Utilization of Egg Quality Biomarkers and Probiotics for the Improvement of Eastern Oyster (*Crassostrea virginica*) Larviculture

by

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ABSTRACT

Shellfish aquaculture is a growing industry in the U.S., with the eastern oyster (*Crassostrea virginica*) being one of the most economically important cultured species. However, seed supply is a limitation to the expansion of oyster aquaculture, as hatcheries often experience high mortality rates. Thus, there is a need to understand egg quality characteristics and improve hatchery production methods. This thesis assessed egg quality biomarkers and the effects of probiotics on eastern oyster larviculture. Results indicate that saturated fatty acids, particularly palmitic acid, are elevated in good-quality eggs and may act as biomarkers for egg quality. Furthermore, supplementing *Bacillus* probiotics to rearing systems improved larval survival and significantly reduced relative abundances of *Vibrio*. Overall, this work identified fatty acid profiles of good-quality eggs to inform which diets may be effective for broodstock conditioning, as well as demonstrated the benefits of probiotics for hatchery-reared eastern oyster larvae.

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TABLE OF CONTENTS

ABSTRACT 2

ACKNOWLEDGMENTS 3

LIST OF TABLES 7

LIST OF FIGURES 8

LIST OF ABBREVIATIONS 9

CHAPTER I: INTRODUCTION TO OYSTERS AND THEIR CULTURE 10

1.0 Ecological Role of Oysters 10

1.1 Oyster Aquaculture 12

1.2 Oyster Biology 13

1.3 Oyster Larviculture 15

1.4 Challenges of Oyster Culture 16

1.4.1 Negative Bacterial Interactions 17

1.4.2 Gamete Quality 18

1.5 Research Objectives 19

1.6 References 20

CHAPTER II: EFFECTS OF PROBIOTICS ON EASTERN OYSTER, CRASSOSTREA VIRGINICA, LARVAE GROWN IN SMALL-SCALE SYSTEMS 32

2.1 Introduction 32

2.2 Material and Methods 39

2.2.1 Experimental Design 39

2.2.2 Spawning 39

2.2.3 Larval Rearing ... 40

2.2.4 Probiotic Supplementation ... 41

2.2.5 Sample Collection ... 41

2.2.6 Data analysis ... 42

2.3 Results ... 44

2.3.1 Water Quality ... 44

2.3.2 Trial 1 ... 44

2.3.2.1 Larval Performance ... 44

2.3.2.2 Microbial Alpha Diversity ... 44

2.3.2.3 Microbial Beta Diversity ... 45

2.3.3 Trials 2 and 3 ... 46

2.3.3.1 Larval Performance ... 46

2.3.3.2 Microbial Alpha Diversity ... 46

2.3.3.4 Microbial Beta Diversity ... 46

2.4 Discussion ... 48

2.4.1 ProFin (Trial 1) ... 48

2.4.2 ProFin+ (Trials 2 and 3) ... 49

2.4.3 Effects of Probiotics on Oyster Larviculture ... 50

2.5 References ... 53

CHAPTER III: MATERNAL EFFECTS AND EGG QUALITY BIOMARKERS IN EASTERN OYSTER, CRASSOSTREA VIRGINICA ... 80

3.1 Introduction ... 80

3.2 Materials and Methods ... 83

3.2.1 Experimental Design 83
3.2.2 Egg Morphometrics 84
3.2.3 Fatty Acid Analysis 84
3.2.4 Data Analysis 85
3.3 Results 86
3.4 Discussion 88
3.5 References 94

LIST OF TABLES

Table 2.1 Water Quality Parameters 67

Table 2.2 Alpha Diversity Metrics from Microbiota Analysis 68

Table 2.3 Differential Taxa and their Relative Abundance (Trial 1) 69

Table 2.4 Differential Taxa and their Average Abundance (Trials 2&3) 70

Table 3.1 Pearson Correlation Coefficients 102

Table 3.2 Total Concentrations of Fatty Acids 103

Table 3.3 Total Percentages of Fatty Acids 104

LIST OF FIGURES

Figure 1.0 Life Cycle of the Eastern Oyster, *Crassostrea virginica* 15

Figure 2.1 Experimental Design 71

Figure 2.2 Trial 1 and 2&3 Survival and Size Results 72

Figure 2.3 Number of Eyed Larvae from each treatment in Trial 1 73

Figure 2.4 PCA from Trial 1 Microbiota Analysis 74

Figure 2.5 Heatmap Showing Abundant OTUS Across Larval Ages 75

Figure 2.6 Relative Abundances of Relevant *Bacillus* and *Vibrio* spp 76

Figure 2.7 Comparison of Survival and Larval Size Across All Trials 77

Figure 2.8 PCA from Trials 2 and 3 Microbiota Analysis 78

Figure 2.9 Heatmap Showing Abundant OTUs Across Larval Ages 79

Figure 3.1 Principal Components Analysis for Fatty Acid Variations 105

Figure 3.2 D-Hinge Survival, Fecundity, Fertilization Rate, And Percent Trochophores Present Between Higher- And Lower-Quality Eggs 106

Figure 3.3 Total Concentrations of Saturated Fatty Acids, Monounsaturated Fatty Acids, And Polyunsaturated Fatty Acids Between Higher- And Lower-Quality Eggs in Eastern Oyster. And the Percent Survival of All Females 107

Figure 3.4 Total Concentrations and Proportions of Saturated Fatty Acids 108

Figure 3.5 Total Concentrations and Proportions of Monounsaturated Fatty Acids 109

Figure 3.6 Total Concentrations and Proportions of Polyunsaturated Fatty Acids 110

Figure 3.7 Ratios of Relevant Polyunsaturated Fatty Acids 111

LIST OF ABBREVIATIONS

U.S.	United States
GoM	Gulf of Mexico
FA	Fatty Acid
SFA	Saturated Fatty Acid
MUFA	Monounsaturated Fatty Acid
PUFA	Polyunsaturated Fatty Acid
UFA	Unsaturated Fatty Acid
DPF	Days post fertilization
OTU	Operational Taxonomic Unit

CHAPTER I

Introduction To Oysters and Their Culture

1.0 Ecological Role of Oysters

Oysters are important marine bivalves that play a crucial economic and ecological role. They are considered keystone species and ecosystem engineers due to their ability to impact entire ecological systems (Gutiérrez et al., 2003; Han et al., 2017) through ecosystem services and reef-forming capabilities. Oysters inhabit salty or brackish coastal areas in intertidal zones, estuaries, and sections of shallow oceans. The most abundant oyster in the northern Gulf of Mexico (GoM) is the eastern oyster (*Crassostrea virginica*), also known as the American cupped or Virginia oyster. The eastern oyster is an invertebrate bivalve belonging to the phylum *Mollusca* and family of true oysters, *Ostreidae*. It has a vast distribution, ranging from the Atlantic Coasts of South and Central America to the Caribbean and the GoM, and as far north as the St. Lawrence River Estuary in Canada (Kennedy and Newell, 1996). The range of the eastern oyster encompasses intertidal areas in brackish or marine waters where they cluster together forming reefs that provide habitat for many aquatic and terrestrial species, including fish, crabs, shrimp, and other marine fauna and flora. These reefs also serve as a nursery for commercially valuable fish and invertebrate crustacean species (Grabowski et al., 2012). Oysters offer a range of services that benefit coastal environments. For example, their filtering ability improves water quality by enhancing light penetration, boosting productivity, and sequestering excess nitrogen and phosphorus, which they incorporate into their shell and tissues (Newell and Mann, 2012; Petersen et al., 2016). Additionally, oysters serve as natural barriers, protecting shorelines from storms, tides, and erosion (Dame et al., 1984; Plunket and La Peyre, 2005).

Oyster reefs have experienced an 85% loss across the Atlantic coast, and more locally, a 50-89% loss in the GoM (Beck et al., 2011), due to unsustainable harvesting and other stressors like disease and predation. In the GoM, *Perkinsus marinus* is a serious parasite that infects oysters, while predators like oyster drills, *Stramonita haemastoma*, and blue crabs, *Callinectes sapidus*, also contribute to their decline (Andrews, 1984; Haskin and Ford, 1982). Furthermore, threats like sedimentation from dredging activities can result in reefs becoming buried, and the elevated concentrations of suspended sediment can have physiological impacts that affect the feeding and respiration rates in oysters as they struggle to filter larger particles (Wilber and Clarke, 2010). Pollution further intensifies the effects of stressors through the introduction of excess nutrients, which fuel algal blooms and create dead zones (areas of low to no dissolved oxygen) that hinder the development of reefs (Beck et al., 2011; MacKenzie, et al., 1997).

Added stress can be attributed to environmental changes and biodiversity loss, the effects of which are interconnected. Shifts in environmental conditions alter native species' ranges, triggering cascading effects on species abundances, composition, and function. Introduction of new species leads to the alteration of species interactions in an ecosystem by impacting competition and predation (Neokye et al., 2024; Pörtner et al., 2023). This causes biodiversity loss, which then exacerbates environmental changes, as a loss of wild species and biomass affects the ecosystem's ability to store and sequester carbon (Pörtner et al., 2023).

Carbon dioxide (CO_2) emissions cause rapid ocean acidification (Scanes et al., 2020) that directly affects coastal ecosystems, since CO_2 absorption is greater on the coast than in the open ocean (Mathis et al., 2024), leading to increased stress and disease susceptibility in oysters (Marcogliese, 2008). Spencer et al. (2020) demonstrated that ocean acidification negatively impacts oyster reproduction and offspring viability. Ocean acidification has also been shown to

impact growth rates, increase the rate of abnormalities, and decrease the survival of various species, including mollusk larvae (Byrne et al., 2011; Byrne and Przeslawski, 2013; Espinel-Velasco et al., 2018; Parker et al., 2013; Ross et al., 2011). Furthermore, environmental conditions impact lipid use in larvae, and these compounds are necessary for bivalve larval development (Gibbs et al., 2021; Talmage and Gobler, 2011). Finally, there is also evidence that varying temperatures can affect the timing of algal blooms, resulting in unpredictable food availability for larvae to meet their energetic needs (Edwards and Richardson, 2004; Winder and Sommer, 2012). Considering these challenges, aquaculture can play a supporting role in the maintenance of oyster populations, alongside providing economic benefits for the local community.

1.1 Oyster Aquaculture

When oyster reefs were healthy and productive, wild harvest was able to provide sufficient supplies of oysters for consumption. However, with the decline of reefs, oyster aquaculture has emerged as a sustainable alternative to meet increasing demands. Aquaculture has experienced significant growth in recent years that can be attributed to the increase in per capita consumption of seafood worldwide, global trade intensification, and urbanization (Bush et al., 2019; Little et al., 2016; Naylor et al., 2021). The increasing demand for seafood products is correlated to an increase in the global human population, which reached 8 billion as of late 2022, with a projected growth of 2 billion by the year 2050 (UN DESA, 2024). Aquaculture has seen greater growth than any other livestock sector worldwide (Little et al., 2016). Currently, aquaculture accounts for more than half of the world’s fish supply (FAO, 2024) with an average annual growth rate of more than 8% over the past 30 years (World Bank Group, 2013), outpacing human population growth.

One of the major advantages of aquatic food over other meat sources is that, on average, it is produced more efficiently and with fewer emissions (Norman et al., 2019). On a global scale, the U.S. is the third-largest consumer and importer of seafood, importing $29.7 billion in edible products to meet domestic demands (NMFS, 2024). To address trade deficits and reduce the pressure on wild stock harvesting, federal and state agencies such as the National Oceanic and Atmospheric Administration (NOAA) and the United States Department of Agriculture (USDA) have promoted shellfish aquaculture along all U.S. coastlines (Gray et al., 2022).

Molluscan aquaculture is one of the most valuable sectors in seafood production, representing the second-largest category of farmed seafood and contributing to 21% of global aquaculture production by weight (Botta et al., 2020; FAO, 2024). Within the realm of Molluscan aquaculture, oysters are the primary species produced and have one of the longest histories of cultivation worldwide. In the Americas, the U.S. is the largest producer of oysters (NMFS, 2022), harvesting over 124.6 million pounds in 2023 (USDA, 2024). The two major species of oysters produced are the Pacific oyster (*C. gigas*), a non-native species grown on the West Coast, and the eastern oyster, which is native to the U.S. and is mainly produced on the East Coast and the GoM. Although the largest production of shellfish in the U.S. occurs on the Atlantic and Pacific coasts, the GoM is the leading source of shellfish by volume, harvesting 15.4 million pounds, with oysters being the number one species produced (NMFS, 2022). Alongside economic and food security benefits, oyster aquaculture provides the same ecological services as natural reefs (Domech et al., 2024; Ozbay and L. Smith, 2019).

1.2 Oyster Biology

Oysters are poikilothermic, meaning their temperatures vary with the ambient environment. The optimal temperature for both larvae and adults ranges from 20-30°C (MacInnes and Calabrese, 1979; Sellers and Stanley, 1984). Additionally, salinity and depth optimums of eastern oyster larvae are 10-30 ppt and 0.5-3 m, respectively. Meanwhile, adult oysters can tolerate a wider range of salinities from 0-42.5 ppt, with optimums between 14-28 ppt. In general, depth profiles for adults coincide with the larvae (Barnes et al., 2007; Carriker, 1951), but they can also survive outside this range.

Eastern oysters are protandrous hermaphrodites, often being born male and changing to female later in life. As a result, younger populations may have a higher number of males, with increasing proportions of females in older and larger oysters (Andrews, 1979; Dinamani, 1974; Harding et al., 2013) which may lead to unbalanced sex ratios (Guo et al., 1998). Like most bivalves, oysters are broadcast spawners. Males and females release their gametes (i.e., sperm and eggs) into the water column where external fertilization occurs. Once fertilized, the larvae develop an active velum, which is the main organ used for swimming and feeding, and remain planktonic (free-swimming) for 14-25 days (Kennedy and Newell, 1996). As filter-feeders, their diet is broad, containing most types of organic matter, like detritus, bacteria, and phytoplankton (Gosling, 2004).

The developmental stages of eastern oyster larvae, as outlined by Wallace et al. (2008), begin with the trochophore stage, occurring within ~5-12 h after fertilization. Trochophores are small, swimming larvae with an indistinct shape that feed on minute algae particles. After ~24 h, they transition into veliger larvae, also known as the D-hinge stage, due to their distinct shape. The veliger stage lasts for 2-3 weeks, during which larval dispersal is dependent on tides, currents, salinity, and site of origin. Following this planktonic period, the larvae metamorphose into pediveliger larvae. At this stage, they are ~300 µm long, with a distinct umbo, an "eyespot" – a

pigmented organ with light sensing capabilities (Kim et al., 2021), and a foot that works to cement or "set" the animals on almost any hard substrate as they complete their metamorphosis into sessile juvenile oysters (Dekshenieks et al., 1996). Studies have demonstrated that oyster larvae prefer to set on conspecific shells from already-developed reefs (Potet et al., 2021; Whitman and Reidenbach, 2012). Given that metamorphosis is a stressful event that often leads to high mortality rates (Prado et al., 2010), this highlights the importance of wild stock populations in providing suitable habitat for larval survival.

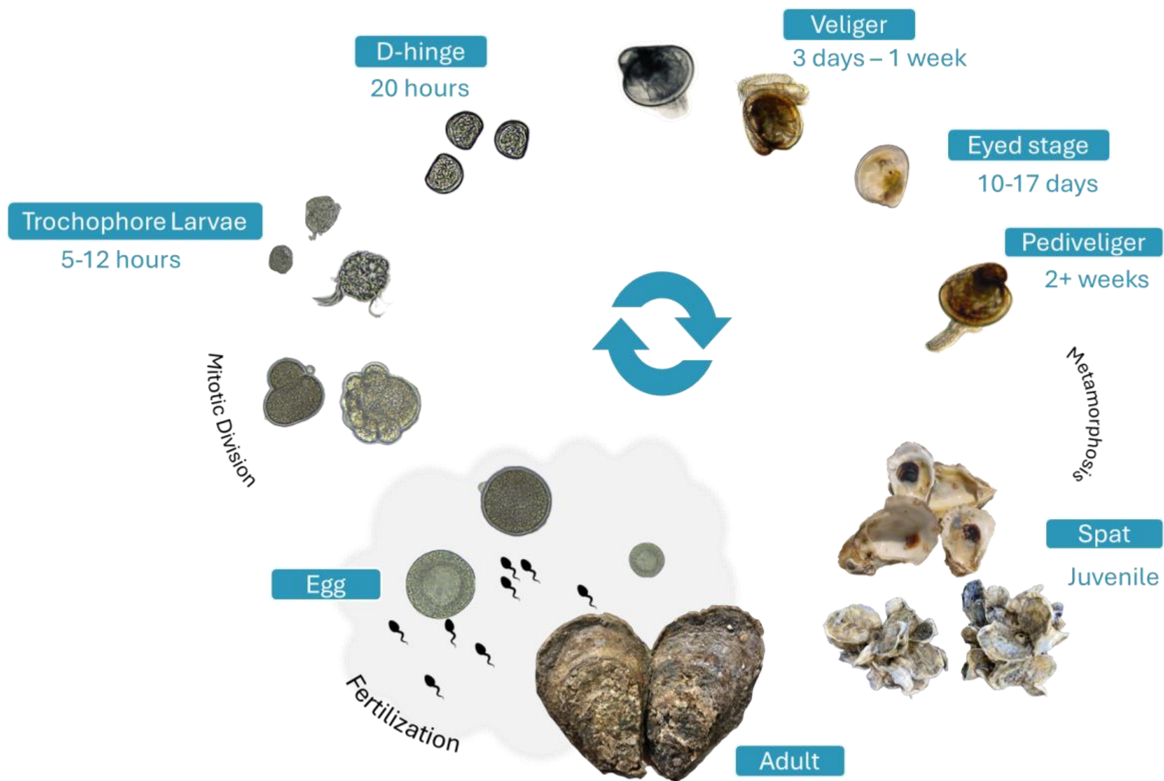

Figure 1.1. Life cycle of the eastern oyster, *Crassostrea virginica*.

