu, Y., Blackburn, H., Taylor, S. S., & Tiersch, T. R. (2019). Development of germplasm repositories to assist conservation of endangered fishes: Examples from small-bodied livebearing fishes. *Theriogenology*, 135, 138–151. https://doi.org/10.1016/j.theriogenology.2019.05.020

Liu, Y., Eskridge, M., Guitreau, A., Beckham, J., Chesnut, M., Torres, L., Tiersch, T.R., Monroe, W.T. (2021). Development of an open hardware 3-D printed conveyor device for continuous cryopreservation of non-batched samples. *Aquac. Eng*. 95, 102202. https://doi.org/10.1016/j.aquaeng.2021.102202

Liu, Y., Todd Monroe, W., Belgodere, J. A., Choi, J.-W., Teresa Gutierrez-Wing, M., & Tiersch, T. R. (2022). The emerging role of open technologies for community-based improvement of cryopreservation and quality management for repository development in aquatic species. *Animal Reproduction Science*, 246, 106871. https://doi.org/10.1016/j.anireprosci.2021.106871

LUMCON. (2021). *Environmental Monitoring – LUMCON Website*. LUMCON Environmental Monitoring. https://lumcon.edu/environmental-monitoring/

Malmquist, D. (2019). Consortium earns funding to enhance oyster breeding. William & Mary. https://www.wm.edu/news/stories/2019/consortium-earns-funding-to-enhance-oyster-breeding.php

Marshall, D. A., Casas, S. M., Walton, W. C., Rikard, F. S., Palmer, T. A., Breaux, N., La Peyre, M. K., Beseres Pollack, J., Kelly, M., & La Peyre, J. F. (2021a). Divergence in salinity tolerance of northern Gulf of Mexico eastern oysters under field and laboratory exposure. *Conservation Physiology*, *9*(1). https://doi.org/10.1093/conphys/coab065

Marshall, D. A., Coxe, N. C., La Peyre, M. K., Walton, W. C., Rikard, F. S., Pollack, J. B., Kelly, M. W., & La Peyre, J. F. (2021b). Tolerance of northern Gulf of Mexico eastern oysters to chronic warming at extreme salinities. *Journal of Thermal Biology*, *100*, 103072. https://doi.org/10.1016/j.jtherbio.2021.103072

MATLAB, 2010. . The MathWorks Inc., Natick, Massachusetts.

Matt, J.L., Allen, S.K. (2021). A classification system for gonad development in triploid *Crassostrea virginica. Aquaculture,* 532, 735994. https://doi.org/10.1016/j.aquaculture.2020.735994

Matt, J. L., Guévélou, E., Small, J. M., & Allen, S. K. (2020). A field test investigating the influence of brood stock origin and ploidy on the susceptibility of *Crassostrea virginica* to "triploid mortality" in the Chesapeake Bay. *Aquaculture*, 526, 735375. https://doi.org/10.1016/j.aquaculture.2020.735375

McCarty, A. J., McFarland, K., Small, J., Allen, S. K., & Plough, L. V. (2020). Heritability of acute low salinity survival in the Eastern oyster (*Crassostrea virginica*). *Aquaculture*, 529, 735649. https://doi.org/10.1016/j.aquaculture.2020.735649

McCausland, W.D., Mente, E., Pierce, G.J., Theodossiou, I., 2006. A simulation model of sustainability of coastal communities: Aquaculture, fishing, environment and labour markets. *Ecol. Model.* 193, 271–294. https://doi.org/10.1016/j.ecolmodel.2005.08.028

Melnik, S., Magnotti, M., Butts, C., Putman, C., & Aqlan, F. (2020). A Concept Relationship Map for Industry 4.0. 1–14.

Meyers, J., Burreson, E., Barber, B., & Mann, R. (1991). Susceptibility Of Diploid And Triploid Pacific Oysters, Crassostrea Gigas (Thunberg, 1793) And Eastern Oysters, *Crassostrea virginica* (Gmelin, 1791), To *Perkinsus marinus*. *Journal of Shellfish Research*, 10(2), 433–437.

