VIRGINIA FISHERY RESOURCE GRANT PROGRAM

FRG 2024 Final Report

Early Season Survival of Oyster Seed During Winter Holding

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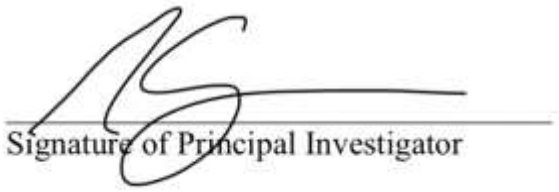

Signature of Principal Investigator

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Date

Introduction

Oyster Seed Holdings, Inc (OSH), a commercial oyster hatchery in Virginia, has encountered a bottleneck that is limiting additional seed production during the early season. Over the past 15 seasons, we have become very familiar with the seasonal variations in water conditions at our site, allowing us to predict periods of good and poor production. With few exceptions, we have designed our hatchery systems to capitalize on these “good” windows or avoid them entirely, as seen in our ongoing efforts to develop a closed recirculating aquaculture system for oyster larvae. One such effort has led to the development of a high-density setting system capable of producing over 30 million 1-mm seed per week.

Our early seed production season now consists of just four weekly spawns from mid-January to mid-February. After this, we suspend spawning until March due to limited capacity to hold seed inside the hatchery. It is not until mid- to late-March that seed can be transferred to outdoor nursery systems. In recent years, early season production has exceeded 100 million 1-mm seed that have to be held in a specially designed holding system waiting for ambient water temperatures to rise. This Cold Holding Duration (CHD) period presents challenges. Seed produced early in the season can be in CHD for nearly 2 months. To prevent overcrowding of inside systems, growth of these early seed needs to be slowed by limiting food and reducing temperatures. These are not normal physiological conditions for newly set spat

CHD seed experience mortality and "stunting," rendering more than 50% unusable. While we have developed effective protocols to weed out mortality and stunting prior to sale, the loss still represents significant untapped production that we want to bring to fruition. Over the past few seasons, we have experimented with various system solutions and adjustments to water flow rates and warm-up protocols, but none have significantly improved the retention of usable seed by the end of the holding period.

This project aimed to better understand the physiological stress experienced by the animals during the CHD and warm-up phases that contribute to the observed mortality and stunting. We recognize that improving seed health during the CHD is an iterative process. The first step, precisely identifying the issues, required expertise beyond the R&D capabilities of OSH alone. As a result, we partnered with the lab at Dr. Emily Rivest at the Virginia Institute of Marine Science (VIMS) where the necessary physiological expertise resides. Based on the results of these investigations, our next step will be to adjust CHD systems, protocols, and husbandry practices to address these physiological issues so that we can significantly improve the robustness of seed during the EHP.

Objectives

The primary goal of this project was to understand the factors affecting seed health during CHD and subsequent warm-up, with the ultimate aim to optimize bottle systems and improve hatchery production. The specific objectives of this project were as follows:

1. Evaluate potential indicators of juvenile health, such as tissue mass, glycogen and lipid content, oxygen consumption rates, and disease prevalence, which can serve as proxies for the health of oyster seed in the bottle system.

2. Determine differences in seed health as a result of temperature acclimation or no temperature acclimation at the end of CHD.

Methods

To evaluate the impact of cold holding duration (CHD) on the physiology and performance of oyster seed, we held oyster seed at 15°C for three CHD lengths: eight, six, and four weeks. For each CHD length, we used seed from 3 distinct commercial cohorts (i.e., separate spawns) produced at OSH. The cohorts were produced sequentially in the early season: mid-January (eight-week CHD, DEBY line), early February (six-week CHD, LOLA line), and mid-February (four-week CHD, DEBY line), allowing a staggered entry into CHD, a two-week interval between cohorts, and exit from the CHD on the same date for all three cohorts. These staggered groups resulted in CHD8, CHD6, and CHD4 cohorts. To evaluate the effects of CHD, multiple metrics of seed physiology were measured before, during, at the end of the CHD and compared between different groups.

Since temperature shock after CHD is hypothesized to cause seed distress, the Rivest lab tested the effects of two warming regimes at the end of CHD on the physiology and performance of oyster seed over 10 days. At the start of this warming period, each cohort was evenly and randomly split into two groups. One half of each cohort experienced fast warming where seed were immediately exposed to 28°C (a delta of 13°C), representing OSH standard operational protocol (SOP), and the other half of each cohort was subjected to a gradual temperature increase (slow warming) to 28°C (~1.2°C/day). We compared multiple physiological responses of seed under these two regimes at the start, middle, and end of the warming period to help us understand the effects of our hatchery's current SOP and whether gradual acclimation could enhance seed health.