1.3 Oyster Larviculture

The process of culturing larval oysters is outlined in Wallace et al. (2008). The spawning season typically begins in spring when water temperatures rise above 25°C and the broodstock is

in spawning condition. Controlled spawning involves placing adult oysters in tanks, where they are stimulated to release gametes by cycling between ambient and warmer water temperatures, along with the addition of 'sperm essence' – stripped sperm that has been inactivated using heat to prevent unwanted fertilization. Eggs are left to hydrate for about 45 min before active sperm is added, and fertilization is checked after 15-20 min. Once enough eggs have been successfully fertilized and identified as zygotes, they are counted and stocked in holding tanks at 10-20 cells mL^{-1}.

Static larval rearing tanks are lightly aerated, and water changes occur every other day until the larvae approach setting, at which point water is changed daily. Between refilling, the tanks are thoroughly cleaned and disinfected, and the animals are restocked at appropriate densities. The animals are fed algae based on larval density and size. This process continues until the desired number of setters is achieved. Once the larvae reach the 'eyed' stage, they are transferred to a nursery and placed in setting tanks containing cultch for settlement. The animals are cared for in the nursery until they grow large enough to be placed into aquaculture bags and transferred to an open water farm, once juveniles reach R6, meaning they are retained on a 6 mm screen.

1.4 Challenges of Oyster Culture

The sustainability of bivalve production relies on a stable and reliable seed supply, as instability in seed availability can significantly hinder industry development (Apines-Amar et al., 2020). Production of seed is dependent on the successful production of oyster larvae. Oyster larviculture faces challenges that affect production stability, including those associated with negative bacterial interactions (Botta et al., 2020) and poor gamete quality (Bertram and Strathmann, 1998; Gallager and Mann, 1986; Kjørsvik et al., 2003; Massapina et al., 1999). These

challenges are described broadly here, and in greater detail in the introductions of *Chapter II* and *Chapter III* of this thesis.

1.4.1 Negative Bacterial Interactions

One of the major bottlenecks affecting successful larval production is negative bacterial interactions that have detrimental effects on the early life history stages of larvae. Larvae are released in early ontogenetic stages, making them particularly sensitive to infections as they have underdeveloped immune systems and are in constant contact with opportunistic bacteria (Prado et al., 2010). Furthermore, introducing food can regularly supply organic matter and bacteria to the rearing environment and affect the microbiological quality of the water (Prado et al., 2010). Thus, the establishment of beneficial bacteria in the larval microbiome could be advantageous to oysters throughout their development, especially as they metamorphose into adults (Arfken et al., 2021).

Bacterial pathogens further affect marine bivalves because of their filter-feeding behavior. As a result, they tend to concentrate diverse bacterial taxa, such as *Vibrio*, *Pseudomonas*, *Acinetobacter*, *Photobacterium*, *Moraxella*, *Aeromonas*, *Micrococcus,* and *Bacillus,* that may be opportunistically pathogenic (Kueh and Chan, 1985; Prado et al., 2010). Pathogenicity depends on the rearing environment, temperature, stocking density, host species, and the bacterial concentration in the rearing environment. Mortalities can reach 100% in static systems, with pathogenic species affecting all life stages of their hosts, from larvae to adults, in various bivalves, like mussels, scallops, clams, oysters, and abalones (Beaz-Hidalgo et al., 2010; Paillard et al., 2004; Zannella et al., 2017).

Pathogenic bacteria are a major cause of large-scale hatchery crashes, with mortalities occurring rapidly and resulting in 100% mortality within a week or more (Comps and Cochennec, 1993; Nicolas et al., 1992; Renault et al., 1994; Gray et al., 2022). One prominent example is

Vibrio tubiashii, a bacterial pathogen that causes vibriosis and is related to large-scale crashes of larvae and juvenile oysters. This pathogen caused losses of up to 59% in 2007 along the Pacific Coast (Elston et al., 2008). In Spain, *V. tubiashii* led to mortality rates of up to 85%. Controlling this pathogen with antibiotics reduced the diversity and presence of *Vibrio* species, but did not prevent larval mortality, which was caused by infection from the egg stage, insinuating broodstock contamination (Dubert et al., 2017). *Vibrio*-related mortalities have also been reported at >80% on the East Coast (Richards et al., 2021). *Vibrio tubiashii* and *V. coralliilyticus* contribute significantly to these losses, as they have been associated with high to absolute mortalities in oyster larvae (Elston et al., 2008; Richards et al., 2021, 2014).

These mortality events have serious economic impacts on hatcheries and nurseries and a rippling effect on the entire industry. Unfortunately, these remain understudied and underexplained (Gray et al., 2022), and they exacerbate ongoing issues like the lack of a steady supply of seed, which is a major concern for farmers (Luckenbach et al., 2008). Thus, improving hatchery techniques and developing preventative measures that control the propagation of harmful bacteria are vital to the success of the industry.

1.4.2 Gamete Quality

Another significant challenge that affects production stability is gamete quality, since the production of high-quality eggs and sperm is essential for effective fertilization and the production of viable offspring in broadcast spawning species. Although environmental factors playing a significant role, egg condition is directly associated with fertilization and the developmental success of the embryo (Bobe and Labbé, 2010). Egg quality, which is the focus of *Chapter III*, can be affected by various factors, such as maternal nutrition, that can impact the health and survival

of larvae (Gallager et al., 1986), emphasizing the importance of understanding maternal effects that could be important for broodstock conditioning.

A common biomarker of egg quality in bivalves is egg morphology, such as shape and size (Myrina et al., 2015). Although a simple, inexpensive way of making predictions, it may not be an accurate technique for determining developmental success (Gallo et al., 2022). Thus, understanding underlying physiological factors may be more relevant, including the involvement of fatty acids (FA), which are one of the main components of lipids in eggs. These lipids serve as an essential energy source for planktonic larvae, influencing survival before exogenous feeding and immune system development (Agh et al., 2019; Bhat et al., 2022; Ferosekhan et al., 2020; Hilbig et al., 2019; Izquierdo et al., 2001; Ljubobratović et al., 2020). Thus, FA profile assessment could potentially be useful for identifying eggs of high quality.

1.5 Research objectives

The purpose of this thesis is to enhance eastern oyster larval production in the GoM by addressing key challenges faced during hatchery operations. *Chapter II* highlights the effects of *Bacillus* probiotics, which were directly supplied to the water of triplicate static systems. Specifically, probiotics' effects on survival, growth, setting rates, and microbiota structure of oyster larvae were quantified, while closely monitoring water quality parameters. *Chapter III* aimed to identify potential biomarkers for egg quality in eastern oysters by exploring egg morphology and fatty acid composition from cohorts experiencing high and low survival.

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CHAPTER II

Effects of Probiotics on Eastern Oyster, *Crassostrea virginica,* Larvae Grown in Small-Scale Systems

Abstract

Seed supply is a limiting factor for the expansion of oyster aquaculture due to incidences of high mortalities in hatcheries. The aim of this study was to evaluate the effects of *Bacillus* probiotics on larval survival, growth, and microbiota in eastern oyster, *Crassostrea virginica*, larviculture. Mixed *Bacillus* probiotics, proven to increase survival and stress resistance in marine fish larvae, were supplemented once daily at 7.0×10^5 CFU mL^{-1} to triplicate static, small-scale oyster larval rearing systems. An additional three systems served as controls that did not receive probiotics. Survival, growth, and number of eyed larvae, along with water quality parameters, were monitored in each of the three 17-day trials. In the first trial, larvae treated with ProFin showed significantly reduced growth and no clear survival benefit. In later trials using ProFin+, probiotic-treated larvae had significantly higher survival. Overall, supplementing certain formulations of *Bacillus* probiotics to rearing systems improved larval survival and significantly reduced the relative abundances of *Vibrio.* This study demonstrated the benefits of probiotics for hatchery-reared eastern oyster larvae.

2.1 Introduction

Shellfish aquaculture plays a key role in sustainable aquaculture production, with eastern oysters, *Crassostrea virginica,* being one of the most important cultured species in the U.S. Oysters are the primary cultured species of shellfish, valued at $199 million (NMFS, 2024) with the Gulf

of Mexico (GoM) and leading production by weight with 13.9 million pounds produced in 2022 (NMFS, 2024). In addition to the economic and food security benefits, oysters grown in aquaculture provide the same ecological services as natural reefs (Domech et al., 2024; Ozbay and L. Smith, 2019), including improvement of water quality and acting as a habitat for numerous coastal vertebrate and invertebrate species.

The recent expansion of this industry increases the demand for a reliable supply of healthy seed; however, larval production and seed supply remain limiting factors due to slow larval growth and/or high mortalities reported by various oyster hatcheries (Walker, 2017). Potential culprits that compromise hatchery conditions and larval survival include poor water quality and negative bacterial interactions (Botta et al., 2020).

Negative bacterial interactions pose significant challenges to successful larval production, with *Vibrio* species being a prominent example, as they contribute to major losses during early life stages (Dubert et al., 2017; Travers et al., 2015; Gray et al., 2022). These bacteria are more abundant during warmer months, from May through October (Green et al., 2019; King et al., 2019), which coincides with the peak of eastern oyster spawning season (Hopkins, 1929). Some *Vibrio* spp. are primary pathogens affecting marine mollusks, and there are several that lead to larval mortalities. *Vibrio tubiashii* has been identified as a pathogen linked to hatchery losses of 59% on the West Coast of the U.S., and greater than 80% mortality rates on the East Coast (Elston et al., 2008; Richards et al., 2021). Another major player is *V. coralliilyticus,* which leads to mortality rates of 56-100% in larval eastern oysters (Richards et al., 2014). Other notable *Vibrio* pathogens affecting bivalve larvae include *V. splendidus*, *V. neptunius*, *V. pectenicida*, and *V. aestuarianus*. Infection characteristics include aggregation around the velum which causes velar damage, cilia detachment, and deterioration of soft tissue (Le Roux, 2002; Galvis et al., 2021; Gómez-León et

al., 2005; Hasegawa et al., 2009; Kesarcodi-Watson et al., 2009; Nicolas et al., 1996). As oyster larvae are sensitive to negative bacterial interactions and *Vibrio* spp. are abundant in coastal environments, these pathogens pose a potential threat to oyster larviculture.

Historically, efforts to mitigate harmful microbial interactions have included the use of antimicrobial compounds (Newaj-Fyzul and Austin, 2015) and water treatment methods, such as chlorine and ultraviolet radiation (UV), aimed at reducing bacterial populations. However, these have limitations and drawbacks that must be considered. Residual chlorine can interfere with normal larval functions like filtering behavior and may interact with organic nitrogen, producing toxic compounds (Prado et al., 2010). UV systems that are effective at treating large volumes of water can be expensive to deploy and could lead to the accidental selection of undesirable organisms. UV radiation may not eliminate all *Vibrio* strains, allowing their proliferation (Prado et al., 2010; Takyi et al., 2024). UV treatment reduces overall bacterial populations, resulting in more resources available to fast-growing, R-strategists such as *Vibrio* spp. Therefore, treatment can lead to blooms of undesirable bacteria that outcompete beneficial bacteria in the system, increasing exposure of larvae to negative bacterial interactions (Vadstein et al., 2018). Although effective at controlling pathogens, prolonged use of antibiotics has resulted in antibiotic-resistant bacteria in aquaculture settings, transfer of resistance traits to bacteria in land animals and human pathogens, and permanent alteration of bacterial communities in sediments and the water column (Verschuere et al., 2000). Thus, developing more sustainable techniques for disease management, focusing on the prevention of negative larval-microbe interactions, is a necessary step for improving hatchery production.

One effective microbial management strategy is the use of beneficial bacteria or probiotics. Probiotics are live microbial supplements that benefit a host by altering host and environmental

microbial communities (microbiota), improving digestion, enhancing the host's defense against disease, and/or improving the quality of the ambient environment (Kesarcodi-Watson et al., 2008; Verschuere et al., 2000). Managing the bacterial communities in oyster larviculture using probiotics is a promising approach to improving oyster hatchery production (Douillet and Langdon, 2020). While the effects of probiotics are widely researched in various marine species, there is a lack of extensive research on their effectiveness in bivalves, especially eastern oysters (Prado et al., 2010; Sumon et al., 2022; Verschuere et al., 2000).

Research indicates that beneficial bacteria can enhance the immune response, growth, and survival of oyster larvae, and maintain healthy microbial environments in rearing tanks (Aguilar-Macías et al., 2010; Gomez-Gil et al., 2000) that are essential for the development of a healthy microbiota in oyster larvae (Arfken et al., 2021). Active probiotics can inhibit pathogenic microorganism activity through competition within the gastrointestinal tract, preventing the adhesion of pathogens to the epithelial cells, inhibiting their growth and colonization by producing antagonistic compounds, and promoting overall health by regulating the immune system (Dahiya and Nigam, 2022; Plaza-Diaz et al., 2019). Gibson et al. (1998) isolated a bacterium that had an inhibitory effect on the growth of various bacterial pathogens, including *Vibrio* spp., which increased survival in Pacific oyster (*Crassostrea gigas*) larvae exposed to *V. tubiashii*. Douillet and Langdon (1994) showed enhanced survival and growth in oysters when using beneficial bacteria as a food supplement. A study by Aguilar-Macías et al. (2010) on the pearl oyster (*Pinctata mazatlanica*) supplemented microalgae laced with several probiotic strains (*Lactobacillus* sp. *NS6.1, Burkholderia cepacian Y021* and *Pseudomonas aeruginosa YC58,* or *Yarrowia lipolytica 020*) for a 21-day trial at 10^6 CFU mL^{-1}. Results showed that the probiotic-treated oysters had significant improvements in shell length, weight, and survival. Studies on *C.*

virginica found that supplementation of probiotics (*Lactobacillus rhamnosus* and *Lactobacillus plantarum*) to larval diets significantly improved the immune function of oyster larvae and resistance to infection. There was a higher expression of immune defense-related genes, such as antimicrobial peptides and other signaling pathways, in larvae that were pre-exposed to probiotics (Modak and Gomez-Chiarri, 2020). Similarly, Hesser et al. (2024) determined that pre-treatment of *C. gigas* larvae with probiotics at a concentration of 3.0×10^5 CFU mL^{-1} significantly improved survival when they were exposed to pathogenic *V. coralliilyticus* by boosting the expression of genes related to inflammatory molecules that support immune response. Probiotics also play a stimulatory role in the settlement and metamorphosis of bivalves (Fitt et al., 1990; Leitz and Wagner, 1993; and Szewzyk et al., 1991). These findings suggest that probiotics could serve as a preventative measure, as well as a cost-effective and sustainable alternative to antibiotics, for disease management in oyster aquaculture (Sumon et al., 2022) when used appropriately.

The filter-feeding behavior of bivalves makes them vulnerable to differences in the composition of bacterial communities present in the water column, so direct supplementation of probiotics to the rearing water has proven effective for oyster aquaculture (Burge et al., 2016; Prado et al., 2010). Ambient environmental conditions and seasonality affect the composition of gut bacteria in aquatic invertebrates (Cahill, 1990; Harris, 1993; Holzapfel et al., 1998; Teng et al., 2022). Additionally, broadcast spawning leads to the release of oyster larvae directly into the environment during early developmental stages when the innate immune system is underdeveloped, making them highly vulnerable to negative bacterial interactions (Prado et al., 2010). Larval microbiota serve as key indicators of oyster health and play a vital role in modulating the impact of probiotics on the host (Le Roux et al., 2016; Sánchez et al., 2017; Stevick et al., 2019; Takyi et al., 2024) as the introduced bacteria must interact with these established bacteria.

This relationship can either facilitate or hinder the establishment of the probiotics through competition for nutrients, production of inhibitory compounds, and/or regulation the immune response in the gut (Dahiya and Nigam, 2022).

Dosage is important to the success of probiotics as overdosing can decrease their effectiveness by reducing the immune response in the nonspecific immune system (Sharifuzzaman and Austin, 2009), while too small a dose may not confer any benefits. Neither 24 h pre-exposure of larvae to probiotics nor daily application of low concentrations of probiotics (10^4 CFU mL^{-1}) in bivalves provided significant or any protection against *Vibrio* sp. (Sohn et al., 2016). A single probiotic strain may not be sufficient to deliver optimal benefits, and its effects may differ based on oyster species or the pathogen encountered. This is supported in Kesarcodi-Watson et al. (2012), where *C. gigas* and flat oyster (*Ostrea edulis*) larvae were supplemented with *Pseudoalteromonas* sp. or *Phaeobacter gallaeciensis* and then challenged with *V. coralliilyticus* or *V. pectenicida*. The probiotics were differently effective in each species, with *C. gigas* showing enhanced survival only against *V. coralliilyticus,* but *O. edulis* having greater survival against both pathogens. On the other hand, the use of multiple strains could have a cooperative effect, enhancing each other's efficacy. Different strains may operate through distinct mechanisms, offering broader protection (McFarland, 2021; Medina et al., 2007; Mileti et al., 2009; Ouwehand et al., 2000). An additional consideration is the need for shelf-stable products, as the probiotic must retain their beneficial properties after storing and processing (Wang et al., 2008). Therefore, the strain(s) used, the targeted pathogen, and the dosage are highly important factors to keep in mind when employing probiotics (Douillet, 1992; Douillet and Langdon, 1994; Gibson et al., 1998; Ribeiro et al., 2014; Yeh et al., 2020).

Various bacterial groups are considered good candidates for aquaculture, with the most common being *Lactobacillus* and *Bacillus* strains. However, *Bacillus* spp. appear more promising because of their ability to form endospores, dormant forms that increase the shelf-life of the probiotic due to their ability to resist high temperatures and pH fluctuations (Amoah et al., 2021; Kuebutornye et al., 2020; Sohn et al., 2016a; Stevick et al., 2019; Tarnecki et al., 2019). *Bacillus* strains produce various enzymes (amylase, trypsin, lipase) and antimicrobial peptides that are beneficial for the enhancement of health, growth, and disease resistance in animal species (Kavitha et al., 2018). Several studies have also shown that members of the *Bacillus* genus can act as protective agents against pathogens, enhancing survival and improving environmental conditions (Arellano-Carbajal and Olmos-Soto, 2002; Boettcher et al., 2005; Hoseinifar et al., 2018). For example, Sohn et al. (2016) showed the efficacy of *Bacillus* spp. at reducing total *Vibrio,* like *V. coralliilyticus,* in the rearing environment of eastern oyster larvae. Karim et al. (2013) demonstrated that *Bacillus* strain probiotics protected larval oysters against *Roseovarius crassostreae* and *V. tubiashii*, increasing the survival by up to 64%. Therefore, *Bacillus* spp. are promising targets for probiotics in oyster larviculture.