Meyhöfer, E. (1985). Comparative pumping rates in suspension-feeding bivalves. *Marine Biology*, *85*(2), 137–142. https://doi.org/10.1007/BF00397432

Miettinen, T. P., Caldez, M. J., Kaldis, P., & Björklund, M. (2017). Cell size control – a mechanism for maintaining fitness and function. *BioEssays*, *39*(9), 1700058. https://doi.org/10.1002/bies.201700058

Millstein, M.A., Martinich, J.S., 2014. Takt Time Grouping: implementing kanban-flow manufacturing in an unbalanced, high variation cycle-time process with moving constraints. *Int. J. Prod. Res*. 52, 6863–6877. https://doi.org/10.1080/00207543.2014.910621

Mizuta, D. D., Wikfors, G. H., Meseck, S. L., Li, Y., Dixon, M. S., Lim, H. J., Hwang, I. J., Bazzano, M., & Pitchford, S. (2021). Use of natural trophic resources by Eastern oysters and Pacific oysters of different ploidy. *Aquaculture and Fisheries*, *6*(1), 75–83. https://doi.org/10.1016/j.aaf.2020.03.011

Moore, S. G., & Hasler, J. F. (2017). A 100-Year Review: Reproductive technologies in dairy science. *Journal of Dairy Science*, 100(12), 10314–10331. https://doi.org/10.3168/jds.2017-13138

Montagnac, V., Guyondet, T., Comeau, L., & Tremblay, R. (2020). Physiological differences between wild and cultured bivalves in Prince Edward Island, Canada. *Aquatic Living Resources*, *33*, 8. https://doi.org/10.1051/alr/2020008

Moroney, D. A., & Walker, R. L. (1999). The Effects of Tidal and Bottom Placement on the Growth, Survival and Fouling of the Eastern Oyster *Crassostrea virginica*. *Journal of the World Aquaculture Society*, *30*(4), 433–442. https://doi.org/10.1111/j.1749-7345.1999.tb00991.x

Munroe, D., Tabatabai, A., Burt, I., Bushek, D., Powell, E. N., & Wilkin, J. (2013). Oyster mortality in Delaware Bay: Impacts and recovery from Hurricane Irene and Tropical Storm Lee. *Estuarine, Coastal and Shelf Science*, *135*, 209–219. https://doi.org/10.1016/j.ecss.2013.10.011

NAGP. (2023, January 21). Animal GRIN Explore the Inventory. https://agrin.ars.usda.gov/tax_inv_drilldown_page_dev?language=EN&record_source=US

Nascimento-Schulze, J. C., Bean, T. P., Houston, R. D., Santos, E. M., Sanders, M. B., Lewis, C., & Ellis, R. P. (2021). Optimizing hatchery practices for genetic improvement of marine bivalves. *Reviews in Aquaculture*, *13*(4), 2289–2304. https://doi.org/10.1111/raq.12568

National Association of Animal Breeders. (2022). Annual Reports of Semen Sales and Custom Freezing. Semen Sales. https://www.naab-css.org/semen-sales

National Marine Fisheries Service. (2020). *Fisheries of the United States. Current Fishery Statistics No. 2018*. NOAA. https://media.fisheries.noaa.gov/dam-migration/fus_2018_report.pdf

Newman, D. L., & Gregory, S. L. (2019). Co-Operation between Aneuploidy and Metabolic Changes in Driving Tumorigenesis. *International Journal of Molecular Sciences*, 20(18), Article 18. https://doi.org/10.3390/ijms20184611

NOAA Fisheries. (2020). *Fisheries of the United States, 2018 | NOAA Fisheries* (Alaska, New England/Mid-Atlantic, Pacific Islands, Southeast, West Coast, National). NOAA.