<u>Oyster husbandry</u>

Oyster larvae were reared in a high-density, flow-through system using standard commercial methods until competent for about two weeks before setting. Eyed larvae were then induced for settlement using proprietary OSH commercial methods. Post-set seed from the three cohorts were maintained separately in OSH's flow-through bottle holding system until the start of the experiment, using two bottles per cohort, with water temperatures averaging 20-23ºC. The seed were continuously fed a live microalgal mix of *Tisochrysis*, *Tetraselmis*, *Pavlova*, and *Chaetoceros*. Prior to entering the CHD phase of the experiment, seed from each cohort were graded, and the 1 mm size class of each cohort were then randomly divided into four replicate

experimental bottles per cohort (~50,000 seed per bottle, totaling 200,000 seed per cohort). The bottle holding system used for this study mirrored our larger commercial production bottle systems at the hatchery, albeit at a smaller scale (Figure 1). During CHD, temperatures were maintained between 15°C and 17°C, with water flow adjusted as needed. Seed health was monitored by assessing shell growth and gut color under a compound microscope.

Figure 1. Photo of the compact bottle holding system used during this project. It consists of 18 bottles, each approximately 44 cm in length from top to bottom, and a 10 L head tank where filtered seawater enters the system.

Before the warm-up period, oyster seed were graded using appropriately sized sieves, and their count was estimated volumetrically. The "middle" size grade from each cohort was selected and randomly split into two groups for the rest of the experiment. Each warming regime was assigned to a separate downweller system (Figure 2) located inside the R&D room at our hatchery, with each system consisting of four cups with mesh bottoms per cohort (12 cups in total), where seed from each replicate bottle corresponding to a distinct cohort were transferred to individual cups. Water flow into each downweller tank was maintained at a rate of of 0.3 L/min and continuously fed the same algal mix mentioned above.

Throughout all phases of this experiment, temperature, salinity, dissolved oxygen, and pH were monitored using a YSI Pro portable multiparameter meter (Yellow Springs, USA).

Figure 2. Photo of the downweller tank setup used in this project. Each tank had a capacity of 200 liters, and each cup measured 5 cm in diameter, fitted with a 500 µm mesh screen at the bottom.

Oxygen consumption rate

Oxygen consumption rate (MO2) in oyster seed was measured using endpoint closed respirometry (Marsh and Manahan, 1999). Oxygen consumption of the seed was recorded at the initial time point before entering the CHD and every other week throughout the holding period for each cohort (5 time points for Cohort 1, 4 for Cohort 2, and 3 for Cohort 3). Additionally, oxygen consumption was measured twice (at the beginning and end) for each warming regime. Prior to measurement, oyster seed were sub-sampled and isolated in separate cups under the current temperature of their experimental group and starved for 18 hours (single cup was assigned to each single replicate bottle). Three to five oysters were then placed into a micro biological oxygen demand (µBOD) vial filled with oxygen-saturated, 0.2 µm-filtered fresh culture water (FCW) corresponding to each cohort's condition. Each cohort replicate cup was measured in triplicate using three µBOD vials, in addition to a blank µBOD vial which contained the same FCW but with no animals to correct for bacterial respiration. µBOD vials, including blanks, were incubated at the respective cohort temperatures for each cohort for approximately 2 to 5 hours in darkness (depending on temperature and seed age) before taking endpoint measurements of oxygen concentration using a PreSens Microx 4 Oxygen Microsensor (Regensburg, Germany). The oxygen microsensor was calibrated using 0% and 100% dissolved oxygen solutions of filtered water.

When each BOD vial was opened, an airtight syringe was used to carefully extract the sample water from each BOD, minimizing potential contamination by atmospheric oxygen. The

sample was then injected into an airtight measurement chamber with a water jacket so that the sample can be maintained at the experimental temperature during the measurement. Three consecutive dissolved oxygen readings were recorded by the oxygen microsensor after a two-minute stabilization period. Both the syringe and chamber were rinsed with 0.2 µm-filtered FCW between samples to prevent cross-contamination. Oxygen consumption rates were then calculated by subtracting microbial oxygen consumption (from blank BOD vials) from oxygen consumption rates of vials with oyster seed, correcting for sensor drift, and factoring in vial volume, incubation time, and total seed biomass. After each respirometry measurement, seed from each vial were counted for standardization purposes, then preserved at -80°C for later total protein analysis. Following protein analysis, oxygen consumption rates were standardized to protein content and expressed as pmol O_2 per gram of total protein.