Greater larval production can improve the stability and availability of high-quality seed in the GoM to support a growing industry. In addition, probiotics are a promising strategy for improving not only survival, but also growth and metamorphosis in oyster larvae. A mixed *Bacillus* probiotic was previously demonstrated to increase survival and transport stress resistance in marine fish larvae reared in recirculating aquaculture systems (Tarnecki et al., 2019); however, its ability to confer benefits in other species and systems is unknown. Consequently, this study aimed to determine the effects of this and a similar *Bacillus* probiotic on the production of larval eastern oysters reared in small, static systems.

2.2 Materials and Methods

The experimental design for this project was based on the Tarnecki et al. (2024) study on eastern oyster larval rearing using small-scale systems, which proved a cost-effective method allowing for replicated studies.

2.2.1 Experimental Design

Small-scale experimental systems consisted of 6, five-gallon buckets filled with 17 L of filtered (1 µm) seawater (FSW). Triplicate systems were set up for control and treatment groups. The systems were aerated using a 5 mL serological pipette inserted into an airline and attached to an air pump set at low aeration, visualized by water rings dissipating as they neared the bucket edge. A pipette was aligned in the center of each bucket and stabilized by inserting the pipette through a rectangular expanded polystyrene (EPS) foam support placed on top of each bucket (Fig. 2.1). All buckets were placed inside a 28°C water bath to maintain a consistent temperature across systems. FSW was conditioned with 0.1 g of ethylenediaminetetraacetate acid (EDTA), which has been shown to improve shellfish production by increasing larval survival through the removal of heavy metals that impede development, as well as reduce *Vibrio* loads (Hassan et al., 2023; McDougall et al., 2019; Qian et al., 2018). Water quality parameters (temperature, pH, dissolved oxygen (DO), salinity, ammonia, nitrate, nitrite) were monitored at every water change during each trial. Temperature, DO, and salinity were measured using a YSI Multi-parameter Digital Water Quality Meter (Xylem Analytics, Washington, DC, USA). pH was measured with an EcoSense® YSI pocket pH meter, and a YSI 9500 photometer was used to measure ammonia, nitrite, and nitrate.

2.2.2 Spawning

Adult diploid oysters were spawned at the Auburn University Shellfish Lab (AUSL) in Dauphin Island, Alabama. The broodstock was placed into individual flow-through tanks (3 L) during spawning to prevent cross-fertilization. Spawning was induced through thermal manipulation and pheromone exposure (Wallace et al., 2008). The pheromones were obtained by stripping male oysters and heating the sperm in 20-second increments until it became inactive. Inactive sperm were added by bulb pipette to each flow-through tank, aiming at the shell opening of each oyster, until spawning occurred. After spawning, the eggs were collected, strained on a 75 µm screen to remove any debris (i.e., feces and pseudo feces), and hydrated before fertilization, which occurred within 60 minutes. Manual fertilization involved collecting, pooling, and adding sperm (25-50 mL) from all available males into batches of pooled eggs. Once fertilization occurred (denoted by the presence of a polar body or mitotic division), the zygotes were counted by placing a 100 µL sample on a Sedgewick-Rafter counting chamber to determine fertilization rates. Counts were repeated three times to ensure accuracy. The experimental systems were volumetrically stocked at 10 larvae mL^{-1}.

2.2.3 Larval Rearing

Three trials were conducted between June and September of 2024. Trials 1 and 2 lasted 17 days post-fertilization (17 dpf), and Trial 3 lasted 13 dpf due to low growth and survival rates. Water changes were performed every other day until the larvae reached the pediveliger (setting) stage, after which water changes occurred daily. The larvae were collected using a 20 µm screen after 2 dpf and on a 40 µm screen after 4 dpf. During trial 1, a 75 µm screen was used to filter out any slow-growing larvae at 6 dpf, and a 105 µm screen was employed at 10 dpf for the same purpose. When pediveliger larvae appeared, they were removed using a 200 µm screen. All veliger larvae were returned to their respective bucket after counts and measurements. For trials

2 and 3, which exhibited overall slower growth and lower survival, larvae were collected using the 40 µm screen at all water changes starting at 4 dpf. Stocking densities were decreased over time according to standard AUSL practices. Densities were reduced to 5 larvae mL^{-1} at 2 dpf and then to 4 larvae mL^{-1} at 6 dpf. All systems were batch fed twice daily using algae concentrate containing a mixture of five marine microalgae—*Isochrysis, Pavlova, Tetraselmis, Thalassiosira weissflogii*, and *Thalassiosira pseudonana* (Shellfish Diet 1800®, Reed Mariculture, Campbell, CA, USA). Feed amounts were calculated based on tank size, larvae size, and stocking density (Kemp Jr. et al., 2006; Rikard and Walton, 2012). Cloram-X (Reed Mariculture, Campbell, CA, USA) was added at 0.12 g per 1.0 mL of feed to remove ammonia, chlorine, and chloramines.

2.2.4 Probiotic Supplementation

Mixed *Bacillus* probiotics were supplemented once daily at 7.0×10^5 CFU mL^{-1} to triplicate static systems, while an additional three systems served as controls that did not receive any probiotics. Probiotics were dissolved in 60 mL of FSW and distributed evenly at 20 mL per treatment tank using a graduated cylinder. Trial 1 was supplemented with ProFin, which is a 1:1 ratio of two *Bacillus* strains (*B. licheniformis* and *B. amyloliquefaciens*) (Tarnecki et al., 2019). Trials 2 and 3 were supplemented with ProFin+, containing a mixture of three *Bacillus* strains (20% *B. amyloliquefaciens, 40% B. licheniformis, 40% Bacillus sp.* GL1). The probiotics used in this study were obtained from Phibro Biotechnics (Sarasota, FL, USA).

2.2.5 Sample Collection

Survival, size, and number of eyed larvae were measured for each trial at each water change. Survival and number of eyed larvae were counted volumetrically, and size (µm) was measured using a reticle on a compound microscope. An initial sample of larvae was collected after fertilization (0 dpf, trials 1 and 2; 2 dpf, trial 3) for high-throughput sequencing of the

microbiota. These samples were placed in 1.5 mL microcentrifuge tubes filled with RNA*later*™ Stabilization Solution (Invitrogen, Carlsbad, CA) and stored in an ultra-low temperature freezer at -80ºC. Eyed larvae from trial 1 and remaining veligers from trials 2 and 3 were also collected for microbiota analysis. DNA was purified in-house using the ZymoBIOMICS DNA Microprep Kit (Orange, California, USA). All samples stored in RNA*later*™ were vortexed, centrifuged, and washed using sterile DI water to remove the RNA*later*™ before proceeding with the protocol. DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Samples were sent to Azenta Life Sciences (South Plainfield, NJ, USA) and were processed according to their proprietary microbiota analysis pipeline, which includes PCR amplification (V3/V4/V5 variable regions of the 16S rDNA), purification of PCR fragments, library construction and quality control, and Illumina MiSeq sequencing.

2.2.6 Data Analysis

Cumulative survival and size were analyzed using SAS statistical software (V.9.4; SAS Institute Inc., Cary, NC, USA) and RStudio 2023.06.1. The cumulative survival rate was calculated by multiplying the probability of survival at each day by the cumulative survival from the previous interval. The size and survival data for trials 2 and 3 were combined and analyzed together since they received the same probiotic treatment. All 0 dpf data points were removed for analysis since the survival (stocking density) and initial size were equal across all systems. Survival and size were checked for normality and variance using Shapiro-Wilk and Levene's tests, respectively. Survival was square root transformed, and size was log transformed to meet normality. Differences between treatments in survival and size were determined using repeated measures ANOVAs. Both analyses included dpf, treatment, and their interaction as factors. Tukey's post-hoc tests were used to determine significant differences in survival and size over

time. Student's T-tests were used for each dpf to determine significance between control and treatment if a significant interaction was present. Water quality parameters, across all days, and the number of eyed larvae were analyzed using Student's T-tests.

DNA sequences were processed at Azenta Life Biosciences according to their bioinformatics pipeline. In brief, forward and reverse sequences were assembled into contigs. Sequences greater than 200 base pairs (bp) were aligned to the UCHIME 'Gold' database for chimera removal. Resulting sequences were grouped into operational taxonomic units (OTUs) at 97% similarity and taxonomically classified to the genus level. OTUs were binned by taxon for further analysis. Taxonomic abundance tables were center log ratio (CLR) transformed (Gloor et al., 2017) in the phyloseq package in RStudio 2024.12.0. ALDEx2 analyses were used to determine taxa that were significantly different between treatments. Student's T-tests were used to compare abundances of *Bacillus* and *Vibrio* between treatments. CLR tables were loaded into PRIMER 7 software. Principal Components Analysis (PCA) was used to visualize patterns in the microbiota community structure. Resemblance matrices were calculated using Euclidean distance, and permutational analysis of variance (PERMANOVA) was used to analyze differences between treatments (control versus probiotics), developmental stage (initial versus eyed larvae (13+ dpf) for trial 1; 0 dpf, 2 dpf, 10 dpf, and 17 dpf for trials 2 and 3), and their interaction (for trial 1 only). CLUSTER analysis was used to determine similarities among samples and taxa for use in generating heatmaps. Alpha diversity measures (Good's coverage, richness (number of taxa), predicted richness using Chao and ACE predictors, Shannon Evenness Index, and Inverse Simpson's Index) were determined using Mothur v1.48.2 after standardizing sequence reads across samples to the lowest number of reads in an individual sample (4,344). Trial 1 alpha diversity measures were compared between larval ages and treatment groups using

T-tests. For trials 2 and 3, differences between treatment groups were analyzed using t-tests, whereas larval ages were analyzed using a one-way ANOVA.

2.3 Results

2.3.1 Water Quality

No significant differences were observed in salinity (ppt) or ammonia (ng L^{-1}) in any of the trials (Table 2.1). In trial 1, pH levels were significantly higher in the probiotic treatments ($p < 0.001$), and nitrite levels were higher in the controls ($p = 0.038$). In trial 2, the controls' nitrate levels were significantly lower ($p < 0.001$). In trials 2 and 3, there was a significant difference in temperature between treatments ($p = 0.029$ and $p < 0.001$, respectively), with the control systems being slightly (average 0.2°C) cooler. Also in trial 3, dissolved oxygen was significantly higher in control treatments than in probiotics treatments, at an average of 0.07 mg mL^{-1}.

2.3.2 Trial 1

2.3.2.1 Larval Performance

For trial 1, there was only a significant difference ($p < 0.001$) in cumulative survival by day, with no significant differences between control and treatment groups (Fig. 2.2A). Larval size showed a day-by-treatment interaction (Fig. 2.2B; $p = 0.014$) with controls being significantly larger at 12 dpf ($p = 0.011$).

Eyed larvae first appeared in both treatments at 13 dpf. At the conclusion of the trial, there was a significant difference between the control and treatment groups ($p = 0.001$), with a greater number of eyed larvae in the controls (Fig. 2.3). The average number of eyed larvae for the control systems was 4,224 which was 3.2 times greater than the probiotic-treated systems.

2.3.2.2 Microbiota Alpha Diversity

Sequencing captured the majority of richness (number of taxa) in the bacterial communities, as indicated by Good's coverage (an estimate of community diversity) exceeding 98% across all samples (Table 2.2). Overall, microbiota analyses identified 195 taxa in the initial samples and 270 taxa in the microbiota of the eyed larvae. The range observed in individual samples ranged from 84-226 taxa. Shannon Evenness and Inverse Simpson Indices were significantly higher in controls, indicating greater diversity and a more even distribution of taxa. No differences were seen between treatment groups among richness, Chao, or ACE indices. On the other hand, comparison between 0 dpf versus 13+ dpf samples demonstrated significant differences in richness only, with older larvae having more taxa.

2.3.2.3 Microbiota Beta Diversity

PCA showed a clear separation between the microbiota structure of initial (0 dpf) and eyed larvae (13-17 dpf), with minimal overlap (Fig. 2.4). It also indicated a separation between control and probiotic treatments, with these differences driven by the fourth principal component (PC4). PC1 explained 18.6% of the variation and PC4 explained 6.9% of the variation in larval microbiota. The PERMANOVA results support these findings, with significant differences between age groups (0 dpf versus eyed larvae, $p = 0.002$) and treatments (control versus probiotic, $p = 0.002$).

Larval microbiota at 0 dpf was mainly dominated by the presence of *Shewanella, Pseudoalteromonas,* and *Vibrio*, with high relative abundances of *Oceanospirillaceae*, *Acinetobacter, Rhodobacteraceae, Photobacterium*, and unclassified *Bacteria.* In eyed larvae, the probiotic treatments were dominated by *Bacillus* but also had high abundances of *Rhodobacteraceae*. In the control eyed larvae, the most prominent taxa were *Rhodobacteraceae, Nautella*, and unclassified *Bacteria*. (Fig. 2.5).

ALDEx2 community composition analyses between 0 dpf larvae and eyed larvae showed the differential taxa to be *Shewanella*, *Photobacterium*, *Marivita*, and unclassified *Alteromonadales* (Table 2.3), with only *Marivita* being enriched in eyed larvae. Between control and treatment groups, *Bacillus* was more abundant in probiotic treatments. Lastly, as determined by t-tests, there was a significant difference between treatment types within the *Bacillus* and *Vibrio* genera ($p = 0.001$, $p = 0.028$, respectively; Fig. 2.6A), with the control groups having a greater abundance of *Vibrio* and the probiotic group having more *Bacillus*.

2.3.3 Trials 2 and 3

2.3.3.1 Larval Performance

Survival was significantly different over time in trials 2 and 3 (Fig 2.2C; $p < 0.001$). Differences between treatments were also observed ($p = 0.024$), with the probiotics having better survival than the controls. There were no differences in larval size at any time between treatments (Fig 2.2D; $p = 0.873$). No eyed larvae were observed for either of these trials, as trials 2 and 3 experienced overall higher mortality rates in both treatments (Fig. 2.7A-B), and trial 2 had stunted growth (Fig. 2.7C-D), when compared to trial 1.

2.3.3.2 Microbiota Alpha Diversity

Good's coverage was > 99% in all samples (Table 2.2). The richness range for individual samples within these trials was 103-182 taxa. Differences in microbiota diversity were only observed in trial 3, with a significantly higher Chao index at 2 dpf than 12 dpf. On the other hand, the 12 dpf samples had higher Shannon Evenness and Inverse Simpson Indices than the 2 dpf samples. No differences in alpha diversity metrics were observed between control and probiotic-treated larvae.

2.3.3.3 Microbiota Beta Diversity

PCA (Fig. 2.8) showed a clear separation between 0 dpf larvae microbiota and those from 2 dpf in trial 3, and from final larvae from trial 2 (17 dpf) and trial 3 (12 dpf). Separation between control and probiotic-treated larvae can also be observed within each time frame for a given trial. The differences observed between 0 dpf and 2+ dpf larvae were driven by PC1 (29.6%), whereas differences between control and treatment groups were driven by PC4 (7.7%). PERMANOVA identified significant differences among microbiota of each age (0, 2, 12, and 17 dpf; $p = 0.001$; all pairwise comparisons $p < 0.05$) and between treatment groups ($p = 0.013$).

Larvae at 0 dpf were dominated by *Pseudoalteromonas*, *Vibrio*, and *Photobacterium* (Fig. 2.9). At 2 dpf, these communities maintained high abundances of *Vibrio*, with shifts towards increasing *Rhodobacteraceae*, *Glaciecola*, *Flavobacteriaceae,* and *Gammaproteobacteria*. At 12 and 17 dpf, dominant taxa were similar and included *Rhodobacteraceae*, *Hyphomicrobiaceae*, *Saprospiraceae*, *Gammaproteobacteria*, and *Flavobacteriaceae*. However, larvae at 12 dpf had increased abundances of *Kordia* and relatively fewer *Rhodobacteraceae* than those at 17 dpf. ALDEx2 confirmed enrichment of *Flavobacteriaceae*, *Gammaproteobacteria*, and *Alteromonas* in all larvae samples at or after 2 dpf. This analysis found an additional 2 genera enriched at 2 dpf and 7 each at 12 dpf and 17 dpf (Table 2.4).

Within probiotic-treated larvae, *Bacillus* was abundant, but ALDEx2 comparisons did not identify any differential taxa between treatments. When examined by a t-test, *Bacillus* was significantly more abundant in the probiotics group in trials 2 and 3 (Fig. 2.6B; $p = 0.001$, $p = 0.004$, respectively). There was no difference between treatments in *Vibrio* abundance ($p = 0.463$).

2.4 Discussion

This experiment showed that mixed *Bacillus* probiotics are beneficial for eastern oyster larviculture, although the effect is dependent on the strains present in the probiotic. ProFin+ (probiotic 2) improved overall survival in two consecutive trials, while ProFin (probiotic 1) showed no benefits in survival, reduced growth, and produced fewer eyed larvae.

Water quality parameters (temperature, DO, salinity, pH, ammonia, nitrite (NO_2^-), and nitrate (NO_3^-)) in our study remained within acceptable levels (temperature < 30ºC, DO > 5.0 mg L^{-1}, salinity < 35 ppt, pH ~8.0, ammonia < 0.1 ppm, nitrite < 0.2 ppm, nitrate < 16 ppm) (Barnes et al., 2007; Carriker, 1951; Laramore, 2015; MacInnes and Calabrese, 1979; Sellers and Stanley, 1984), with the exception of ammonia in trial 3, which was likely due to high mortalities in this trial. In trial 1, pH levels were lower, and nitrite levels were higher in the controls, which could be attributed to the larger larvae in the control group producing more waste. In trials 2 and 3, there was a significant difference in temperature between treatments, with the control systems being slightly cooler. Although the position of buckets was randomized, proximity to the pump attached to the heating unit and the A/C vent may have influenced temperatures. However, due to these temperature differences being small, it is unlikely they impacted the results of the study.

2.4.1 ProFin (trial 1)

Supplementation of probiotics had no effect on survival or growth, as larvae were significantly smaller than the controls and did not increase in size beyond 12 dpf, indicating a growth disadvantage in the probiotic-treated larvae. Although extensive studies show positive correlations between probiotic supplementation and increased growth rates, studies by Hoseinifar et al. (2018) and Batista et al. (2016) have shown decreased growth rates in fish supplemented with probiotics. Other possibilities are outlined in Knobloch et al. (2022), who mentioned

decreased growth rates could be due to competition for nutrients between the bacteria and the host, changes in gastrointestinal pH affecting nutrient absorption, crowding out of beneficial resident bacteria, or causing negative host immune reactions. Although our data does not allow us to identify the exact mechanisms behind reduced growth rates, the microbiota analysis may provide some insight.