Nwanya, S. C., Udofia, J. I., & Ajayi, O. O. (2017). Optimization of machine downtime in the plastic manufacturing. *Cogent Engineering*, 4(1), 1335444. https://doi.org/10.1080/23311916.2017.1335444

Paniagua-Chavez, C. G., Buchanan, J. T., Supan, J. E., & Tiersch, T. R. (2000). Cryopreservation of Sperm and Larvae of the Eastern Oyster. In *Cryopreservation in Aquatic Species* (pp. 230–239). World Aquaculture Society.

Paniagua-Chavez, C. G., & Tiersch, T. R. (2001). Laboratory Studies of Cryopreservation of Sperm and Trochophore Larvae of the Eastern Oyster. *Cryobiology*, 43(3), 211–223. https://doi.org/10.1006/cryo.2001.2346

Perdue, J.A., Beattie, J.H. & Chew, K.K. (1981). Some relationships between gametogenic cycle and summer mortality phenomenon in the Pacific oyster (*Crassostrea gigas*) in Washington state. *Journal of Shellfish Research* 1, 9–16.

Petrolia, D. R., Walton, W. C., & Cebrian, J. (2022). Oyster Economics: Simulated Costs, Market Returns, and Nonmarket Ecosystem Benefits of Harvested and Non-harvested Reefs, Off-Bottom Aquaculture, and Living Shorelines. *Marine Resource Economics*, 37(3), 325–347. https://doi.org/10.1086/719969

Piferrer, F., Beaumont, A., Falguière, J.-C., Flajšhans, M., Haffray, P., & Colombo, L. (2009). Polyploid fish and shellfish: Production, biology and applications to aquaculture for performance improvement and genetic containment. *Aquaculture*, *293*(3), 125–156. https://doi.org/10.1016/j.aquaculture.2009.04.036

Powell, E. J., & Keim, B. D. (2015). Trends in Daily Temperature and Precipitation Extremes for the Southeastern United States: 1948–2012. *Journal of Climate*, *28*(4), 1592–1612. https://doi.org/10.1175/JCLI-D-14-00410.1

Proestou, D. A., Vinyard, B. T., Corbett, R. J., Piesz, J., Allen, S. K., Small, J. M., Li, C., Liu, M., DeBrosse, G., Guo, X., Rawson, P., & Gómez-Chiarri, M. (2016). Performance of selectively-bred lines of eastern oyster, *Crassostrea virginica*, across eastern US estuaries. *Aquaculture*, 464, 17–27. https://doi.org/10.1016/j.aquaculture.2016.06.012

Protzman, C., McNamara, J., Protzman, D. (2017). One-Piece Flow vs. Batching: A Guide to Understanding How Continuous Flow Maximizes Productivity and Customer Value. CRC Press, Boca Raton, FL.

Purdy, P. H., Wilson, C. S., Spiller, S. F., Blackburn, H. D., Purdy, P. H., Wilson, C. S., Spiller, S. F., & Blackburn, H. D. (2016). Biobanking genetic resources: Challenges and implementation at the USDA National Animal Germplasm Program. *Reproduction, Fertility and Development*, 28(8), 1072–1078. https://doi.org/10.1071/RD15399

Quintana, R., Burnside, W. M., Supan, J. E., Lynn, J. W., & Tiersch, T. R. (2011). Rapid Estimation of Gonad-to-Body Ratio in Eastern Oysters by Image Analysis. *North American Journal of Aquaculture*, *73*(4), 451–455. https://doi.org/10.1080/15222055.2011.630258

R Core Team. (2020). *R: A Language and Environment for Statistical Computing* (4.0.3) [Computer Software]. R Foundation for Statistical Computing. https://www.R-project.org/

Ragone-Calvo, L. M., Dungan, C. F., Roberson, B. S., & Burreson, E. M. (2003). Systematic evaluation of factors controlling *Perkinsus marinus* transmission dynamics in lower Chesapeake Bay. *Diseases of Aquatic Organisms*, *56*(1), 75–86. https://doi.org/10.3354/dao056075

Riisgård, H. (1988). Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. *Marine Ecology Progress Series*, *45*, 217–223. https://doi.org/10.3354/meps045217