Biochemical assays

The Rivest lab employed various biochemical assays to assess the physiology of oyster seed during both the CHD and warming periods at multiple time points. For each assay, one sample per bottle or cup replicate per timepoint was analyzed, with each sample processed in technical replicates. Total protein content was measured in the same samples used for each assay, allowing us to normalize the assay results to seed biomass. This standardization ensured that physiological measurements reflect responses to treatment conditions and not variation associated with differences in size among the oyster seed.

Total protein content

To measure the total protein content (TP) of oyster seed, the Rivest lab used the Pierce™ Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, 23225; Smith et al., 1985) following the manufacturer's instructions. Oyster samples (5-10 seed per sample depending on size) were homogenized in 200 µL of Milli-Q ultrapure water (Millipore Sigma) using a metal pestle and then sonicated on ice at 20% amplitude. Dilution of the sample homogenate was performed when needed, and samples were analyzed in technical triplicates. Duplicate standard curves of 9 known concentrations of bovine serum albumin (0-2,000 µg mL^{-1}) were included on each plate. Absorbance was measured at 562 nm and 28°C using a SpectraMax® iD3 (Molecular Devices) microplate reader. Total protein content was calculated as µg total protein per individual.

Data analysis

The Rivest lab used linear mixed-effect models to analyze the effects of CHD and warming regime treatments on various physiological traits, employing the lmer function from the lme4 package in R. In two separate analyses, CHD and time point were included as fixed factors to evaluate the CHD's impact on oyster physiology, while CHD, time points, and warming regime were considered as fixed factors to assess the effect of warming regime. The bottle number was included as a random effect in all analyses to account for variability between bottles. A stepwise model reduction based on likelihood ratios (using the anova function in R) and AIC values was used for model simplification. ANOVA (Type III sum of squares) was conducted to

test for differences in mean values of physiological traits, including MO_2 (metabolic rate), TP (total protein), TG (total glycogen), TAC (total antioxidant capacity), LP (lipid peroxidation), and survival rates. Post-hoc differences between levels were determined using Bonferroni corrections. Model residuals were visually inspected to verify normality and homogeneity of variance, transformation of data was conducted when violation of assumptions occurred. All statistical analyses were performed using the core stat package in R (v4.3.1, R Core Team, 2023), with a significance threshold set at $p < 0.05$. The 0 W time point was not included in the analysis of the CHD effects; similarly, the end of CHD time point was not included in the statistical analysis of the effect of the warming regime. However, both points were included in data visualization as reference of change.