The separations observed in the PCA analysis for the microbiota between treatments suggest that probiotics result in distinct microbial communities with differing functions that could influence larval health and development. The microbiota of control larvae showed higher evenness and diversity than the probiotic-treated larvae. This suggests that probiotic supplementation resulted in the suppression of some bacterial taxa. This shift may indicate dysbiosis, a shift from normal bacterial community structure, potentially impacting host health (Verschuere et al., 2000). This difference in microbiota structure may explain the reduced growth and limited number of eyed larvae in probiotic-treated larvae. The enrichment of *Bacillus* in the probiotic treatments decreased the relative abundance of *Vibrio* and *Rhodobacteraceae*. However, members of the *Rhodobacteraceae* family have been positively correlated with improved host fitness in oysters (Cram et al., 2024; Fallet et al., 2022). In addition, while various *Vibrio* species are known to cause significant mortality during the early life stages (Dubert et al., 2017; Travers et al., 2015; Gray et al., 2022), not all strains are pathogenic as some non-pathogenic *Vibrio* strains have been shown to control disease outbreaks in shellfish (Hossain et al., 2024; Lim et al., 2011). Therefore, these changes in the microbiota may in part explain the lack of benefits demonstrated by ProFin.

2.4.2 ProFin+ (Trials 2 and 3)

Trials with ProFin+ experienced high mortalities and slow growth in comparison to the trial with ProFin. Both the controls and treatments were plagued by these issues. Survival decreased quickly after 2 dpf in trial 2 and after 6 dpf in trial 3. We are not able to conclusively identify the reason(s) behind this. Temperature and salinity were similar across trials. Interestingly, the relative abundance of *Photobacterium* was much higher in trial 2 larvae than in larvae of the same age from trial 1 (0 dpf; 43.8% of total sequences in trial 2 versus 8.6% in trial 1). As this bacterial group decreased with age, it may indicate a negative bacterial interaction where the survivors were those associated with fewer *Photobacterium*. Strains belonging to the genus *Photobacterium*, a close relative of *Vibrio* (Urbanczyk et al., 2011), have been identified as highly virulent as well as a potential cause of disease in marine animals (Pira et al., 2022). Despite the challenges in trials 2 and 3 with larval development, probiotic-treated larvae had significantly higher survival than controls. The strain of *B. amyloliquefaciens* used in ProFin and ProFin+ showed inhibitory activity against a strain of *Photobacterium* isolated from an aquaculture system (Tarnecki, unpublished data). We hypothesize that the benefits of ProFin+ may be, at least in part, due to its ability to influence host-bacteria interactions. This benefit was observed in significant differences in microbiota structure between control and probiotic larvae, including a reduction of *Vibrio* spp.

2.4.3 Effects of Probiotics on Oyster Larviculture

Across all trials, the control groups experienced lower survival rates than the probiotic-treated larvae, although this difference was only significant for ProFin+. These results could be related to the higher relative abundance of *Vibrio* in control larvae. This is expected as *Vibrio* species are most prominent during peak summer months (Green et al., 2019; King et al., 2019), which correlates with the timeframe of our study. This genus is a normal member of the

microbiota of bivalve larvae (Beaz-Hidalgo et al., 2010; Romalde et al., 2014). However, some *Vibrio* spp. are a major contributor to high mortality rates in bivalve larvae (Dubert et al., 2017; Gray et al., 2022; Travers et al., 2015), which could explain the lower survival rates in the controls. This is supported by the literature, where studies have demonstrated reduced mortalities from *Vibrio* spp. in *Crassostrea* larvae through the addition of probiotics (Gibson et al., 1998; Kesarcodi-Watson et al., 2012). Active probiotics can prevent negative pathogenic interactions through competition for space and resources. They block harmful microorganisms from becoming established, inhibit their growth and colonization by producing antagonistic compounds, and support overall health by regulating the immune system of the larvae (Dahiya and Nigam, 2022; Plaza-Diaz et al., 2019). Unpublished data from our group indicate that *B. amyloliquefaciens* and *B. licheniformis* strains used in ProFin and ProFin+ are antagonistic towards some strains of *V. alginolyticus*, *V. coralliilyticus*, and *V. rotiferianus*, which are pathogenic in bivalves, including oysters (Gómez-León et al., 2005; Kesarcodi-Watson et al., 2012; Romalde et al., 2014). Therefore, the mechanism for the benefits of the probiotics used in this study may be through the reduction of harmful bacterial interactions.

Variability in bacterial community composition between trials could be related to the bacterial composition of the incoming seawater, affected by seasonal variations (Arfken et al., 2021; Gray et al., 2022; Takyi et al., 2024), as well as host-microbe interactions affected by environmental fluctuations, differences in host genetics, and the health status of the larvae at each trial period (Gray et al., 2022; King et al., 2019; Unzueta-Martínez et al., 2021). In addition, variability across trials could potentially be explained by shifts in husbandry procedures, introduction of microbes to the rearing environment (splashing, handling), or environmental effects due to small variations in tank position (Stevick et al., 2019; Wegner et al., 2013). We did

not sample water in this study, but previous research indicates oyster larvae maintain a microbiota that is different from the surrounding water (Arfken et al., 2021). Although our trials differed by time of the year, the dominant bacterial taxa were similar among trials. Larval microbiota at 0 dpf were characterized by *Pseudoalteromonas, Vibrio, Shewanella, Photobacterium, Acinetobacter,* and *Rhodobacteraceae*. These bacterial taxa are common members of the microbiota of marine organisms (Fallet et al., 2022; Vignier et al., 2021) and may represent core or shared groups within eastern oyster larvae.

Bacillus have been reported to enhance health and disease resistance, as well as enhance environmental conditions through the improvement of water quality by breaking down organic matter and outcompeting pathogenic species in the rearing environment (Arellano-Carbajal and Olmos-Soto, 2002; Boettcher et al., 2005; Hoseinifar et al., 2018; Kavitha et al., 2018). Supplementation of probiotics directly into the water column appears to have a beneficial effect on larval survival and microbial composition, as was observed in some of our trials. The stunted growth and reduced number of eyed larvae of the probiotic-treated larvae in trial 1 deserve more attention. As highlighted by Yeh et al. (2020), probiotic strains obtained outside of the target host may be unsuccessful at adapting to the host's immune system when compared to those obtained from the actual species, due to incompatibilities in the host environment. However, ProFin+ was beneficial for survival, indicating the third strain may be the driver of these effects. It is possible that the probiotic increased immune function in the host, reallocating energy from growth. There may also be optimization needed for the larval rearing. We did not measure turbidity, but oyster larvae behavior is influenced by light (Kennedy and Newell, 1996; Wheeler et al., 2017), and the addition of probiotics may have reduced light penetration through the water column. This project also only included a single dosage of probiotics. This concentration was based on preliminary

experiments, which demonstrated low survival and stunted growth at twice the dose used in this study, but the optimum may lie between these two concentrations or even below 7.0×10^5 CFU mL^{-1}. Thus, the benefits of these probiotics may be increased with protocol optimizations.

In conclusion, this study showed that mixed *Bacillus* probiotics improve larval survival in eastern oysters. When supplemented at the same concentration, ProFin+ improved larval survival, while ProFin hindered larval growth and development. Both probiotics altered bacterial communities, reducing the relative abundance of *Vibrio* species, which may indicate a mechanism for enhanced survival. However, our latter trials suffered from overall high mortalities and stunted growth, indicating undetermined challenges with these cohorts. These results demonstrate the need to optimize larval culture and probiotic supplementation in small-scale systems. Regardless, the probiotics used in this study show promise for the improvement of eastern oyster larviculture.

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Table 2.1. Average (± standard deviation) water quality parameters for all trials. N/A represents missing data due to backorder of testing kits.

			Parameters						
Month	**Trial**	**Treatment**	**Temperature (°C)**	**DO (mg/L)**	**Salinity (ppt)**	**pH**	**Ammonia (mg/L)**	**Nitrite (mg/L)**	**Nitrate (mg/L)**
July	T1	Control	27.72 ± 0.52	6.86 ± 0.50	22.46 ± 1.93	7.75 ± 0.16	0.01 ± 0.03	0.04 ± 0.02	N/A
		Probiotic	27.66 ± 0.42	6.77 ± 0.39	22.47 ± 1.93	7.84 ± 0.08	0.01 ± 0.03	0.03 ± 0.02	N/A
August	T2	Control	27.63 ± 0.48	6.92 ± 1.10	23.92 ± 2.18	7.93 ± 0.16	0.06 ± 0.19	0.03 ± 0.02	0.63 ± 0.41
		Probiotic	27.74 ± 0.27	6.98 ± 0.28	23.96 ± 2.14	7.96 ± 0.13	0.04 ± 0.14	0.05 ± 0.09	0.98 ± 0.38
September/ October	T3	Control	27.76 ± 0.55	6.88 ± 0.09	23.31 ± 2.52	8.13 ± 0.09	0.21 ± 0.15	0.04 ± 0.02	0.73 ± 0.19
		Probiotic	28.04 ± 0.52	6.81 ± 0.05	23.29 ± 2.54	8.14 ± 0.04	0.20 ± 0.16	0.04 ± 0.02	0.67 ± 0.19
Averages across all trials									
		Control	27.70 ± 0.06	6.89 ± 0.03	23.23 ± 0.73	7.94 ± 0.19	0.09 ± 0.10	0.04 ± 0.00	0.68 ± 0.07
		Probiotic	27.81 ± 0.07	6.85 ± 0.03	23.24 ± 0.73	7.98 ± 0.19	0.08 ± 0.10	0.04 ± 0.01	0.83 ± 0.07
		Combined	27.76 ± 0.15	6.87 ± 0.08	23.24 ± 0.66	7.96 ± 0.16	0.09 ± 0.09	0.04 ± 0.01	0.75 ± 0.16

Table 2.2. Alpha diversity metrics from microbiota analysis. Values represent average ± standard deviation. For trials 1 and 2, metrics were analyzed using T-tests between ages (days post fertilization, dpf) and treatment (control, probiotic). Significant p values are represented by bolded text.

Trial	Factor	Coverage	Taxonomic Richness	Chao	Ace	Inverse Simpson Index	Shannon Evenness Index
Trial 1	0 dpf	0.992 ± 0.0003	125 ± 14	186 ± 6	227 ± 22	6.31 ± 1.03	0.511 ± 0.033
	13+ dpf	0.989 ± 0.004	157 ± 43	207 ± 54	221 ± 69	8.29 ± 5.33	0.556 ± 0.146
	Age p-value	0.085	**0.027**	0.594	0.725	0.1	0.273
	Control	0.989 ± 0.005	159 ± 36	199 ± 50	208 ± 72	10.66 ± 5.42	0.623 ± 0.092
	Probiotic	0.988 ± 0.003	155 ± 49	214 ± 58	233 ± 66	5.92 ± 4.17	0.489 ± 0.161
	Treatment p-value	0.149	0.763	0.471	0.301	**0.012**	**0.008**
Trial 2	0 dpf	0.991 ± 0.002	131 ± 24	214 ± 46	254 ± 53	3.91 ± 1.00	0.414 ± 0.031
	17 dpf	0.994 ± 0.001	124 ± 10	162 ± 19	164 ± 13	3.89 ± 1.74	0.458 ± 0.080
	Age p-value	0.074	0.673	0.133	0.094	0.985	0.275
	Control	0.994 ± 0.001	118 ± 8	154 ± 10	164 ± 8	2.73 ± 0.58	0.408 ± 0.053
	Probiotic	0.994 ± 0.001	130 ± 9	169 ± 26	163 ± 19	5.05 ± 1.78	0.509 ± 0.074
	Treatment p-value	0.943	0.156	0.429	0.891	0.143	0.136
Trial 3	2 dpf	0.992 ± 0.001	154 ± 9	211 ± 29	228 ± 37	7.02 ± 2.12	0.534 ± 0.056
	12 dpf	0.995 ± 0.002	131 ± 31	167 ± 36	174 ± 50	10.16 ± 2.17	0.634 ± 0.035
	Age p-value	**0.012**	0.101	**0.044**	0.074	**0.050**	**0.010**
	Control	0.993 ± 0.002	153 ± 27	202 ± 41	210 ± 52	8.27 ± 2.68	0.578 ± 0.073
	Probiotic	0.994 ± 0.002	132 ± 18	176 ± 35	193 ± 52	8.91 ± 2.78	0.589 ± 0.070
	Treatment p-value	0.355	0.120	0.194	0.536	0.648	0.716

Table 2.3. Differential taxa and their average relative abundance (percent of sequences ± SD) identified by ALDEx2 for trial 1 by development stage (top) and treatment (bottom).

Phylum	Class	Order	Family	Genus	Initial	Eyed Larvae	Adjusted p value
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*	27.8 ± 7.68	0.10 ± 0.28	0.006
Proteobacteria	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Photobacterium*	8.64 ± 1.95	0.03 ± 0.07	0.019
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Marivita*	0.01 ± 0.01	1.69 ± 1.96	0.005
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	unclassified	unclassified	3.42 ± 0.95	0.22 ± 0.53	0.034
Phylum	**Class**	**Order**	**Family**	**Genus**	**Control**	**Probiotic**	**Adjusted p value**
Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*	*Bacillus*	1.96 ± 3.30	42.5 ± 24.6	< 0.001
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*OM60*	unclassified	0.12 ± 0.09	1.00 ± 0.94	0.047

Table 2.4. Differential taxa and their average relative abundance (percent of sequences ± SD) identified by ALDEx2 for trials 2 and 3 by development stage (days post fertilization, dpf). Bold values indicate significant differences as compared to 0 dpf.

Phylum	Class	Order	Family	Genus	0 dpf	2 dpf	Adjusted p value	12 dpf	Adjusted p value	17 dpf	Adjusted p value
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Anaerospora*	0.05 ± 0.01	2.16 ± 1.24	**< 0.001**	0.38 ± 0.18	0.299	1.31 ± 0.62	**< 0.001**
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Alteromonadaceae*	*Glaciecola*	0.04 ± 0.04	8.39 ± 6.52	**< 0.001**	1.71 ± 1.22	**0.045**	1.08 ± 0.80	0.118
Bacteroidetes	*Flavobacteriia*	*Flavobacteriales*	*Flavobacteriaceae*	unclassified	0.12 ± 0.10	2.23 ± 1.15	**0.002**	6.01 ± 2.12	**< 0.001**	3.60 ± 1.90	**< 0.001**
Proteobacteria	*Gammaproteobacteria*	unclassified	unclassified	unclassified	0.25 ± 0.20	2.72 ± 1.58	**0.012**	5.25 ± 2.30	**< 0.001**	4.92 ± 1.35	**< 0.001**
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Alteromonadaceae*	*Alteromonas*	0.04 ± 0.05	1.69 ± 0.35	**0.033**	2.85 ± 3.64	**0.025**	2.18 ± 2.24	**0.038**
Proteobacteria	*Gammaproteobacteria*	*Marinicellales*	*Marinicellaceae*	*Marinicella*	0.00 ± 0.00	0.15 ± 0.11	0.274	0.70 ± 0.57	**0.011**	0.47 ± 0.27	**0.020**
Bacteroidetes	*Saprospirae*	*Saprospirales*	*Saprospiraceae*	unclassified	0.09 ± 0.08	0.52 ± 0.26	0.807	1.64 ± 1.46	**0.036**	2.28 ± 1.51	**0.003**
Proteobacteria	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Photobacterium*	43.8 ± 12.0	2.17 ± 1.25	0.837	0.03 ± 0.04	**0.011**	0.20 ± 0.37	0.053
Proteobacteria	*Alphaproteobacteria*	unclassified	unclassified	unclassified	0.08 ± 0.04	0.36 ± 021	0.897	2.94 ± 1.54	**< 0.001**	0.74 ± 0.31	0.059
Proteobacteria	*Gammaproteobacteria*	*Oceanospirillales*	unclassified	unclassified	0.05 ± 0.02	0.22 ± 0.13	0.999	2.08 ± 1.29	**0.013**	0.12 ± 0.05	1.000
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	unclassified	5.68 ± 1.99	24.9 ± 6.90	0.050	21.6 ± 3.63	0.084	50.6 ± 13.4	**< 0.001**
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Marivita*	0.02 ± 0.03	0.27 ± 0.12	0.166	0.23 ± 0.15	0.207	1.28 ± 0.60	**0.005**
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Hyphomicrobiaceae*	unclassified	0.23 ± 0.16	0.61 ± 1.01	1.000	2.33 ± 1.86	**< 0.001**	6.75 ± 3.94	**0.007**

Figure 2.1. Experimental system design. **(A)** materials used per system, **(B)** a close-up of two experimental systems, **(C)** experimental setup including all six systems placed inside a water bath.

