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Larval assessment and infection etiology of cultivated and wild eastern oysters, Crassostrea virginica, of Great Bay Estuary, NH

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LARVAL ASSESSMENT AND INFECTION ETIOLOGY OF CULTIVATED AND WILD EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*, OF GREAT BAY ESTUARY, NH

BY

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ABSTRACT

Eastern oyster, *Crassostrea virginica*, populations have been declining steadily over the past several decades across the North American east coast. Great Bay Estuary (GBE), located in New Hampshire, is experiencing this loss and restoration efforts have been put into effect. This work addresses restoration needs by characterizing abundances of settled spat and two early stages of *C. virginica*, D-hinge and veliger, in GBE from June 2020 through November 2020. Abundances are compared based on the collection site and the physicochemical data recorded on each sampling date. It was found that there were no differences in larval abundance at different sites in GBE. Although physicochemical factors are known to play a role in larval abundance, very little significance was found, suggesting future study may need to be modified to include a broader range of sampling. This study indicates that both D-hinge, veliger, and spat settlement occur in GBE prior to the time when sampling traditionally has started (June), suggesting an earlier than previously thought first spawn of *C. virginica* in GBE. This finding can be used to enhance restoration efforts as it suggests that spat for restoration are present earlier in the season and that recruitment devices should be deployed before the previously thought first spawn of each season.

Disease analysis was performed on eastern oysters as diseases are currently contributing greatly to the decline of eastern oysters in GBE. Presence of *Haplosporidium nelsoni* and *Perkinsus marinus*, organisms that cause MSX and Dermo (respectively) was examined in the water column, and it was found there were no significant differences between abundances of these disease-causing organisms at oyster farms and oyster reefs. A molecular diagnostic test, QCPCR, was compared to histological classifications of MSX and Dermo and it was found that MSX could be identified

and quantified as well using QCPCR as with histology, but work remains to be done on the Dermo method to improve accuracy. Both MSX and Dermo levels were compared in farmed and wild oysters (as farmed oysters are thought to be MSX-tolerant). This study showed that MSX levels were significantly lower in aquacultured oysters than wild oysters, but that Dermo levels were comparable between the two. Although there were varying levels of disease in eastern oysters in GBE (especially between wild and aquacultured oysters), similar amounts of the infectious agents were found throughout GBE waters, indicating that location does not have an impact on disease susceptibility.

INTRODUCTION

Eastern oyster history, morphology, and environmental impact

Since estuary formation thousands of years ago, eastern oysters *Crassostrea virginica* have been a native and critical species to the development of marine life along the Atlantic coast of North America. When English colonists came to the Chesapeake Bay region in the early 17th century, they discovered hundreds of oyster reefs throughout the Bay (Hargis & Haven 1999). At the time, oysters were a common food that stayed local to the areas in which they were harvested. In the early 19th century, fishermen realized the value of the oyster and began shipping large supplies of Delaware and Chesapeake Bay oysters to markets throughout the United States (Ford & Haskin 1982). In 1886, the first recorded case of oyster farming occurred, where small farmers would purposefully plant oyster seeds to create simulated reefs. Individuals began to realize the economic importance of oysters, leading to more oyster farming. Small farmers and large enterprises began to participate in oyster aquaculture, but other causes, such as adverse weather, channel dredging, and disease caused the already diminished populations to decline (MacKenzie Jr. 2007). Once farmers and businesses began to realize the potential of oysters in creating a widespread industry, the oyster economy boomed and businesses all over the country were purchasing oysters at alarmingly high rates. In the 1800s and early 1900s, the oyster industry was one of the most valuable industries in the United States, with an annual return to fishermen of nearly $15 million in 1919 (Churchill 1920). In the Great Bay region, the oyster industry was steady from about the 1930s through the 1960s with steady increases in the price of oyster bushels (Matthiessen 1970). Since then, the oyster demand has continued to increase. In 2015, oyster production was worth $234 million, which was a significant increase from the year prior (NOAA Fisheries 2019). The

demand for oysters across the United States has contributed to the price increase as well as the difficulty in maintaining good harvests each year.