Robinson, S. (2005). Discrete-event simulation: from the pioneers to the present, what next? *J. Oper. Res. Soc.* 56, 619–629. https://doi.org/10.1057/palgrave.jors.2601864

Rodhouse, P. G., & Gaffney, P. M. (1984). Effect of heterozygosity on metabolism during starvation in the American oyster *Crassostrea virginica*. *Marine Biology*, *80*(2), 179–187. https://doi.org/10.1007/BF02180185

Rørtveit, R., Lilienthal, R.E. (2017). *Discrete-Event Simulation of a Multimodal Downstream Supply Chain for Future Norwegian Aquaculture* (Master's Thesis). Norwegian University of Science and Technology, Trondheim, Norway.

Russell, M. L., & Meller, R. D. (2003). Cost and Throughput Modeling of Manual and Automated Order Fulfillment Systems. *IIE Transactions*, 35(7), 589–603. https://doi.org/10.1080/07408170304358

Rutledge, S. D., & Cimini, D. (2016). Consequences of aneuploidy in sickness and in health. *Current Opinion in Cell Biology*, *40*, 41–46. https://doi.org/10.1016/j.ceb.2016.02.003

Ruzicka, L., Bradford, Y. M., Frazer, K., Howe, D. G., Paddock, H., Ramachandran, S., Singer, A., Toro, S., Van Slyke, C. E., Eagle, A. E., Fashena, D., Kalita, P., Knight, J., Mani, P., Martin, R., Moxon, S. A. T., Pich, C., Schaper, K., Shao, X., & Westerfield, M. (2015). ZFIN, The zebrafish model organism database: Updates and new directions. *Genesis*, 53(8), 498–509. https://doi.org/10.1002/dvg.22868

Rybovich, M., Peyre, M. K. L., Hall, S. G., & Peyre, J. F. L. (2016). Increased Temperatures Combined with Lowered Salinities Differentially Impact Oyster Size Class Growth and Mortality. *Journal of Shellfish Research*, 35(1), 101–113. https://doi.org/10.2983/035.035.0112

Samain, J.F. (2011). Review and perspectives of physiological mechanisms underlying genetically-based resistance of the Pacific oyster *Crassostrea gigas* to summer mortality. *Aquatic Living Resources*, *24*(3), 227–236. https://doi.org/10.1051/alr/2011144

Samain, J. F., Dégremont, L., Soletchnik, P., Haure, J., Bédier, E., Ropert, M., Moal, J., Huvet, A., Bacca, H., Van Wormhoudt, A., Delaporte, M., Costil, K., Pouvreau, S., Lambert, C., Boulo, V., Soudant, P., Nicolas, J. L., Le Roux, F., Renault, T., … Boudry, P. (2007). Genetically based resistance to summer mortality in the Pacific oyster (*Crassostrea gigas*) and its relationship with physiological, immunological characteristics and infection processes. *Aquaculture*, *268*(1), 227–243. https://doi.org/10.1016/j.aquaculture.2007.04.044

Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/nmeth.2089

Schriber, T.J., Brunner, D.T., Smith, J.S. (2013). Inside discrete-event simulation software: How it works and why it matters, in: 2013 Winter Simulations Conference (WSC). Presented at the 2013 Winter Simulations Conference (WSC), pp. 424–438. https://doi.org/10.1109/WSC.2013.6721439

Shamkhalichenar, H., Choi, J.-W., & Tiersch, T. R. (2019). Three-dimensional printing can provide customizable probes for sensing and monitoring in cryobiology applications. *Cryobiology*, 88, 64–69. https://doi.org/10.1016/j.cryobiol.2019.03.010

Sheltzer, J.M., Amon, A. (2011) The aneuploidy paradox: costs and benefits of an incorrect karyotype. *Trends Genetics, 27*: 446–453. https://doi.org/10.1016/j.tig.2011.07.003

Shumway, S. E., Cucci, T. L., Newell, R. C., & Yentsch, C. M. (1985). Particle selection, ingestion, and absorption in filter-feeding bivalves. *Journal of Experimental Marine Biology and Ecology*, 91(1), 77–92. https://doi.org/10.1016/0022-0981(85)90222-9

Simio (2021).version 4.0.3.Simio LLC, Sewickley, PA.