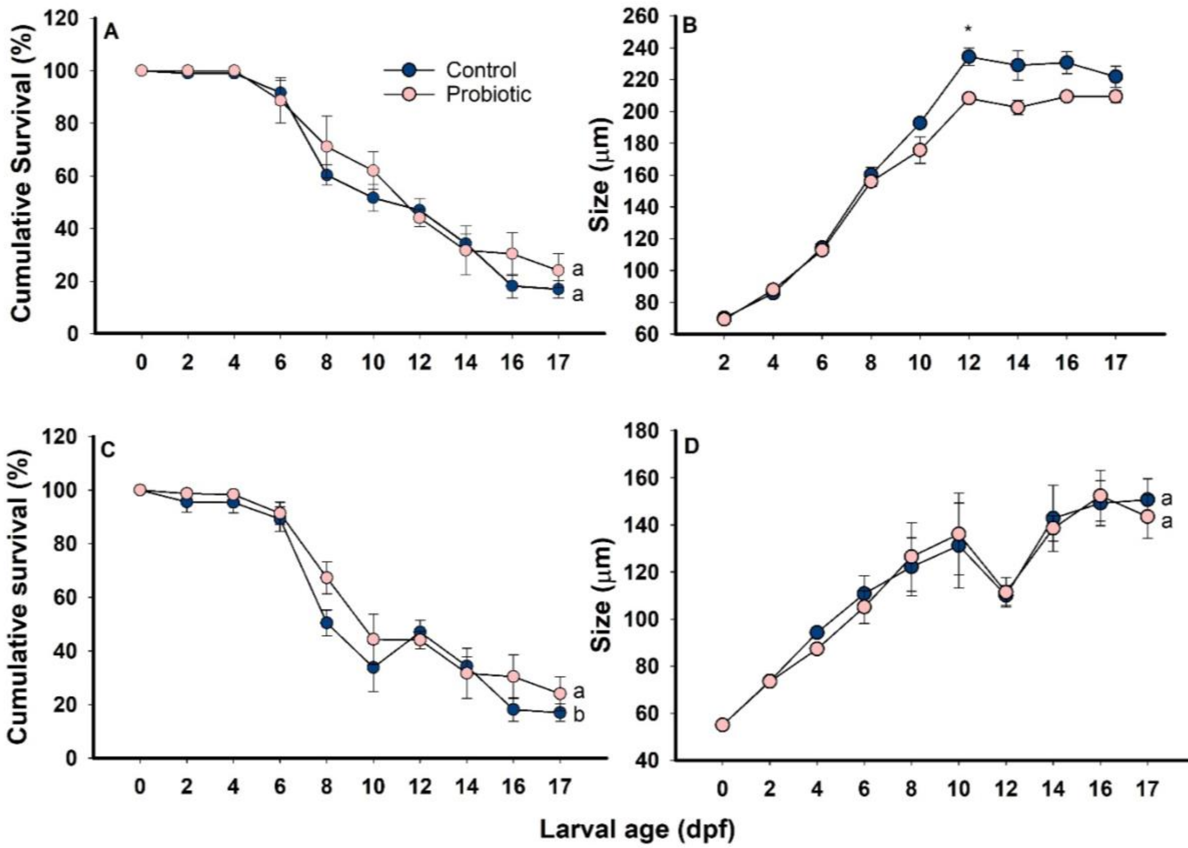

Figure 2.2. Trial 1 and trials 2 and 3 survival and larval size results. Trial 1 **(A)** cumulative survival, **(B)** larval size. Trials 2and 3 **(C)** cumulative survival, **(D)** larval size.

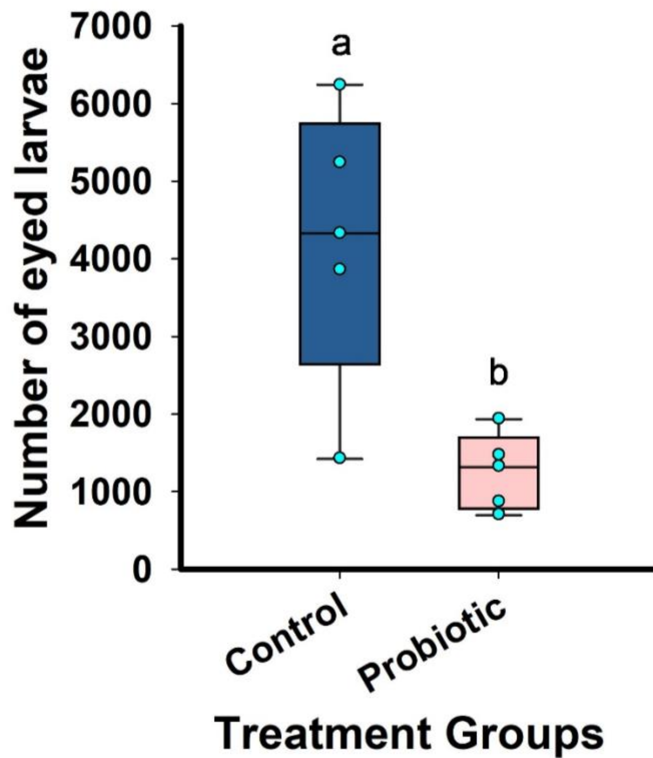

Figure 2.3. Number of eyed larvae from each treatment in trial 1. Distribution of data points (larval age from 13 dpf to 17 dpf) is represented in blue for each plot. 50% of the data fell within the interquartile range for both treatments. 13 and 17 dpf fell in the upper and lower 25% above quartile 3 and below quartile 1, respectively, for the control and probiotic groups. The solid line in the box plot indicates the median. Letters indicate a significant difference between treatments.

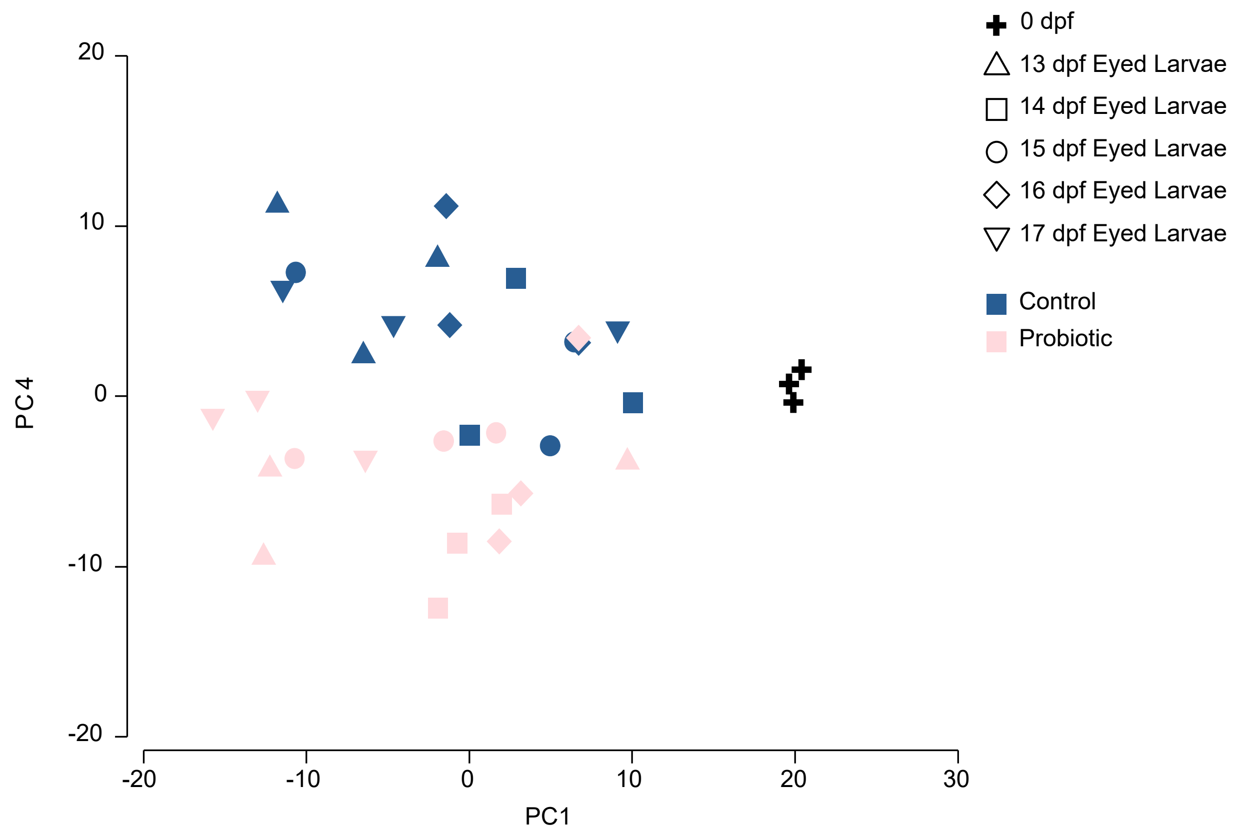

Figure 2.4. PCA from trial 1 microbiota analysis. The development stage is indicated by shapes and treatment is indicated by color.

Figure 2.5. Heatmap showing abundant OTUs across larval age (shapes) and probiotic treatment (colors).

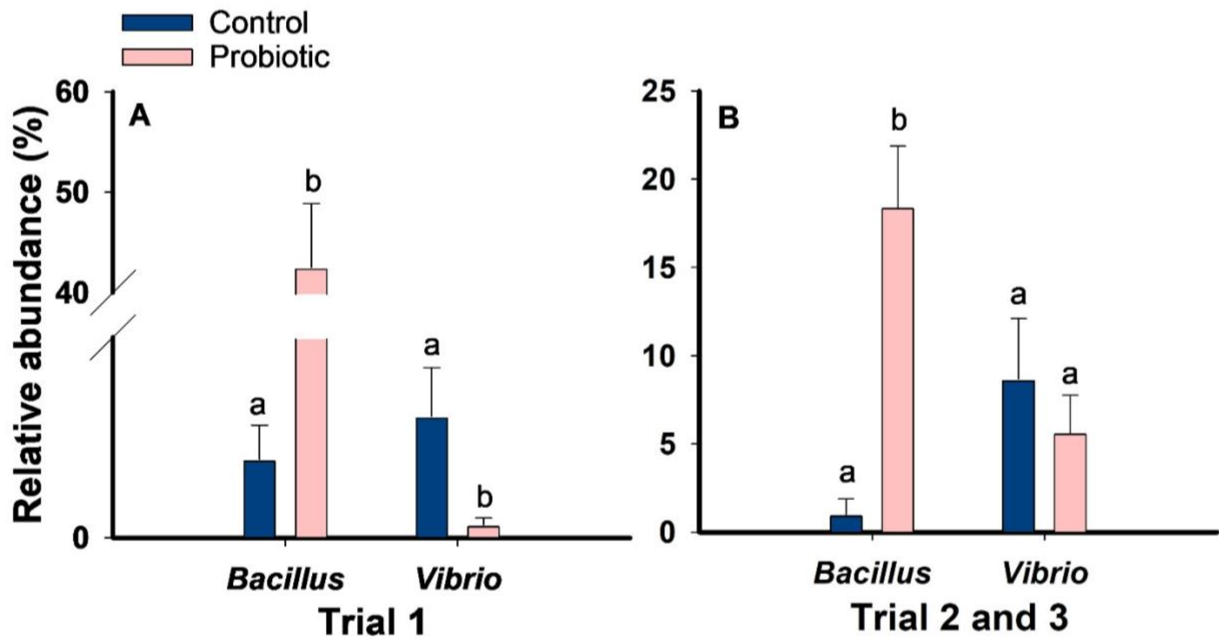

Figure 2.6. Relative abundances of relevant *Bacillus* and *Vibrio* spp. between control and treatment groups across all trials. **(A)** trial 1, **(B)** trials 2 and 3 combined.

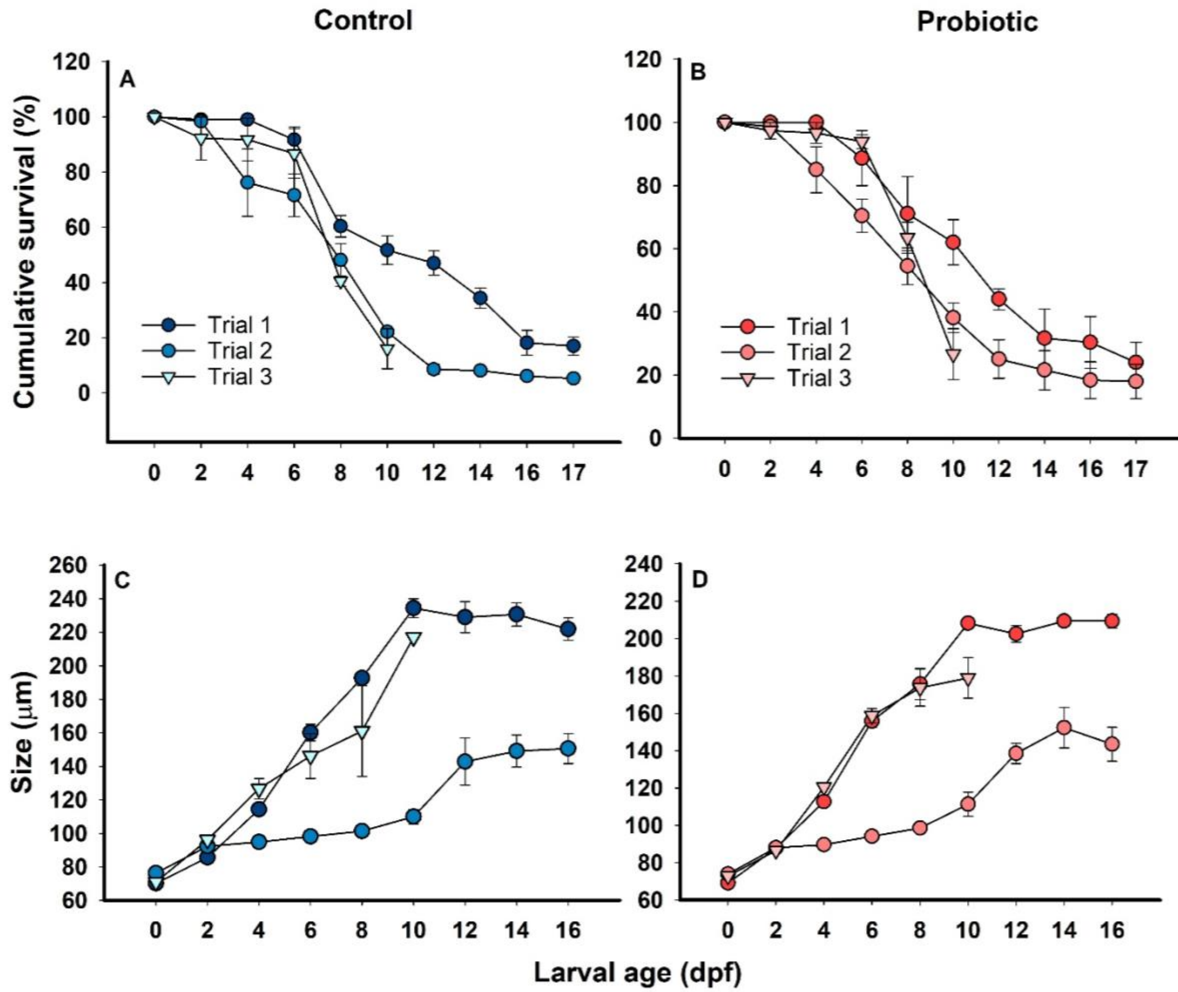

Figure 2.7. Comparison of survival and larval size across trials. **(A)** survival in control treatments; **(B)** survival in probiotic treatments; **(C)** size in control treatments; **(D)** size in probiotic treatments.

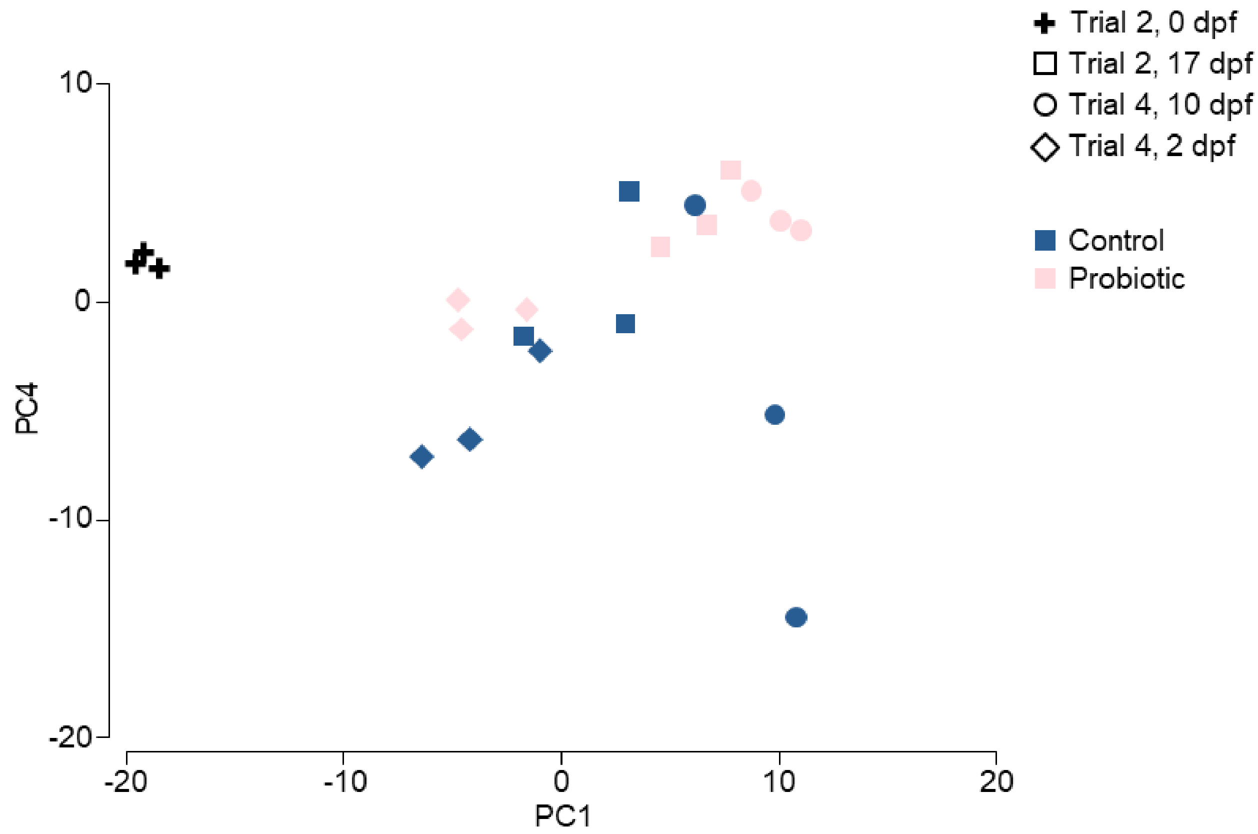

Figure 2.8. PCA from trials 2 and 3 microbiota analysis. The development stage is indicated by shapes and treatment is indicated by color.

Distance

20 15 10 5 0

Escherichia
Photobacterium
Shewanella
Acinetobacter
Rhodobacteraceae
Kordia
Winogradskyella
Pseudoalteromonas
Vibrio
Bacillus
Glaciecola
Alteromonas
Gammaproteobacteria
Flavobacteriaceae
Alphaproteobacteria
Hyphomicrobiaceae
Saprospiraceae
Oleibacter
Bacteria

Distance

20 15 10 5 0

✚ Trial 2, 0 dpf
□ Trial 2, 17 dpf
○ Trial 3, 10 dpf
◇ Trial 3, 2 dpf

Control
Probiotic

Figure 2.9. Heatmap showing abundant OTUs across larval age (shapes) and probiotic treatment (colors).