The decline in eastern oyster populations is important in "ecosystem services." Eastern oysters play an important and quantifiable environmental role in the marine ecosystems to which they belong. van der Schatte Olivier et al. 2018 estimated, for bivalve species as a whole, a global, non-food worth of $6.47 billion ($2.95 billion–9.99 billion) per annum. Oyster reefs serve as a sanctuary for many forms of marine life in ways very similar to the well-known coral reef habitats in that various marine organisms can seek protection and support themselves throughout the reef. Juvenile fish, which include young Atlantic cod, bluefish, and white flounder, are some of the many species of marine life that can call oyster reefs their home (NOAA Fisheries 2019). Oyster reefs also play an important role in buffering the effects of weather on the shoreline. Brandon et al. (2016) conducted a study where wave energy was recorded from a 1992 Nor'easter. They found a 183% reduction in wave energy when the waves hit an oyster reef, demonstrating that the reefs provide significant coastal protection. They further studied wave energy from Hurricane Sandy in 2012 and found a 75% reduction. Without the oyster reefs providing a barrier between the wave energy and the shore, the shoreline becomes vulnerable to over-wash from storms.

Eastern oysters also function as water filterers, so much that in estuaries they historically most likely were able to filter the water from entire estuarine systems in a few days (Newell 1988). Presently, the universal oyster filtration potential is reduced by at least 80%, resulting in significant negative effects to the ecosystem (zu Ermgassen et al. 2013). Oysters filter to consume their food source, phytoplankton, but in the process, they remove other microbes, improve water clarity, and

transfer biomass to the estuary floor promoting more favorable nutrient cycling and ultimately decreasing levels of nitrogen pollution (Newell et al. 2005). In GBE, it is estimated that the valuation of eastern oyster nitrogen removal is between $1.12 million and $1.28 million per year, with a maximum potential of between $4.3 million to $5.0 million per year (Bricker et al. 2015). Studies have documented the reduction in nitrogen levels due to oyster filtration (Kellogg et al. 2014, Bricker et al. 2018), bringing forward the idea that bivalve species, such as the Eastern oyster, could be "used" to improve nutrient levels in the estuaries where they are found.

Great Bay Estuary (GBE) as an estuary system

Due to water mixing, estuaries experience a large range of salinities throughout, as well as varying levels of tides, waves, and river fluxes depending on location in the estuary system (Pritchard 1967). Because of their conditions, estuaries are common areas for fisheries production and nutrient enrichment, allowing coastal systems to thrive with large marine life populations and nutrient-dense water conditions (Nixon et al. 1986, Bianchi 2006). The tidal range is 2-4 m, tidal currents are > 2 m s^{-1} in the channels at maximum ebb and flood, and at low tide, as much as 50% of GBE is exposed as low-lying mudflats. The surface area of GBE is approximately 55 km^2 (Trowbridge 2007), the volume is 156×10^6 m^3 and 235×10^6 m^3 for low and high tides, respectively, and the tidal prism is 79×10^6 m^3 (Trowbridge 2007, Swift and Brown 1983). The Bay is fed small volumes of fresh water by seven rivers where fluxes are determined by precipitation and runoff, and except during high flow events (storms and ice off), freshwater input only contributes 2% of the tidal prism (Short 1992).

The GBE system, located along the New Hampshire-Maine border within the Gulf of Maine region, has unique characters that make it difficult to simply extend knowledge from other North American mid-Atlantic estuaries. The generally small freshwater fluxes and strong tidal mixing result in weak or negligible stratification (except very close to the river mouths) and during periods of little rainfall, the salinities at the center of GBE are nearly equal to the Gulf of Maine proper, contributing to the unique ecological dynamics of GBE (Mills et al. 2009). In addition, water temperatures in the Gulf of Maine are rising faster than 99% of the world's oceans (Poppick 2018). This is due to proximity to two major gulf streams (Labrador current and from Atlantic to Arctic), which in combination with one another, allow for rapid change to water conditions. Because of the rapid change, marine species native to the Gulf of Maine must respond quickly to sustain their populations or perish. Mills et al. (2013) studied the effects of fisheries management in a changing climate, effects that are exacerbated in the Gulf of Maine. Because estuary systems have unique characteristics, restoration and protection efforts, such as the National Estuarine Research Reserve System (NERRS), are important in recognizing and protecting estuarine systems.