Şimşit, Z.T., Günay, N.S., Vayvay, Ö. (2014). Theory of Constraints: A Literature Review. Procedia - Soc. Behav. Sci., 10th International Strategic Management Conference 2014 150, 930–936. https://doi.org/10.1016/j.sbspro.2014.09.104

Smaal, A. C., & Widdows, J. (1994). The scope for growth of bivalves as an integrated response parameter in biological monitoring. *K.J.M. Kramer (Ed.), Biomonitoring of Coastal Waters and Estuaries, CRC Press, Boca Raton, FL (1994), Pp.*, 247–262.

Sokolova, I. M., Frederich, M., Bagwe, R., Lannig, G., & Sukhotin, A. A. (2012). Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. *Marine Environmental Research*, *79*, 1–15. https://doi.org/10.1016/j.marenvres.2012.04.003

Storchova, Z. (2012). *Aneuploidy in Health and Disease*. IntechOpen. https://doi.org/ 10.5772/2155

Sullivan, W. G., McDonald, T. N., & Van Aken, E. M. (2002). Equipment replacement decisions and lean manufacturing. *Robotics and Computer-Integrated Manufacturing*, 18(3), 255–265. https://doi.org/10.1016/S0736-5845(02)00016-9

Sumaila, U.R., Tai, T.C. (2020). End Overfishing and Increase the Resilience of the Ocean to Climate Change. *Front. Mar. Sci.,* 7.

Swam, L. M., La Peyre, M. K., Callam, B. R., & La Peyre, J. F. (2022). Local Populations of Eastern Oyster from Louisiana Differ in Low-Salinity Tolerance. *North American Journal of Aquaculture*, naaq.10248. https://doi.org/10.1002/naaq.10248

Tervit, H. R., Adams, S. L., Roberts, R. D., McGowan, L. T., Pugh, P. A., Smith, J. F., & Janke, A. R. (2005). Successful cryopreservation of Pacific oyster (*Crassostrea gigas*) oocytes. *Cryobiology*, 51(2), 142–151. https://doi.org/10.1016/j.cryobiol.2005.06.001

Thunes, M. (2018). *Discrete-Event Simulation of Vessel Response Time for Acute Pollution in Aquaculture* [Master's Thesis, Norwegian University of Science and Technology]. https://ntnuopen.ntnu.no/ntnu-xmlui/handle/11250/2564497

Tiersch, T.R., Green, C., World Aquaculture Society (2011). Cryopreservation in aquatic species. The World Aquaculture Society, Baton Rouge, Louisiana.

Tiersch, T. R., & Jenkins, J. A. (2001). Biosecurity and regulatory considerations for transfer of cryopreserved sperm and early life stages of aquatic species. World Aquaculture Society 171–198. https://pubs.er.usgs.gov/publication/70207252

Torres, L., Hu, E., Tiersch, T. R., Torres, L., Hu, E., & Tiersch, T. R. (2016). Cryopreservation in fish: Current status and pathways to quality assurance and quality control in repository development. *Reproduction, Fertility and Development*, 28(8), 1105–1115. https://doi.org/10.1071/RD15388

Torres, L., Liu, Y., Guitreau, A., Yang, H., & Tiersch, T. R. (2017). Challenges in Development of Sperm Repositories for Biomedical Fishes: Quality Control in Small-Bodied Species. *Zebrafish*, 14(6), 552–560. https://doi.org/10.1089/zeb.2017.1426

Torres, L., Tiersch, T.R. (2018). Addressing Reproducibility in Cryopreservation, and Considerations Necessary for Commercialization and Community Development in Support of Genetic Resources of Aquatic Species. *J. World Aquac. Soc*., 49, 644–663. https://doi.org/10.1111/jwas.12541