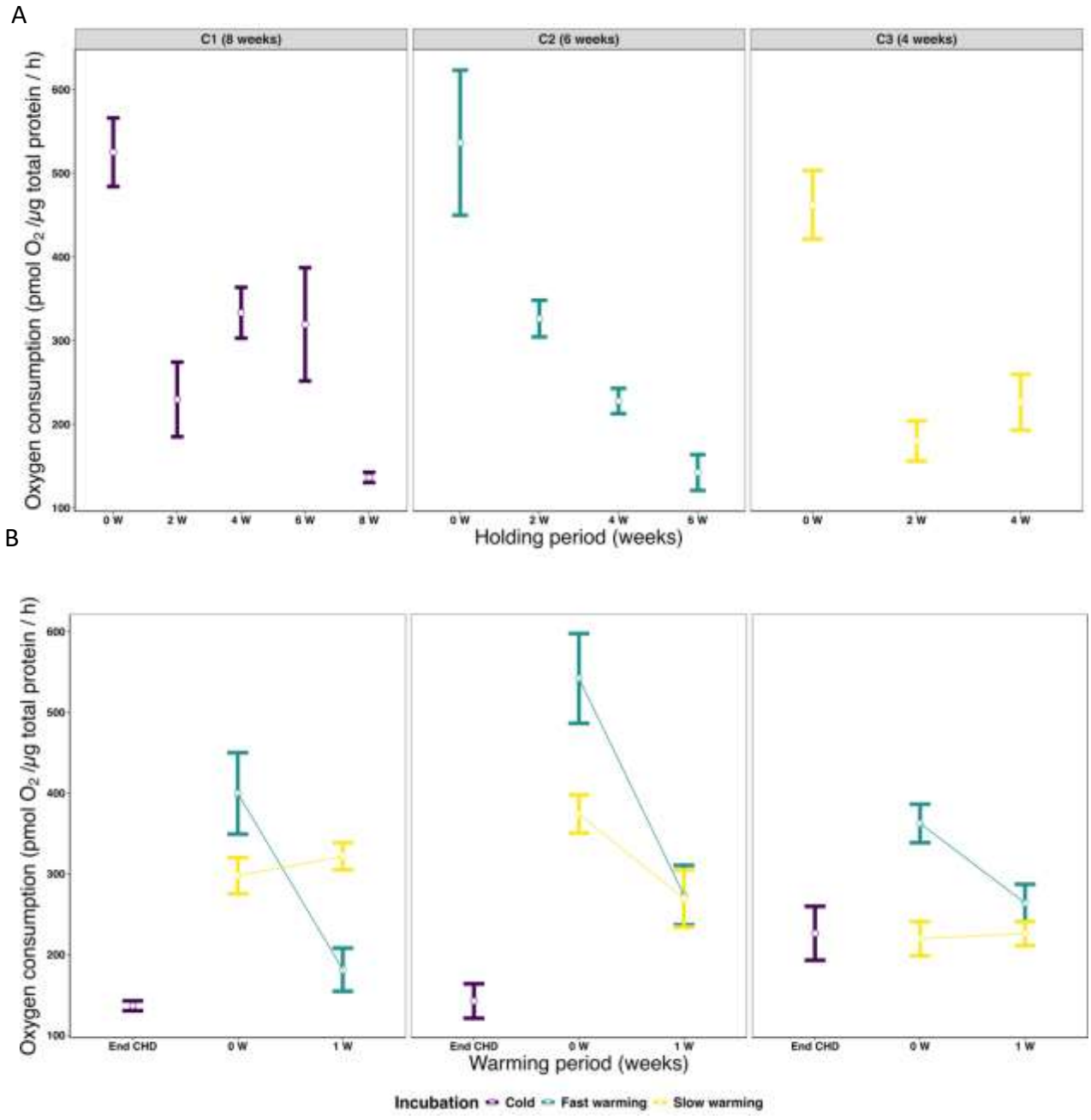
Results

Oxygen consumption rate

Under CHD conditions, there was a significant interaction between CHD duration and timepoint on oxygen consumption rates of seed (Table 1, Fig. 3A). Oxygen consumption rates of seed in the CHD8 and CHD6 groups declined towards the end of the CHD period, whereas oxygen consumption rates of seed in the CHD4 group maintained throughout the CHD period. By the end of the CHD, oxygen consumption rates were similar among all seed groups.

Under warming conditions, interactions between CHD and timepoint as well as timepoint and warming regime explained a significant portion of the variation in oxygen consumption rates of oyster seed (Table 1, Fig 3B). During fast warming, oxygen consumption rates in all CHD groups peaked on the first day and were similar. Following this peak, oxygen consumption rates declined significantly across all groups. In contrast, under slow warming, oxygen consumption rates of seed in the CHD6 group showed a similar peak-and-decline pattern as seen in fast warming, whereas rates of seed in CHD4 and CHD8 groups remained stable throughout the period. By the end of the warming period, oxygen consumption rates for seed in CHD6 and CHD4 were comparable under both fast and slow warming conditions. However, seed in the CHD8 group under fast warming had significantly lower oxygen consumption rates than seed from the same group under slow warming.

Figure 3. Oxygen consumption rates of *Crassostrea virginica* seed. (**A)** Rates of seed exposed to three different CHD durations (8 weeks, 6 weeks, 4 weeks). (**B)** Rates of seed from each CHD group subjected to two different warming regimes (fast vs. slow warming). Data are presented as means ± standard error.



Total protein content

Under cold holding conditions, total protein content (TP) of oyster seed was influenced by a significant interaction between the cold holding duration (CHD) and timepoint (Table 1). For seed in all CHD groups, TP increased over time, reaching its peak at the final time point for CHD 4, and at the last two time points for CHD 6 and CHD 8 groups. By the conclusion of the cold holding period, CHD 4 and CHD 8 groups exhibited similar TP levels, both surpassing that of the CHD 6 group (Fig. 4A).