Eastern oysters in GBE

Oyster populations in GBE have varied both spatially and temporally since the NH Fish and Game Department started monitoring oyster reefs in 1991 (Eckert 2016). Eastern oyster populations have been on a dramatic decline (Beck et al. 2011) and are estimated to be about 10% of what they were in the 1980s (NH Fish and Game Department unpublished data). While eastern oysters are still extremely important in ecosystem services, declines are currently being caused by overharvesting, changes in water conditions, and diseases (Odell et al. 2006; Grizzle et al. 2006; Konisky et al. 2014). Majority of eastern oysters are limited on substrate to set on, so research has focused on

providing suitable material for larval setting (Brown et al. 2014). Eastern oysters in GBE, though, are limited on substates *and* larval abundance, so restoration efforts focus on both suitable substrate and sufficient spat for settlement (Grizzle et al. 2013).

A study by Grizzle and Ward (2016) assessed the different recruitment methods in GBE in an attempt to optimize eastern oyster recruitment on artificial reefs. Video surveying was used to determine the reef height, size, and shape. In addition, any significant bottom conditions were noted that could potentially affect settlement. Next, a bottom sub-sample of oysters from each reef was taken and oysters were analyzed by counting and measuring shell height of all live oysters, as well whether they occurred on a clam shell or oyster shell. These two methods were analyzed to determine how successful oyster reefs were in GBE. Conclusions showed the two factors that most influenced restoration success were sedimentation and site location relative to a natural reef. While restoration efforts of eastern oysters have been strong, this study allowed for more precise methodology to get the best recruitment levels. Temporal dynamics play a large role in the development of eastern oysters in GBE, as other studies that found correlations among spawning activity, settlement, water temperature, and salinity (Carriker 1951, Eckert 2016). Studies have shown that a better understanding of larval dispersal (Rodriguez-Perez et al. 2020) and temporal dynamics (Carriker 1951) of eastern oysters in GBE will lead to more focused restoration efforts.

Disease in eastern oysters

The first known epizootic mortality of oysters occurred off the coast of Prince Edward Island, Canada in 1915. To this day, the causative pathogen is unknown, but this preceded epizootic outbreaks in oyster populations along the eastern coast of North America. In 1957, Delaware Bay

experienced massive oyster mortality, with death tolls recorded up to 95%. Two years later, in Chesapeake Bay there was another massive oyster mortality. The causative agent was discovered to be *Haplosporidium nelsoni* (Ewart & Ford 1993), which causes the disease MSX in oysters. It is a protozoan parasite that infects the gill epithelium in primarily young oysters or spat. Proliferation of the parasite causes the epithelial layer to detach from the basement membrane, which allows the parasites to break through the basement membrane and circulate through the circulatory system (Ford & Haskin 1982). *Haplosporidium nelsoni* is primarily influenced by temperature, but also by salinity. The pathogen thrives during warmer seasons, having the heaviest infection rates during the end of May or beginning of June in the Delaware and Chesapeake Bays (Burreson & Ford 2004), and has been similarly found in the Great Bay Estuary (Grout 2014). Once waters cool, the pathogen has significantly lower infection rates. Generally, the colder the preceding winter, the less prevalent *H. nelsoni* infections will be. In a high salinity environment, the pathogen can infect hosts more efficiently (Burreson & Ford 2004).

The pathogen continues to thrive in coastal and estuarine waters and there is no known mechanism for the transmission between oysters. By 1962, scientists knew that *H. nelsoni* was not capable of transmitting directly from oyster to oyster (Barrow & Taylor 1966); however, the intermediate host is still unknown. After tens of thousands of zooplankton were tested for the presence of *H. nelsoni*, including copepods, shrimp, and crab larvae, there was no evidence of the pathogen in any of the samples (Ford et al. 2018). There are two life stages of the pathogen: the plasmodial stage and the spore stage. It has been found that spat, when infected with *H. nelsoni*, release spores (Burreson & Ford 2004); however, spore distribution from infected oysters has not been conclusively shown as the method for infection, as the abundance of spores released never

exceeded the infection threshold necessary to infect another oyster. The spore stage also has been hypothesized to be the completion of the life cycle in *H. nelsoni*, but without adequate levels of spores, no conclusions can be drawn (Ford et al. 2018).