Tran, N., Rodriguez, U.-P., Chan, C. Y., Phillips, M. J., Mohan, C. V., Henriksson, P. J. G., Koeshendrajana, S., Suri, S., & Hall, S. (2017). Indonesian aquaculture futures: An analysis of fish supply and demand in Indonesia to 2030 and role of aquaculture using the AsiaFish model. *Marine Policy*, 79, 25–32. https://doi.org/10.1016/j.marpol.2017.02.002

Turan, G., Neori, A., 2010. Intensive Sea Weed Aquaculture: A Potent Solution Against Global Warming, in: Seckbach, J., Einav, R., Israel, A. (Eds.), Seaweeds and Their Role in Globally Changing Environments, Cellular Origin, Life in Extreme Habitats and Astrobiology. Springer Netherlands, Dordrecht, pp. 357–372. https://doi.org/10.1007/978-90-481-8569-6_20

University of Maryland. (2022). Oyster Larvae Sales – Horn Point Oyster Hatchery. University of Maryland - Center for Environment Science - Horn Point Oyster Hatchery. https://hatchery.hpl.umces.edu/oyster-larvae-sales/

USGS Water Data for Louisiana. (2021). https://waterdata.usgs.gov/la/nwis

Varga, Z. M. (2011). Chapter 24—Aquaculture and Husbandry at the Zebrafish International Resource Center. In H. W. Detrich, M. Westerfield, & L. I. Zon (Eds.), *Methods in Cell Biology* (Vol. 104, pp. 453–478). Academic Press. https://doi.org/10.1016/B978-0-12-374814-0.00024-0

Voss, S. R., Contreras, M., Magnani, D., Murphy, S., Murray, K., O'Connor, D., Reinholdt, L., Rougvie, A., Sánchez, E. E., & Whitworth, C. (2021). Innovating, Adapting, and Sustaining Resources for a Dynamic Biomedical Landscape. Thirteenth Comparative Medicine Resource Directors Meeting, 53. https://orip.nih.gov/sites/default/files/13th-CMRDM-Final-Report.pdf

Wachtel, S. S., & Tiersch, T. R. (1993). Variations in genome mass. Comparative Biochemistry and Physiology Part B: *Comparative Biochemistry*, 104(2), 207–213. https://doi.org/10.1016/0305-0491(93)90360-H

Wadsworth, P., Casas, S., La Peyre, J., Walton, W. (2019). Elevated mortalities of triploid eastern oysters cultured off-bottom in northern Gulf of Mexico. *Aquaculture,* 505, 363–373. https://doi.org/10.1016/j.aquaculture.2019.02.068

Wallace, R. K., Waters, P., & Rikard, F. S. (2008). Oyster Hatchery Techniques. *Southern Regional Aquaculture Center,* 4302, 6.

Walton, W. C., Davis, J. E., Chaplin, G. I., Rikard, F. S., Hanson, T. R., Waters, P. J., & Swann, D. L. (2012). Off-Bottom Oyster Farming. Alabama Cooperative Extension System, Fisheries & Aquaculture Series. http://fisheries.tamu.edu/files/2013/09/Off-Bottom-Oyster-Farming.pdf

Walton, W. C., Davis, J. E., & Supan, J. E. (2013). Off-Bottom Culture of Oysters in the Gulf of Mexico. *National Oceanic and Atmospheric Administration*, 6.

Walton, W. C., & Swann, L. (2021). Role of Sea Grant in Establishing Commercial Oyster Aquaculture through Applied Research and Extension. *Journal of Contemporary Water Research & Education*, 174(1), 171–179. https://doi.org/10.1111/j.1936-704X.2021.3367.x

Wang, H., Chen, Q., La Peyre, M. K., Hu, K., & La Peyre, J. F. (2017). Predicting the impacts of Mississippi River diversions and sea-level rise on spatial patterns of eastern oyster growth rate and production. *Ecological Modelling*, *352*, 40–53. https://doi.org/10.1016/j.ecolmodel.2017.02.028