During the warming period, the interaction between CHD and timepoint had a significant effect on TP content, whereas the warming regime alone did not. Under fast warming conditions, TP content slightly increased over time in both CHD 4 and CHD 6 groups. By the end of the warming period, all CHD groups displayed comparable TP levels across both warming regimes, except for the CHD 8 group, where oysters subjected to fast warming had lower TP content (Table 1; Fig. 4B).

Figure 4. Total protein content (TP) of *Crassostrea virginica* seed. (**A)** TP for seed exposed to three different CHD durations (8 weeks, 6 weeks, 4 weeks). (**B)** TP for seed from each CHD group subjected to two different warming regimes (fast vs. slow warming). Data are presented as means ± standard error.

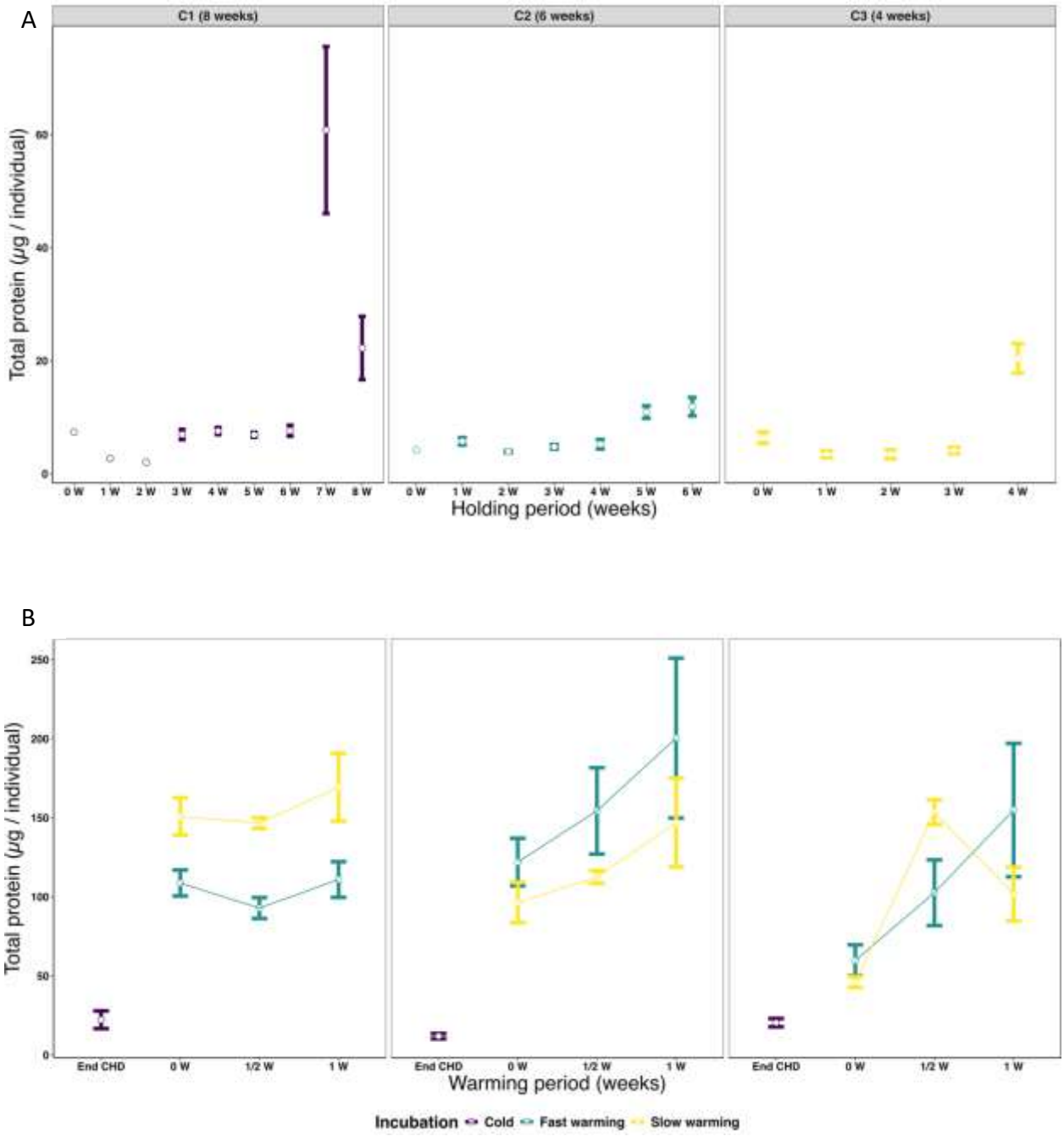


Table 1. Generalized linear mixed effects model results for comparisons of oxygen consumption and total protein content for *Crassostrea virginica* seed. For all behavior response variables, the bottle number was included as a random effect. Test statistic (Chisq), degrees of freedom (DF), and p-values (*p*) are reported. Bold text indicates statistical significance below an alpha threshold of 0.05.