Chapter III

Maternal Effects and Egg Quality Biomarkers in Eastern Oyster, *Crassostrea virginica*

Abstract

Oyster aquaculture in the Gulf Coast is restricted by a limited supply of seed and high mortalities in hatcheries. This study aimed to identify biomarkers for egg quality in eastern oysters (*Crassostrea virginica*) to improve hatchery efficiency. From July to September 2023, five cohorts of oysters were spawned using natural methods. Eggs were collected from nine females per spawn, with subsets analyzed for morphometrics and fatty acid profiles. Fertilization rates were determined, and survival rates were assessed at 24 hours post-fertilization, with larvae reaching the D-hinge stage. Females were categorized as "higher-quality" (>81% survival) or "lower-quality" (<62% survival) based on survival rates. Results showed no significant differences in egg morphometrics between good and bad eggs, but fatty acid profiles differed, with higher levels of saturated fatty acids in higher-quality spawns. Docosahexaenoic acid (DHA) levels varied by season, being higher in September. These findings identify egg quality biomarkers linked to survival for improving hatchery production.

3.1 Introduction

Shellfish aquaculture plays a crucial role in the advancement of sustainable aquaculture production, with the eastern oyster, *Crassostrea virginica*, standing out as one of the most commercially important marine species in the U.S. As of 2023, molluscan aquaculture has reached a record high, making up 75% of total production (FAO, 2024). At the species level, cupped oyster harvest reached 29,765,311 pounds, representing the second highest aquaculture-

produced species in the U.S., highlighting its importance to the aquaculture industry (NMFS, 2024). However, the expansion of oyster aquaculture faces numerous challenges, including limited availability of high-quality seed that grows quickly and has high survival rates. This demand for high-quality seed necessitates improvement in the consistency of larval production to increase commercial hatchery efficiency.

Production of high-quality gametes is needed for successful fertilization in broadcast-spawning species, like the eastern oyster. Although environmental conditions play a role in larval production, egg health is directly correlated to fertility and offspring viability (Andriantahina et al., 2012; Reading et al., 2018; Utting and Millican, 1997). As such, egg quality is a critical variable affecting reproductive fitness across taxa (Yilmaz et al., 2017). Therefore, identifying underlying physiological mechanisms that influence egg quality is essential to increase seed supply for this growing industry. Although there is extensive research describing egg-quality biomarkers in fish (Ienaga et al., 2021; Jaelen N. Myers et al., 2020; Reading et al., 2018; Ren et al., 2023), few studies have focused on egg quality in oysters.

There is substantial variation in egg quality between females, which is often influenced by body condition (Walne, 1964), age, size, and fecundity (Dupoué et al., 2024; Glandon et al., 2016; Mann et al., 2014). When looking specifically at bivalves, oocyte size and shape are often used as assessment criteria (Myrina et al., 2015). Unfortunately, categorizing egg quality based solely on these morphological analyses can be troublesome, as results are often subjective and do not always reflect hatch results and developmental success (Gallo et al., 2022).

Maternal nutrition plays a significant role in the determination of egg quality. Animals without postzygotic parental care, including broadcast spawners like eastern oyster, are dependent on yolk reserves for nutrition prior to first-feeding (Gallager et al., 1986), where the composition

of yolk is typically determined by maternal diet (Bertram and Strathmann, 1998; Yilmaz et al., 2017) and is crucial for reproductive success, offspring survival, and overall broodstock health (Agh et al., 2019; Bhat et al., 2022; Ferosekhan et al., 2020; Henrotte et al., 2010; Hilbig et al., 2019; Ljubobratović et al., 2020). Broodstock diets that are deficient in fatty acids (FA) could affect fecundity and result in larval abnormalities (Soudant et al., 1996). Polyunsaturated FA (PUFAs), such as omega-3 and omega-6, have been identified as essential lipids for growth, survival, and gonadal development in mollusks. They serve as precursors of eicosanoid molecules related to innate immune responses, such as inflammatory responses (induction and reduction), immune cell activation, and tissue repair (Hendriks et al., 2003; Iverson, 2009; Soudant et al., 1996). Dietary FA can be incorporated into cell membranes as polar lipids or stored in tissues as neutral lipids that are used for energy reserves, membrane integrity, fertilization, and signaling related to oocyte maturation (Hulbert et al., 2014; Martin-Creuzburg et al., 2012; Mathieu-Resuge et al., 2020). Arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n-3), and docosahexaenoic acid (DHA; 22:6n-3) play a key role in cellular membrane functions (i.e., fluidity, stability, immune response) in bivalves with EPA and DHA being most prominent in oysters (Delaporte et al., 2005; Moniruzzaman et al., 2021).

Identification of egg quality biomarkers in the eastern oyster, such as egg morphometrics and FA composition could deepen our understanding of underlying physiological mechanisms regulating egg quality. Early identification of higher- and lower-quality eggs could allow for focused spawning efforts that target high-quality gametes, thereby improving and reducing larval mortalities and increasing production efficiency. Thus, the objective of this study was to identify egg quality biomarkers in the eastern oyster.

3.2 Materials and Methods

3.2.1 Experimental design

Diploid (2N) oysters were produced at the Auburn University Shellfish Lab (AUSL) in Dauphin Island, Alabama (30.248451°N and -88.077982°W). Five spawns (n = 9 females per spawning event) were conducted from July through September 2023. Adult oysters (AUBIE ’22 line, ~95 mm) were placed into individual 3 L flow-through tanks to prevent cross-fertilization. Spawning was stimulated through cyclical water exchanges and the addition of pheromones (sperm-essence). Water exchanges were between ambient water (average 28.3ºC) and warm water (average 34.1ºC) with at least a 4ºC difference. The sperm essence was extracted by stripping the gonad of male oysters and heating the sperm in 20 s increments until the sperm were no longer viable and no motility was observed (Wallace et al., 2008). Within 1 h of spawning, the eggs were collected from females, strained with a 75 µm screen to remove debris, and resuspended in 2.4 L of filtered seawater. The eggs were separated into 4 aliquots. One aliquot was used to collect unfertilized eggs, and the remaining three were fertilized.

Immediately after splitting the eggs into aliquots, the aliquot with unfertilized eggs was concentrated using a 15 µm sieve. From the sieve, a subset of unfertilized eggs (n = 100) was resuspended in filtered seawater for egg morphometrics. The remaining unfertilized eggs (average 173 mg) were split into two aliquots and stored directly at -80°C for FA analyses. The remaining eggs from the three aliquots per female were fertilized with a separate male (n = 3). The same three males were used for each female, resulting in 27 crosses per spawning event. Sperm were added until ~6 to 10 sperm surrounded each egg. If fewer sperm were present per egg, more sperm were added. Fertilization was checked using a microscope every 15 to 20 min. Once >60% of the eggs were fertilized, the eggs were collected on a 15 µm screen to remove

excess sperm. The three aliquots were then recombined, and embryos diluted to ~2.4 L. Fertilization rate and fecundity were determined for each female using volumetric counts on a Sedgewick Rafter Counting Cell, with fertilization denoted by either the presence of a polar body or mitotic division. Fertilization rates were used to calculate the volume needed to stock triplicate 1 L beakers filled to 800 mL at 10 larvae/mL. This resulted in a total of 8,000 larvae per replicate beaker. Each beaker was batch-fed 0.054 mL at 0 days post fertilization (dpf) and 0.243 mL at 1 dpf before counting, using Shellfish Diet 1800® (Reed Mariculture, Campbell, CA, USA). Feed amounts were calculated based on larval size (average ~65 µm), stocking density, and beaker volume (Rikard and Walton, 2012).

Surviving larvae were collected on a 20 µm screen after 24 h and resuspended in 100 mL of filtered seawater. Total survival was determined using volumetric counts under a compound microscope. The number of trochophores was also recorded. A subsample of D-hinge larvae (n = 10) per beaker were measured using a reticle.

3.2.2 Egg morphometrics

Unfertilized eggs in filtered seawater were digitally imaged (n = 100 per female) using a Zeiss Axiolab 5 microscope (Carl Zeiss Microscopy, LLC, New York, USA) equipped with an Axiocam 208 color microscope camera (ZV-AxC208). Egg morphometrics (area, diameter, perimeter, width, height, and circularity) were processed using ImageJ 1.54d (Schindelin et al., 2012).

3.2.3 Fatty acid analysis

Unfertilized egg samples were frozen at -80°C and sent to the Harbor Branch Oceanographic Institute (Fort Pierce, FL, USA) on dry ice for FA analysis. Samples were rinsed with DH_20 and freeze-dried for 18 h to remove all moisture content. Lipid extraction methods

followed Folch et al. (1957) and were modified for aquatic animals by Parrish (1999). The resulting extracts were methylated using methods described by Lepage and Roy (1984) to produce FA methyl esters (FAMEs) for analysis with gas chromatography-mass spectrometry (GC-MS). An internal C19:0 standard was added to all samples for quantification. Analyses of samples were conducted on a Clarus 680/600 T GC-MS (Perkin-Elmer; Waltham, MA, USA) equipped with a 15 m Supelco Omegawax column with a 250 μm diameter. Hydrogen was used as a carrier gas at a flow rate of 1.5 mL/min with GC inlets maintained at a constant temperature of 250ºC. The samples were injected into the column using a 144-vial autosampler at 1.0 μL with a 61.5/1 split ratio. The oven temperature was programmed from 120 to 180°C for 2 min at a rate of 15°C/min and then heated to 250°C at a rate of 10°C/min. Quantification was based on the total ion chromatogram or single ion recording. All FA values were recorded as relative percentages and total concentrations for further analysis.

3.2.4 Data analyses

All data were analyzed using SAS statistical software (V.9.4; SAS Institute Inc., Cary, NC, USA). Using all females, a correlation matrix was generated between the following parameters: D-hinge survival, egg morphometrics, D-hinge larval size, percent of trochophores present, and fecundity. Next, high- and low-quality eggs were determined by choosing the ten females that produced larvae with the highest survival (higher-quality group) and the ten females that produced larvae with the lowest survival (lower-quality group). This resulted in higher-quality eggs having survival >81% and lower-quality eggs having survival <65% (Fig. 1AB). A series of students' T-tests were then used to compare survival, egg morphometrics, D-hinge larval size, percentage of trochophores present, and fecundity between the higher- and lower-quality eggs. Relative percentages of FAs were then analyzed using PRIMER 7 software, and a

PCA was used to visualize FA structure between the higher- and lower-quality eggs. A one-way PERMANOVA was then used to determine if this structure was significantly different. Lastly, students' T-tests were used to compare individual FAs between the higher- vs. lower-quality egg groups. Values represent mean ± SEM (standard error of the mean). Alpha was set at 0.05.

3.3 Results

No significant correlations were detected between the egg morphometrics (i.e., circularity, area, perimeter, width, and height) and D-hinge survival, fecundity, and percent of trochophores present (Table 3.1). Meanwhile, significant positive correlations were detected between D-hinge size and most of the egg morphometric traits. Specifically, egg area, perimeter, width, and height were all positively correlated ($P \leq 0.008$; $r \geq 0.41$), while egg circularity was negatively correlated to the other egg morphometric traits ($P \leq 0.022$; $r \geq -0.36$).

D-hinge survival differed between the egg quality treatments ($P = 0.002$), with the higher-quality eggs having 95.5 ± 1.4% survival and lower-quality eggs having 47.6 ± 4.4% survival (Fig. 3.1A). Mean egg circularity, egg area, egg perimeter, egg width, egg height, fecundity (Fig. 3.1B), fertilization rate (Fig. 3.1C), and percent of trochophores present (Fig 3.1D) were 0.89 ± 0.004, 1780 ± 50 μm^2, 158.4 ± 2.4 µm, 48.0 ± 0.7 µm, 48.0 ± 0.7 µm, 43,984,762 ± 10,424,621 eggs, 47.6 ± 0.4%, and 5.8 ± 2.7%, respectively. For all these hatchery traits, no significant differences were detected between the higher- and lower-quality egg groups ($P \leq 0.941$).

Complete divergence in the concentration (Fig. 3.2A; PERMANOVA; $P = 0.006$) and percentage (Fig. 3.2B; PERMANOVA; $P = 0.002$) of FA were detected between the higher- and lower-quality egg groups. The first two principal components drove variations in overall FA

profiles. Differences between the higher- and lower-quality eggs were mainly driven by PC1, explaining 85.3% and 87.4% of the variation, while PC2 explained 12.0% and 6.4% of the variation in concentrations and percentages, respectively.

In general, when looking at the percent of FA, we showed an equal distribution of MUFAs, PUFAs, and SFAs across egg quality groups. The FA C16:0, EPA, and ARA were prominent, in addition to C18:1. Furthermore, FA concentrations of eggs also showed that palmitic acid (C16:0) and, to a lesser extent, stearic acid (C18:0) were dominant SFAs.

The total concentration (µg/mg) of saturated FA (SFA) was significantly different between the higher- and lower-quality eggs ($P = 0.004$; Fig. 3.3). Regarding specific SFA, lauric acid (C12:0; $P < 0.001$) was at higher concentrations in the higher-quality eggs, while tridecanoic acid (C13:0; $P < 0.001$) was at a lower concentration (Fig. 3.4A). Myristic acid (C14:0; $P = 0.005$; Fig. 3.4B), palmitic acid (C16:0; $P = 0.032$; Fig. 3.4C), and stearic acid (C18:0; $P = 0.040$; Fig. 3.4C) were also at higher concentrations in the higher-quality eggs, with C16:0 and C18:0 being the most prominent. The higher-quality eggs also had a higher percentage of several SFA, including C12:0 ($P = 0.004$; Fig. 3.4D), C13:0 ($P = 0.011$; Fig. 3.4D), C14:0 ($P = > 0.001$; Fig. 3.4E), C16:0 ($P = 0.002$; Fig. 3.4E), behenic acid (C22:0; $P = 0.034$; Fig. 3.4F), and tricosanoic acid (C23:0; $P = 0.034$; Fig. 3.4F).

The total concentration of monounsaturated FA (MUFA) did not differ between the egg quality groups (Fig. 3.3). On the contrary, when looking at specific MUFA, the higher-quality eggs had a lower concentration of pentadecanoic acid (C15:1; $P = 0.018$; Fig. 3.5A) and a lower percentage of eicosenoic acid (C20:1; $P = 0.004$), myristoleic acid (C14:1; $P = 0.009$), nervonic acid (C24:1; $P = 0.028$), and oleic acid (C18:1; $P = 0.002$) (Fig. 3.5B).

The total concentration of polyunsaturated FA (PUFAs) also did not differ between the egg quality groups (Fig. 3.3). Meanwhile, concentrations of two specific PUFAs were different, where dihomogamma linoleic acid omega-6 (C20:3n6, $P = 0.004$; Fig. 3.6A) and eicosadienoic acid (C20:2n6; $P = 0.045$; Fig. 3.6B) were at lower concentrations in the higher-quality eggs. The percentage of seven individual PUFA, including linoleic acid (C18:2n6; $P = 0.029$); alpha-linoleic acid (C18.2n6; $P = 0.024$), arachidonic acid (C20:4n6; $P = 0.003$), docosadienoic acid (C22:2n6; $P = 0.013$), eicosapentaenoic acid (C20:5, $P = 0.012$), dihomogamma linoleic acid (C20:3n6; $P < 0.001$), and eicosadienoic acid (C20:2n6; $P = 0.036$) were also notably lower in the higher-quality eggs (Fig. 3.6CD). The ratio between EPA:ARA was elevated in higher-quality eggs ($P = 0.029$), while the DHA:EPA ratio did not differ (Fig. 3.7).

3.4 Discussion

This study showed that FA may act as biomarkers of egg quality in eastern oysters. Specifically, we saw that (1) there was a clear distinction in FA profiles among individual females with higher- and lower-quality eggs; (2) SFA concentrations were elevated in the higher-quality eggs when looking at concentrations and percentages, with C16:0 having a dominant presence, and (3) the higher-quality eggs had an elevated EPA:ARA ratio.

Results of this study indicate that egg morphometrics and fecundity do not serve as reliable predictors of egg quality in eastern oysters, which coincides with reports by Helm et al. (2004) in the hatchery culture of bivalves. Regarding morphometrics, these findings align with the literature, as there appears to be no clear predictor of egg-quality based on size and shape across aquatic taxa (Migaud et al., 2013). Even so, morphometrics can still be used as a general predictive tool for performance, especially in very apparent cases, as has been highlighted by the

literature (Kjørsvik et al., 2003; Desai et al., 2000; Ezra et al., 1992; Migaud et al., 2013). Furthermore, significant differences were observed in survival and development in our study, where the higher-quality eggs had higher overall survival and faster development into D-hinge larvae after 24 h post-fertilization, suggesting other indicators may play a role in egg quality.

Making predictions based on egg morphometric traits is complex as these traits are often confounded by egg/embryonic developmental sampling stage (Segers et al., 2012), environmental incubation conditions (Nash et al., 2019), handling procedures (Mileva et al., 2011), maternal age (Mann et al., 2014), paternity (Lalancette et al., 2008), and the actual level of hatching and survival that was assessed (Migaud et al., 2013). Nevertheless, these egg morphometric relationships do exist for specific species, like European eel (Sørensen et al., 2016). Additionally, reproductive traits like egg buoyancy (Ramos-Júdez et al., 2025), cleavage abnormalities (Avery et al., 2009), egg weight (Craik and Harvey, 1984), ovarian fluid (Myers et al., 2020), and egg biochemistry (Utting and Millican, 1997) also appear to provide some predictive power for deciphering egg quality in certain species.

Fecundity also has a role in determining egg quality (Migaud et al., 2013). In our study, the trend towards higher fecundity in the low-quality eggs could be attributed to trade-offs in energy allocation. As nutrient input is limited, the amount that goes into each egg decreases when fecundity is high, whereas more nutrients can be allocated to each egg with lower fecundity (Moran, 2004). As such, like egg morphology, fecundity did not serve as a predictor of egg quality in our study.