Wang, Z., Guo, X., Allen, S. K., & Wang, R. (1999). Aneuploid Pacific oyster (Crassostrea gigas Thunberg) as incidentals from triploid production. *Aquaculture*, 173(1), 347–357. https://doi.org/10.1016/S0044-8486(98)00457-8

Wang, Z., Guo, X., Allen, S. K., & Wang, R. (2002). Heterozygosity and body size in triploid Pacific oysters, *Crassostrea gigas* Thunberg, produced from meiosis II inhibition and tetraploids. *Aquaculture*, *204*(3), 337–348. https://doi.org/10.1016/S0044-8486(01)00845-6

Ward, M., Spalding, A. K., Levine, A., & Wolters, E. A. (2022). California shellfish farmers: Perceptions of changing ocean conditions and strategies for adaptive capacity. *Ocean & Coastal Management,* 225, 106155. https://doi.org/10.1016/j.ocecoaman.2022.106155

Watson, P. F. (2000). The causes of reduced fertility with cryopreserved semen. *Animal Reproduction Science*, 60–61, 481–492. https://doi.org/10.1016/S0378-4320(00)00099-3

Widdows, J. (1985). Physiological responses to pollution. *Marine Pollution Bulletin*, *16*(4), 129–134. https://doi.org/10.1016/0025-326X(85)90002-5

Widdows, J., & Johnson, D. (1988). Physiological energetics of Mytilus edulis: Scope for Growth. *Marine Ecology Progress Series*, *46*(1/3), 113–121.

Willer, D.F., Aldridge, D.C. (2019). Microencapsulated diets to improve bivalve shellfish aquaculture for global food security. *Glob. Food Secur.,* 23, 64–73. https://doi.org/10.1016/j.gfs.2019.04.007

Yang, H. (2022). Performance and Fecundity of Triploid Eastern Oysters *Crassostrea virginica* (Gmelin, 1791) and Challenges for Tetraploid Production. *Journal of Shellfish Research*, 40(3), 489–497. https://doi.org/10.2983/035.040.0305

Yang, H., Hu, E., Buchanan, J.T., Tiersch, T.R. (2018). A Strategy for Sperm Cryopreservation of Atlantic Salmon, *Salmo salar*, for Remote Commercial-scale High-throughput Processing. *J. World Aquac. Soc.,* 49, 96–112. https://doi.org/10.1111/jwas.12431

Yang, H., Hu, E., Cuevas-Uribe, R., Supan, J., Guo, X., Tiersch, T.R. (2012). High-throughput sperm cryopreservation of eastern oyster *Crassostrea virginica. Aquaculture,* 344–349, 223–230. https://doi.org/10.1016/j.aquaculture.2012.03.018

Yang, H., Huo, Y. (2021). Review of molluscan larval cryopreservation and application to germplasm cryobanking and commercial seed production. *Aquaculture,* 547, 737491. https://doi.org/10.1016/j.aquaculture.2021.737491

Yang, H., Supan, J., Guo, X., & Tiersch, T. R. (2013). Nonlethal Sperm Collection and Cryopreservation in the Eastern Oyster *Crassostrea virginica*. *Journal of Shellfish Research*, *32*(2), 429–437. https://doi.org/10.2983/035.032.0223

Yang, H., Wang, Y., Guo, X., & Tiersch, T. R. (2015). Production of inbred larvae through self-fertilization using oocytes and cryopreserved sperm from the same individuals after sex reversal in eastern oyster *Crassostrea virginica. Aquaculture Research*, 46(9), 2153–2165. https://doi.org/10.1111/are.12371

Zouros, E., & Foltz, D. W. (1983). Minimal selection requirements for the correlation between heterozygosity and growth, and for the deficiency of heterozygotes, in oyster population. *Developmental Genetics*, *4*(4), 393–405. https://doi.org/10.1002/dvg.1020040413