Effect of CHD on oxygen consumption rate of oyster seed			
	Chisq	DF	*p*
CHD	13.650	2	**0.001**
Time	19.709	3	**<0.001**
CHD*Timepoint	21.715	3	**<0.001**
Effect of warming regime on oxygen consumption rate of oyster seed			
CHD	6.686	1	**0.035**
Warming regime	2.945	1	0.086
Time	34.924	3	**<0.001**
CHD*Warming regime	2.539	2	0.280
CHD*Timepoint	6.908	2	**0.032**
CHD*Warming regime*Timepoint	5.598	2	0.608
Effect of CHD on total protein content of oyster seed			
CHD	7.127	2	**0.028**
Time	201.180	7	**<0.001**
CHD*Timepoint	65.161	8	**<0.001**
Effect of warming regime on total protein content of oyster seed			
CHD	14.114	2	0.001
Warming regime	2.540	1	0.111
Timepoint	0.854	2	0.652
CHD*Warming regime	4.903	2	0.086
CHD*Timepoint	9.616	4	**0.047**
CHD*Warming regime*Timepoint	5.330	4	0.255

Conclusion

OSH has observed declines in oyster seed health as a presumed result of our existing cold holding and warming approaches. This project revealed that the CHD did not affect the size and metabolism of oyster seed in predictable ways. If the length of CHD was a driver of seed mortality and poor performance, we would have expected to see correlations between CHD and both oxygen consumption and total protein content. Instead, CHD did not affect final oxygen consumption rate amongst cohorts by the end of cold holding, and total protein content at the end of holding did not correlate with CHD.

While CHD did not significantly affect oxygen consumption rates at the end of cold holding between cohorts, oxygen consumption was consistently lower across all treatments compared to the beginning of CHD. This overall decline could suggest that CHD does influence oxygen consumption, and by extension, seed metabolism over time. It is possible this trend supports the idea that CHD has a cumulative effect on metabolic activity. To more clearly verify this information, higher-resolution measurements, including increased frequency - taken several times a week rather than every two weeks, and more narrow selection of seed of like size, would likely help reduce variability and better capture the gradual changes occurring throughout the CHD period.

The warming regime used as seed emerged from cold holding significantly influenced seed health and growth, and this effect was influenced by the CHD. First, rapid warming caused thermal stress in oyster seed, as shown by the spike in oxygen consumption rate at the onset of fast warming. During this period of stress, any additional sources of stress may compound to cause seed mortality. By the end of the warming period, seed metabolism was resilient and oxygen consumption rates partially recovered, suggesting that at least for the oyster strains used in this study, the rapid warming regime may not have long-term consequences on the energy budgets of oyster seed. Total protein content of oyster seed, a proxy for tissue biomass, increased over the warming period under both warming regimes, specifically for two of the three groups of oysters studied. Tissue growth under both warming regimes suggests that the type of warming regime did not negatively impact seed growth. However, for oyster seed that experienced the longest CHD (8 weeks), total protein content was significantly lower in the fast-warming treatment than in the slow-warming treatment, and tissue mass did not increase over time in seed in either warming regime. These results suggest that some CHD may have negative impacts on oyster seed growth (e.g., stunting) that are only observable after the cold holding period ends. In this case, the longest CHD tested had negative impacts on oyster seed growth during warming (i.e., no growth over time). This study also confirmed that total protein and oxygen consumptions rates are informative indicators of oyster seed health.

To build on these results, we plan to apply for future funding opportunities to conduct more rigorous sampling during cold holding. Our goal is to reduce variability between the current bi-weekly time points and determine whether the observed decrease in oxygen consumption reflects a broader, time-dependent effect on seed metabolism. More frequent measurements—taken several times per week rather than biweekly—will provide higher-resolution data, offering clearer insights into the relationship between CHD, oxygen consumption, and long-term seed metabolism. More frequent sampling may also help identify differences between metabolism of healthy and unhealthy seed within the bottles. The insights gained will guide improvements to our cold holding systems, potentially including the

development of new holding chambers, as well as the optimization of warming protocols to minimize physiological stress and support seed growth.

Literature Cited

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