When looking at total concentrations of FA, there was a clear distinction in profiles between the higher- and lower-quality eggs, although some overlap did occur in our PCA analyses. This variation, however, was not due to seasonality, as females from the different trials

were intermixed between our higher- and lower-quality groups. The general trend in individual FA profiles showed that the higher-quality eggs had higher concentrations of SFAs, whereas the abundance of MUFAs and PUFAs were not significantly different. However, significantly different EPA:ARA ratios were observed. These PUFAs are critical structural components of cell membranes and precursors to important signaling and regulating molecules (eicosanoids). These molecules play a key role in regulating immune response, inflammation, and various physiological processes (Hendriks et al., 2003; Iverson, 2009; Soudant et al., 1996). Our results show that this may be the case, with this specific balance of EPA:ARA having a potential anti-inflammatory effect, since elevated concentrations of ARA may be detrimental as they can lead to chronic inflammation (Bao et al., 2023), while EPA acts as an anti-inflammatory molecule, improving egg quality (Calder, 2015).

The main lipid reserves in bivalves include MUFAs, PUFAs, and SFAs, accounting for ~76% of total lipids (Myrina et al., 2015). These are important for sexual maturation and embryogenesis, as well as for survival and metamorphosis, because SFAs are an energy source and serve as temporary reservoirs for PUFAs within triglycerides to serve as protective agents from oxidation since FAs are more stable (Myrina et al., 2015). In addition, both SFAs and MUFAs are known for their resistance to oxidation and oxidative stress, which could be related to increased egg viability as they prevent cellular or membrane degradation (Hulbert et al., 2014; Pamplona and Costantini, 2011). This was observed in a study by Istomina et al. (2023), where a relationship was detected between FA mitochondrial stability in bivalves and lifespan. The study found correlations between lifespan and the presence of SFAs and MUFAs, as these are more resistant to peroxidation. This resistance can mitigate oxidative stress, which is negatively correlated with longer lifespans in various species (Munro and Blier, 2012).

Furthermore, higher concentrations of SFAs in the higher-quality egg group could influence egg membrane fluidity as SFAs, which lack double bonds, can pack tightly together, increasing rigidity (Hulbert et al., 2014; Weijers, 2016). Although not relevant in our study, this trend could also be related to seasonal changes, as has been shown for various taxa. SFAs generally increase in warmer seasons, suggesting that lower SFAs may destabilize egg membranes, affecting fluidity (Bowden et al., 1996; Hulbert et al., 2014; Sajjadi and Mooraki, 2016). This could be beneficial in the Gulf Coast (Southern United States), where warmer temperatures are prevalent, preventing the membrane from becoming too fluid and affecting its function, especially in bivalves that are sensitive to temperature fluctuations. This is confirmed by Hulbert et al. (2007), among others, who highlighted that higher concentrations of PUFAs increase membrane fluidity due to their lower melting points than SFAs.

FA profiles showed an equal distribution of MUFA, PUFA, and SFA across our egg quality groups, with C16:0, the EPA:ARA ratio, and C18:1 being most prominent. C16:0 was the most dominant FA, followed by lower levels of C18:1. This coincides with other studies on marine species, where C16:0 was similarly abundant (Bowden et al., 1996; Hulbert et al., 2014; Sajjadi and Mooraki, 2016). The prominence of C16:0 and C18:0 could be due to their relation in de novo lipogenesis (DNL), where acetyl-coenzyme A (acetyl-CoA), a central metabolite and signaling molecule essential for energy production, is polymerized to form FA, with C16:0 being the primary FA produced during lipid synthesis, followed by C18:0 after elongation (Wu et al., 2011). This is important for egg quality as SFAs can aid in maintaining membrane stability, support cellular integrity, protect against oxidative stress, and serve as an energy source for normal development.

In addition, these variations could suggest seasonal differences, as previously demonstrated by the literature, where an increase in PUFAs and a decrease in SFAs during winter, and vice versa, were observed in fish species. For example, Bowden et al. (1996) saw an increase in SFA content and a decrease in PUFAs in the summer season, with a statistically significant reduction of 25% in C16:0, with reductions in ambient temperature. Similarly, Sajjadi and Mooraki (2016) also noted seasonal variations in FA compositions, suggesting that temperature can significantly influence lipid composition in bivalves. They also found that SFA were more prevalent than unsaturated FA, with C16:0 having a notable presence. Also, SFA reached levels of up to 90% in the summer, while unsaturated FA increased by 22% in winter, and SFA reached a minimum of nearly 10% in colder temperatures. Additionally, Sajjadi and Mooraki (2016) highlighted invertebrates' ability to adjust enzyme activity and membrane fluidity to maintain physiological functions in response to temperature changes. This could explain the changes in FA composition observed in these species and in our study. Bowden et al. (1996) also proposed another explanation for these variations by emphasizing that caution is needed when interpreting these changes, since variations in batches or differences in feeding behavior between seasons may also contribute to these fluctuations.

Moreover, FA fluctuations in mollusks can be affected by exogenous factors, like environmental conditions related to food availability and temperature changes, as well as endogenous factors, like physiological and metabolic activities (Elkin et al., 2011). FA profiles in females also vary depending on maturity, phylogeny, salinity, and temperature (Ricardo et al., 2021). Although these factors play a role in FA compositions, our study showed high variability among higher- and lower-quality eggs across the spawning season.

To further understand the underlying mechanisms involved in egg quality, future studies should investigate gene expression to identify key genes involved in specific processes (i.e., gametogenesis, maturation, etc.) as suggested by Baptista et al. (2014) and Corporeau et al. (2012). In addition, amino acids and protein composition of oocytes should be analyzed as studies have shown a link between differentially accumulated proteins and egg quality (Baptista et al., 2014; Corporeau et al., 2012). Garcıa-Esquivel et al. (2001) also pointed out that post-larval survival was directly linked to protein utilization during metamorphosis, indicating that proteins were a limiting factor for survival, with total lipids present in pre-metamorphic larvae exerting little effect. Hence, the inclusion of protein composition could provide deeper insights into egg quality and the underlying biological processes related to this.

In conclusion, the results of our study showed that while egg morphometrics and fecundity alone were not reliable indicators of egg quality in eastern oysters, FA profiles, specifically high concentrations of SFAs in the higher-quality eggs, show promise as biomarkers for egg quality. FA play a role in maintaining membrane stability and rigidity as well as provide protection from oxidative stress, which could contribute to better egg viability and larval development. The significant differences in survival and faster development in the higher-quality eggs place further importance on focusing on other molecular mechanisms, as well as FA composition, as predictors of reproductive success. These findings ultimately highlight the potential for the optimization of broodstock diets with specific FA profiles to enhance egg quality. As various studies have shown, FA are essential for reproductive success, offspring survival, and broodstock health. In addition, they serve as the main source of energy needed for normal cell functions (Agh et al., 2019; Bhat et al., 2022; Ferosekhan et al., 2020; Hilbig et al.,

2019; Ljubobratovic et al., 2020). Overall, FA play a significant role in improving larval production efficiency and minimizing larval mortalities in a hatchery setting.

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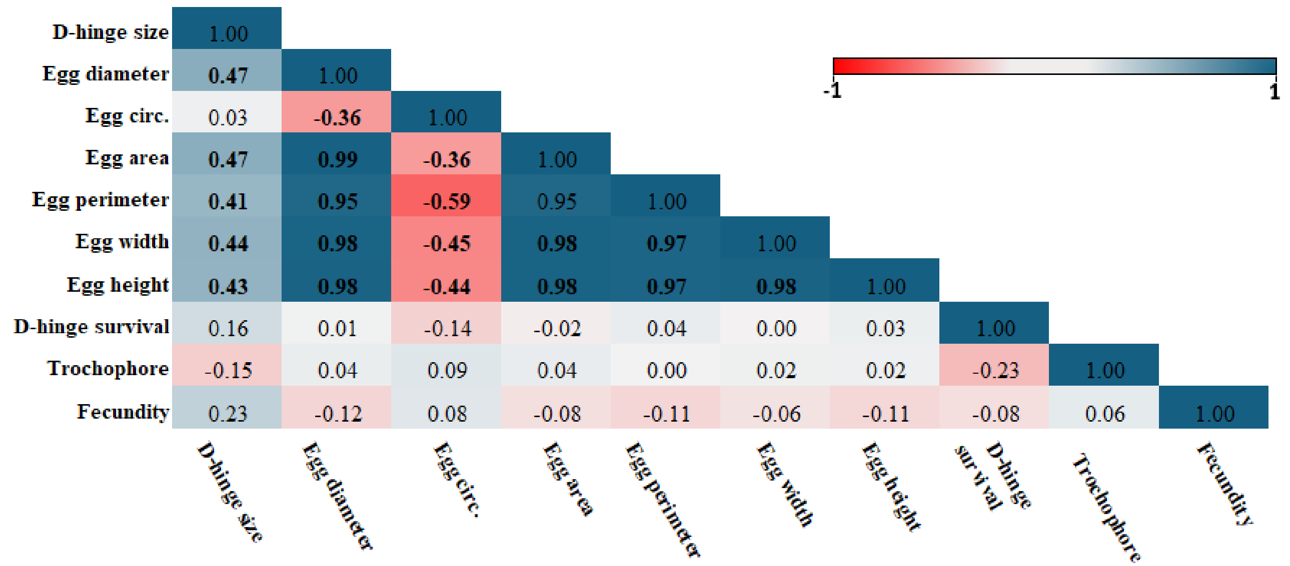
Table 3.1. Pearson correlation coefficients between D-hinge survival, egg morphometrics, D-hinge larval size, percent of trochophores present, and fecundity in eastern oyster, *Crassostrea virginica*. The color represents the strength of the correlation and significant correlations are bolded.



	D-hinge size	Egg diameter	Egg circ.	Egg area	Egg perimeter	Egg width	Egg height	D-hinge survival	Trochophore	Fecundity
D-hinge size	1.00									
Egg diameter	**0.47**	1.00								
Egg circ.	0.03	**-0.36**	1.00							
Egg area	**0.47**	**0.99**	**-0.36**	1.00						
Egg perimeter	**0.41**	**0.95**	**-0.59**	0.95	1.00					
Egg width	**0.44**	**0.98**	**-0.45**	**0.98**	**0.97**	1.00				
Egg height	**0.43**	**0.98**	**-0.44**	**0.98**	**0.97**	**0.98**	1.00			
D-hinge survival	0.16	0.01	-0.14	-0.02	0.04	0.00	0.03	1.00		
Trochophore	-0.15	0.04	0.09	0.04	0.00	0.02	0.02	-0.23	1.00	
Fecundity	0.23	-0.12	0.08	-0.08	-0.11	-0.06	-0.11	-0.08	0.06	1.00

Table 3.2. Total concentrations (μg/mg) of fatty acids (FA) between the higher-quality and lower-quality eggs in eastern oyster, *Crassostrea virginica*. The t-statistics, *P*-value, mean, and standard error of the mean (SEM) are reported for each FA and egg quality group. Higher- and lower-quality FA with empty t-statistic cells and NA valves were compared using non-parametric statistics.

Fatty Acids	t	*P*	Good		Bad	
			Mean	SEM	Mean	SEM
C12:0	-1.632	0.115	0.229	0.037	0.109	0.009
C13:0		0.032	0.176	0.029	0.089	0.006
C14:0		*NA*	1.559	0.306	0.663	0.080
C14:1	0.418	0.679	0.614	0.076	0.678	0.071
C15:0	0.340	0.733	0.872	0.147	0.813	0.085
C15:1		0.029	0.021	0.014	0.050	0.009
C16:0	3.110	0.004	19.071	5.543	4.764	1.392
C16:1	1.390	0.173	5.622	0.739	4.857	0.528
C17:0	0.770	0.447	0.938	0.118	0.873	0.086
C17:1		0.890	0.729	0.323	0.739	0.210
C18:0	1.900	0.068	3.689	0.467	2.491	0.242
C18:1	0.270	0.782	2.866	0.387	3.167	0.266
C18:2n6 (L	1.030	0.310	1.489	0.186	1.387	0.096
C18:3n6 (GLA)		*NA*	0.004	0.004	0.000	0.000
C18:3n3 (L	0.370	0.713	2.746	0.444	2.918	0.288
C20:0	1.260	0.217	0.167	0.024	0.137	0.006
C20:1	0.480	0.628	1.496	0.193	1.613	0.090
C20:2N6		*NA*	0.006	0.005	0.071	0.027
C20:3n6	-1.510	0.140	0.045	0.015	0.122	0.017
C21:0		0.173	0.039	0.008	0.031	0.002
C20:4N6 (A	-0.560	0.576	3.150	0.426	3.843	0.303
C20:3n-3		0.850	0.009	0.005	0.018	0.009
C20:5 (EPA	1.170	0.250	11.697	1.521	10.788	0.843
C22:0		0.344	0.000	0.000	0.016	0.011
C22:1	-0.170	0.859	0.275	0.045	0.311	0.030
C22:2N6	0.830	0.408	1.996	0.276	2.056	0.144
C23:0		0.439	0.000	0.000	0.003	0.002
C24:1	1.880	0.072	0.509	0.070	0.444	0.025
C22:6N3 (D	1.450	0.157	16.840	2.423	13.399	0.823
SFA	2.990	0.006	26.740	6.266	9.989	1.677
MUFA	0.890	0.378	12.132	1.545	11.858	0.911
PUFA	1.390	0.175	37.982	4.874	34.601	1.987
Ratios						
DHA/EPA	1.440	0.174	1.434	0.046	1.263	0.109
EPA/ARA	2.360	0.029	3.766	0.227	2.907	0.285

Table 3.3. Total percentages of fatty acids (FA) between the higher-quality and lower-quality eggs in eastern oyster, *Crassostrea virginica*. The t-statistics, *P*-value, mean, and standard error of the mean (SEM) are reported for each FA and egg quality group. Higher- and lower-quality FA with empty t-statistic cells and NA valves were compared using non-parametric statistics.

Fatty Acids	t	*P*	Good		Bad	
			Mean	SEM	Mean	SEM
C12:0	-3.280	0.004	0.327	0.044	0.196	0.015
C13:0	-2.870	0.011	0.247	0.030	0.161	0.010
C14:0	4.040	0.001	2.057	0.208	1.150	0.083
C14:1	-2.970	0.009	0.853	0.066	1.209	0.100
C15:0	-0.880	0.393	1.298	0.233	1.435	0.109
C15:1		0.021	0.031	0.019	0.089	0.016
C16:0	3.740	0.002	22.728	3.445	7.752	2.040
C16:1	-1.020	0.321	7.671	0.521	8.392	0.468
C17:0	-1.460	0.160	1.313	0.127	1.561	0.124
C17:1	1.290	0.214	1.061	0.414	1.300	0.350
C18:0	1.550	0.138	4.982	0.249	4.381	0.296
C18:1	-3.660	0.002	3.787	0.239	5.702	0.465
LA C18:2n6	-2.430	0.029	1.999	0.096	2.523	0.192
GLA C18:3n6		*0.368*	0.003	0.003	0.000	0.000
LNA C18:3n3	-2.540	0.024	3.601	0.301	5.369	0.625
C20:0	-0.710	0.487	0.232	0.022	0.251	0.017
C20:1	-3.250	0.004	2.050	0.164	2.965	0.228
C20:2N6		0.036	0.007	0.007	0.143	0.057
C20:3n6	-4.370	<0.001	0.057	0.018	0.229	0.043
C21:0	-0.730	0.469	0.052	0.005	0.057	0.005
ARA C20:4N6	-3.390	0.003	4.423	0.489	6.932	0.555
C20:3n-3		0.809	0.012	0.006	0.036	0.018
EPA C20:5	-2.940	0.012	15.641	1.073	19.041	0.422
C22:0		0.037	0.000	0.000	0.033	0.020
C22:1	-1.570	0.133	0.424	0.068	0.563	0.056
C22:2N6	-2.780	0.013	2.750	0.277	3.685	0.190
C23:0		0.034	0.000	0.000	0.007	0.004
C24:1	-2.430	0.028	0.672	0.026	0.806	0.049
DHA C22:6N3	-1.400	0.180	21.720	1.318	24.034	0.996

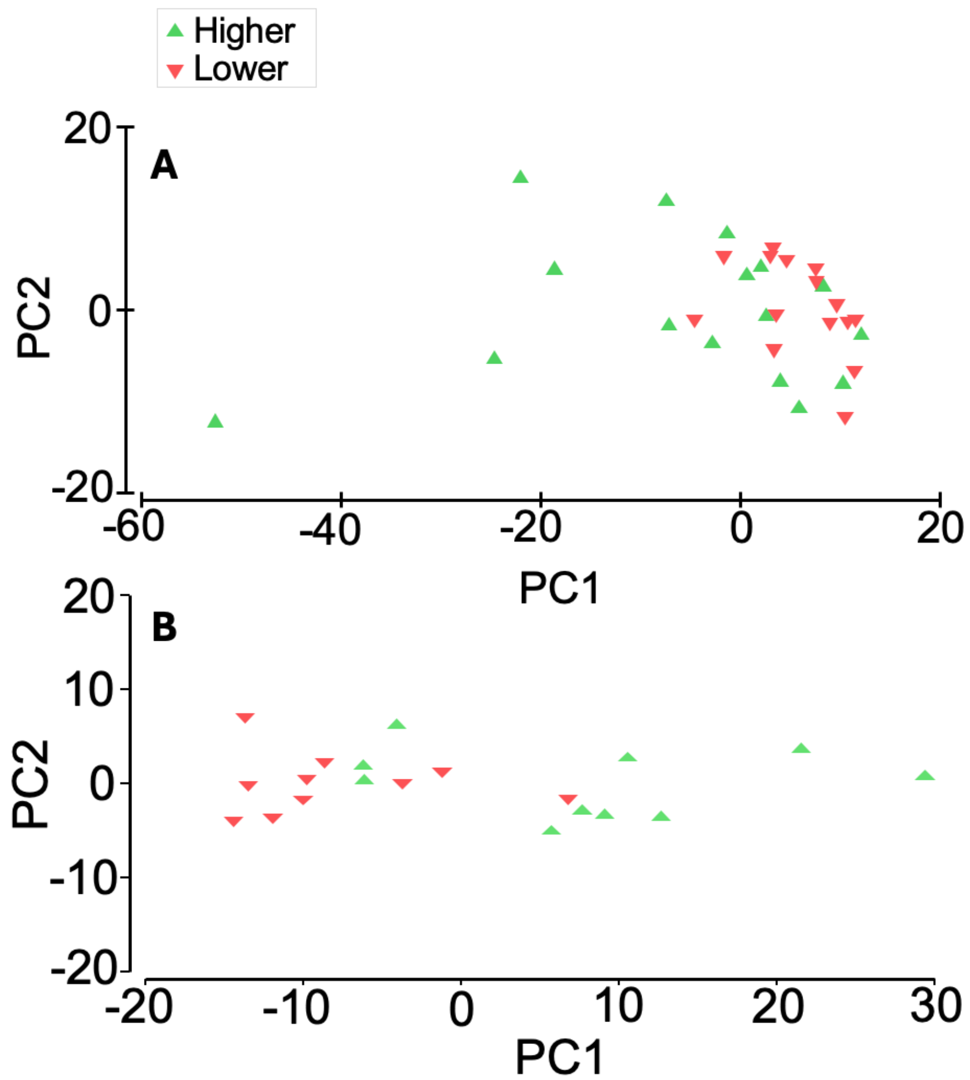


Figure 3.1. Principal Component Analysis (PCA) for variations in concentration (µg/mg) (A) and percent (B) of fatty acids from higher- (green) and lower-quality eggs (red) in eastern oyster, *Crassostrea virginica*.