Vita

Sarah Bodenstein is originally from Chicago, IL. She received her Bachelor's degree in Marine Science and Biology from the University of Miami in 2016. In 2017 Sarah began her Master's degree in Fisheries, Aquaculture, and Aquatic Sciences from Auburn University in Alabama under Dr. William C. Walton. At Auburn she worked at the Auburn University Shellfish Lab (a research hatchery on Dauphin Island, AL) and studied the potential causes of differential mortality rates between triploid and diploid oysters. Sarah successfully defended her Master's thesis titled *Comparing responses of triploid and diploid eastern oysters, Crassostrea virginica, to common farm stressors* in 2019. One month later she started her PhD under Dr. Terrence R. Tiersch and Dr. Jerome F. La Peyre in the Aquatic Germplasm and Genetic Resources Center (AGGRC), part of the Louisiana State University Agricultural, School of Renewable Resources in Baton Rouge, LA. Sarah is currently a PhD candidate for the degree of Doctor of Philosophy in Fisheries and Aquaculture in March 2023. At the AGGRC, Sarah's research focused on developing processes to build germplasm repositories into existing oyster aquaculture systems using industrial engineering tools and principles. This project involved aspects of aquaculture, field research, physiology, process mapping, and discrete event simulation modeling. Sarah is a member of the U.S. Chapter of the World Aquaculture Society and the Louisiana Chapter of the American Fisheries Society.

Publications

Bodenstein, S., Callam, B. R., Walton, W. C., Rikard, F. S., Tiersch, T. R., & La Peyre, J. F. (2023). Survival and growth of triploid eastern oysters, Crassostrea virginica, produced from wild diploids collected from low-salinity areas. Aquaculture, 739032. https://doi.org/10.1016/j.aquaculture.2022.739032

Bodenstein, S., Nahmens, I., & Tiersch, T. R. (2022). Simulation Modeling of a High-Throughput Oyster Cryopreservation Pathway. Journal of Shellfish Research, 41(2), 209–221. https://doi.org/10.2983/035.041.0206

Bodenstein, S., Walton, W. C., & Steury, T. D. (2021). Effect of farming practices on growth and mortality rates in triploid and diploid eastern oysters Crassostrea virginica. Aquaculture Environment Interactions, 13, 33–40. https://doi.org/10.3354/aei00387

Manuscripts submitted for publication or in preparation

Bodenstein, S., Abdullayeva, F., Varga, Z. M., Tiersch, T.R. (2023) Simulation Modeling of Zebrafish Cryopreservation Development and Operation at an Aquatic Biomedical Repository. Journal of the American Association for Laboratory Animal Science. *Cryobiology* (In Preparation)

Bodenstein, S., Casas, S., Tiersch, T.R., and La Peyre, J.F. (2023) Energetic budget of diploid and triploid eastern oysters during a summer die-off. *Frontiers in Marine Science* (Under Review)

Bodenstein, S., Nahmens, I., Callam, B.R., Tiersch, T.R. (2023) Simulation analysis of high-throughput oyster cryopreservation at three scales of production. *Aquaculture International* (Under Review)

Conference Presentations

Date	Title	Chapter	Format	Conference	Location
2021	Using germplasm repositories to support Eastern oyster *Crassostrea virginica*, aquaculture: the biological information component	4	Oral	American Fisheries Society-Louisiana Chapter	Online
2021	Characterizing Eastern oysters, *Crassostrea virginica*, bred from native Louisiana broodstock under different salinity regimes: effect of stock and cohort	4	Oral	National Shellfish Association	Online
2022	Effect of ploidy and cohorts produced at two hatcheries on the physiology of the Eastern oyster, *Crassostrea virginica*	5	Oral	World Aquaculture Society	San Diego, CA
2022	Using simulation modeling of high-throughput Eastern oyster cryopreservation to develop germplasm repositories	2	Oral	World Aquaculture Society	San Diego, CA
2022	Simulation modeling of high-throughput cryopreservation facilitates oyster germplasm repository development	2	Oral	American Fisheries Society-Louisiana Chapter	Thibodaux, LA
2023	Simulation analysis of high-throughput oyster cryopreservation at three scales of production	3	Oral	World Aquaculture Society	New Orleans, LA