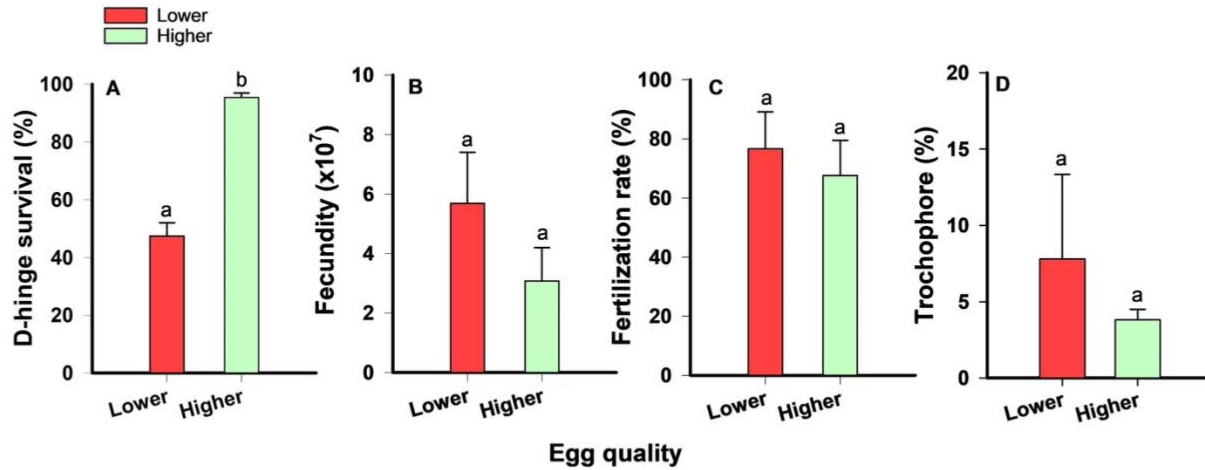


Figure 3.2. D-hinge survival (A), fecundity (B), fertilization rate (C), and percent trochophores present (D) between higher- (green) and lower-quality eggs (red) in eastern oyster, *Crassostrea virginica*. T-tests were used to compare individual FAs between the higher- (green) vs. lower-quality (red) egg groups. Values represent mean ± SEM (standard error of the mean).

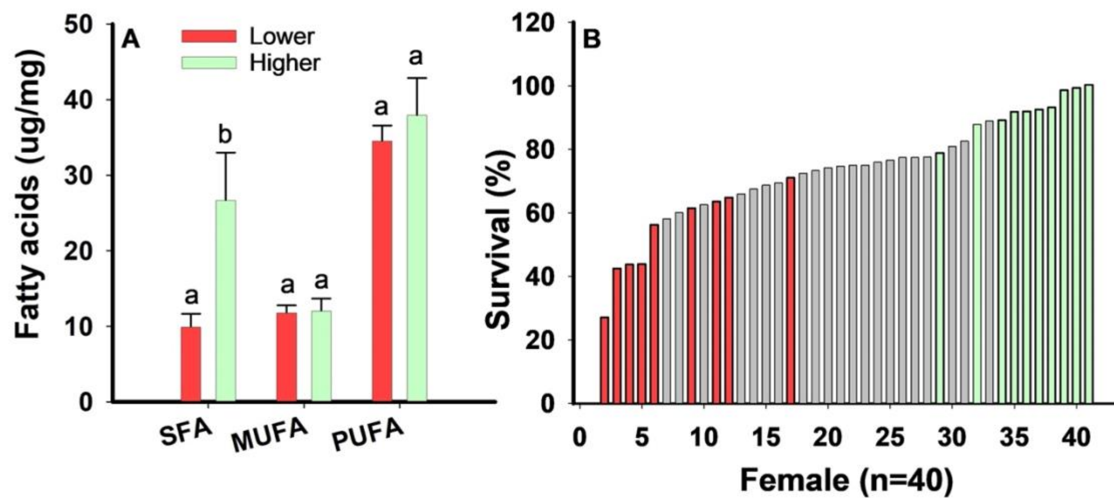


Figure 3.3. Total concentrations of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) between higher- and lower-quality eggs in eastern oyster, *Crassostrea virginica* (A). Percent survival of all females (n = 40) is reported (B), showing the females with the higher- (green) and lower-quality eggs (red). T-tests were used to compare individual FAs between the higher- vs. lower-quality egg groups. Values represent mean ± SEM (standard error of the mean).

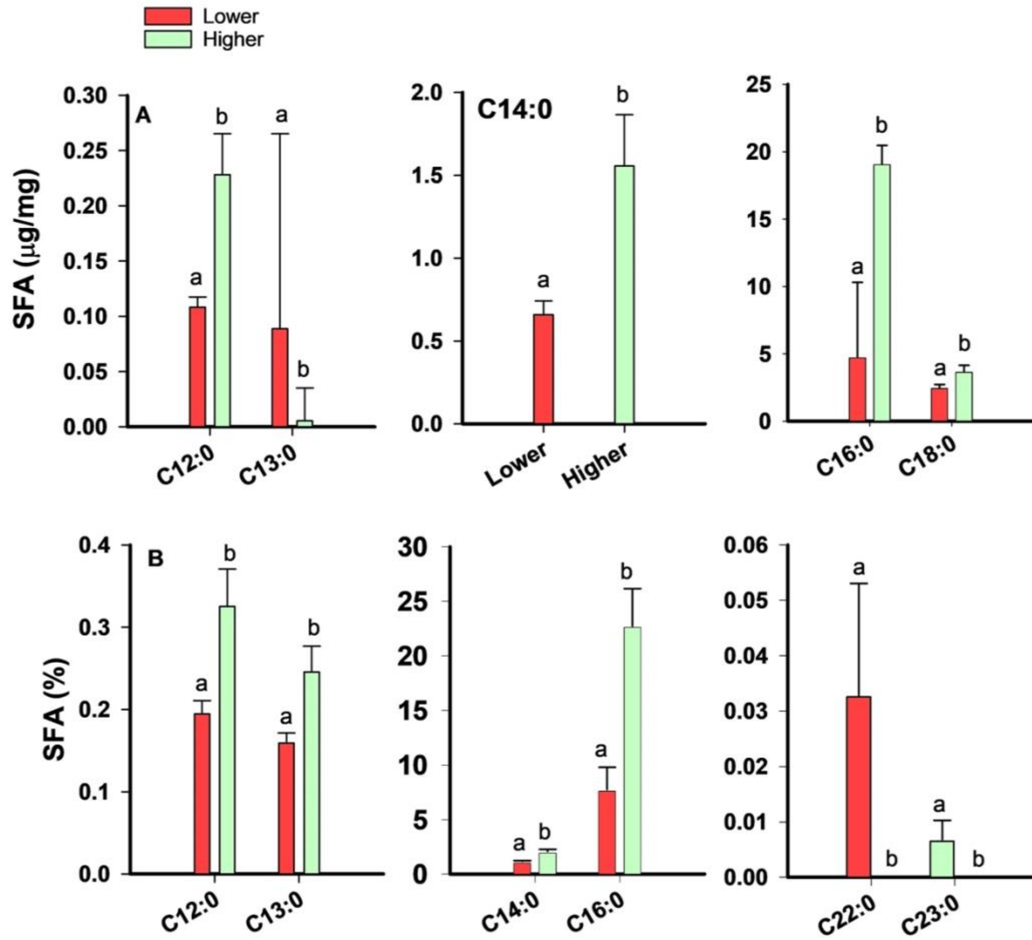


Figure 3.4. Total concentrations of saturated fatty acids (SFA) (A) and concentration percentages (B) between higher- and lower-quality eggs in eastern oyster, *Crassostrea virginica*. T-tests were used to compare individual FAs between the higher- vs. lower-quality egg groups. Values represent mean ± SEM (standard error of the mean).

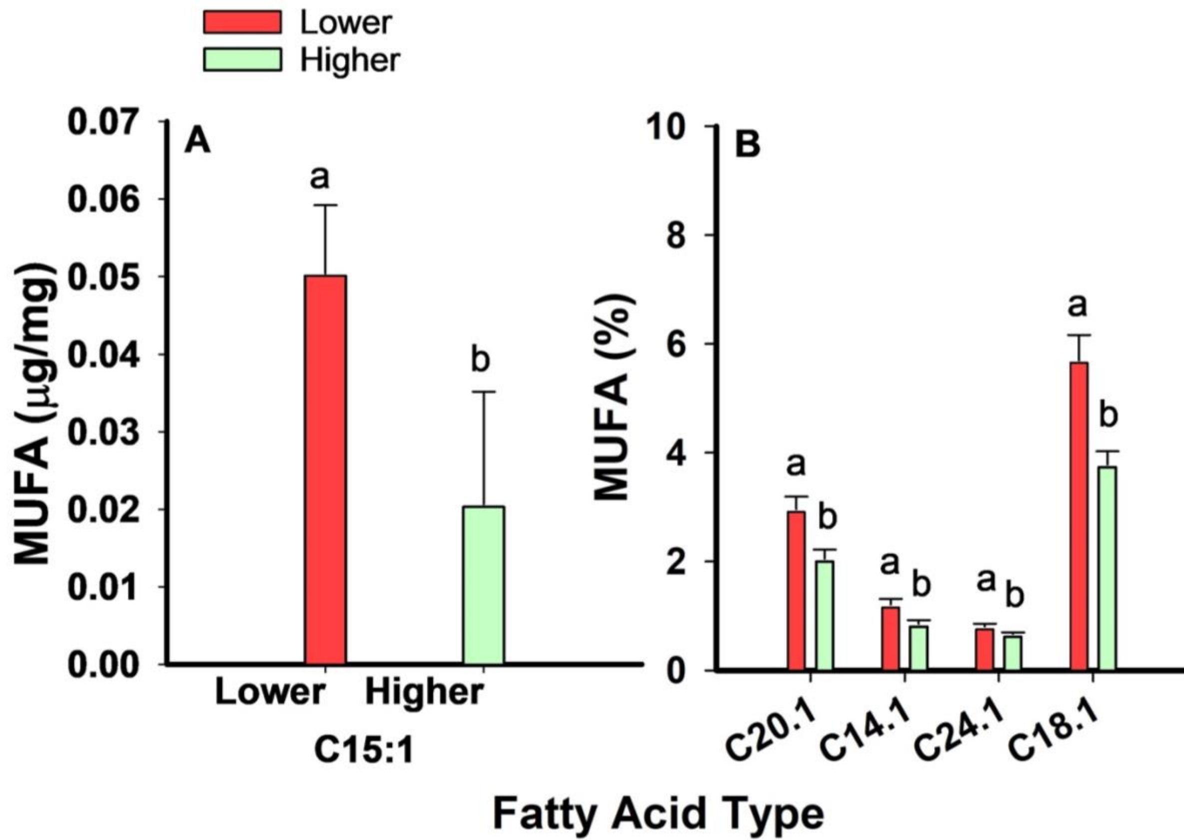


Figure 3.5. Total concentrations of monounsaturated fatty acids (MUFA) (A), and concentration percentages (B) between higher- and lower-quality eggs in eastern oyster, *Crassostrea virginica*. T-tests were used to compare individual FAs between the higher- vs. lower-quality egg groups. Values represent mean ± SEM (standard error of the mean).

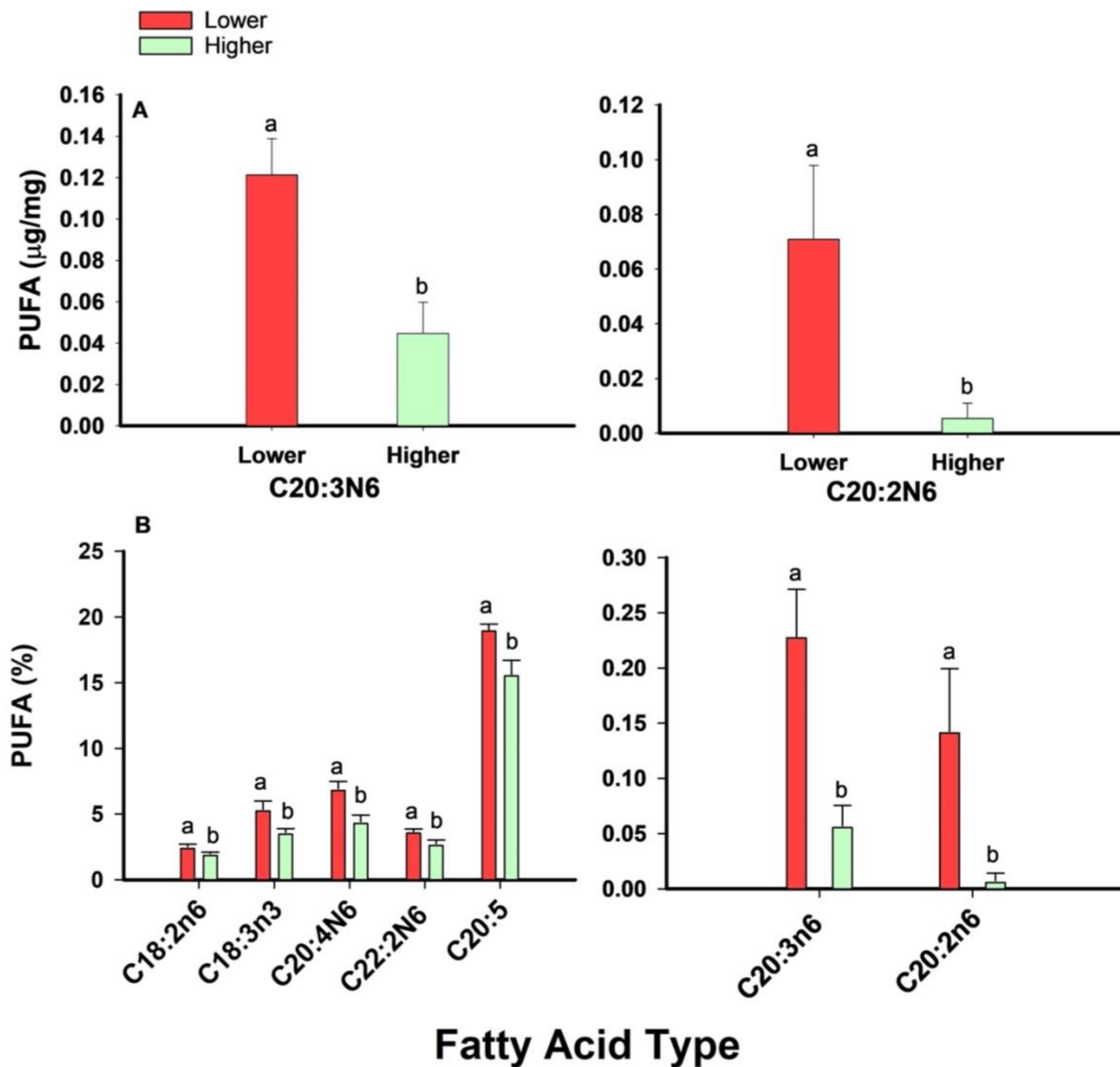


Figure 3.6. Total concentrations of polyunsaturated fatty acids (PUFA) (A), and concentration percentages (B) between higher- and lower-quality eggs in eastern oyster, *Crassostrea virginica*. T-tests were used to compare individual FAs between the higher- vs. lower-quality egg groups. Values represent mean ± SEM (standard error of the mean).

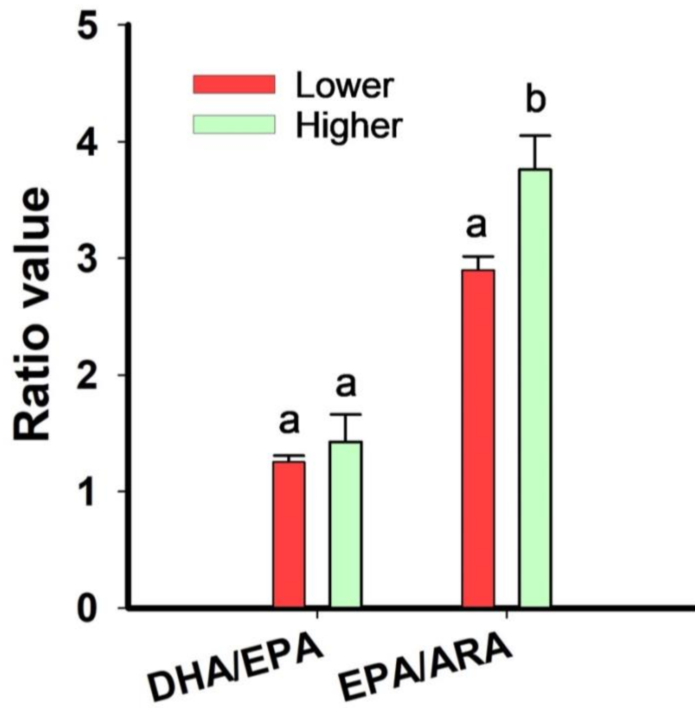


Figure 3.7. Ratios of relevant polyunsaturated fatty acids (PUFA) including DHA:EPA and EPA:ARA between higher- and lower-quality eggs in eastern oyster, *Crassostrea virginica*. T-tests were used to compare individual FAs between the higher- vs. lower-quality egg groups. Values represent mean ± SEM (standard error of the mean).