Several decades after Delaware Bay experienced its massive oyster mortality from MSX, the Bay once again experienced a large-scale oyster mortality, this time due to a different parasite. *Perkinsus marinus* was introduced to Delaware Bay in the 1950s; however, the weather at the time was too cold for the pathogen to thrive, and there were no signs of infection. In the 1990s, after a series of warm winters, the pathogen began infecting oysters throughout the Bay and another mass mortality occurred (Ewart & Ford 1993). The outbreak continued up and down the east coast as water temperatures rose, and to this day *P. marinus*, which causes Dermo, is still a large issue in the oyster population (Audemard et al. 2004). Contrary to *H. nelsoni*, the transmission of *P. marinus* is very well understood. Trophozoites use the hemocyte surface receptor galectin CvGal to adhere to the surface of and enter oyster hemocytes (Tasumi & Vasta 2007). Then, the trophozoite forms an eccentric vacuole within itself, expanding and reproducing. When it reaches the sporangia stage, it lyses and releases the newly formed trophozoites, which spread throughout the entirety of the oyster. Once the oyster dies from infection, trophozoites develop into zoospores, which are flagellated, and are released into the water. This causes subsequent infection in other oysters within the same water column (Alavi et al. 2009).

Project goals

The overall goal of this project was to elaborate on major causes of the decline of eastern oysters in GBE. Chapter 1 of this thesis focuses on the abundance of eastern oyster larvae in GBE in and

around both reefs and oyster farms. These abundances were compared with physicochemical data collected from NOAA buoys to assess potential relationships between larval abundance and factors such as temperature, salinity, pH, and chlorophyll. These preliminary data provided a basis for overall health of eastern oyster larval abundance in GBE.

Chapter 2, broken into 2a and 2b, describes use of PCR-based methods to identify and quantify parasitic infections in GBE oysters. Chapter 2a focuses on the quantity of infectious agents in the water column, and 2b focuses on differences in infection intensity between wild and farmed oysters. The amplification-based methods allowed quantification of infection by two known disease agents, *H. nelsoni* and *P. marinus*. This method was used to detect and quantify infectious agents in the water column throughout the sampling season and to determine if there was a difference in infection between wild and farmed oysters, as one would expect assuming most cultivated oysters in Great Bay are derived from MSX tolerant stocks.

Using QCPCR, I examined infection in eastern oysters to monitor parasite abundance throughout spring, summer, and fall (it is known that disease intensity is low during winter (Brown et al. 2005)). This study focused on two specific infections, *H. nelsoni* and *P. marnius*, to determine whether the infections potentially are interrelated. Specific objectives included:

1. Assess eastern oyster larval abundance and determine if physicochemical factors play an important role in abundance in GBE.
2. Quantify the levels of *H. nelsoni* and *P. marinus* in the water column throughout the growing season.

3. Quantify infection by *H. nelsoni* and *P. marinus* in *C. virginica* throughout the growing season.
4. Compare infection rates between wild and farmed *C. virginica*.

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CHAPTER 1 – LARVAL ASSESSMENT AND TEMPORAL DYNAMICS OF EASTERN OYSTER LARVAE IN GBE

INTRODUCTION

Drastic declines in eastern oyster, *Crassostrea virginica*, abundance (Kirby 2004, Beck et al. 2011, Rick et al. 2016) have led to a loss of ecosystem services provided throughout its North American range. As "ecosystem engineers," services provided by *C. virginica* have been estimated to be worth \$5500 - \$99,000 ha^{-1} yr^{-1} (Grabowski et al. 2012). Specifically, healthy oyster reefs aid in mediating nutrient cycles through nitrogen removal (Higgins et al. 2011, 2013), provide complex habitats for an array of organisms (Beck et al. 2011), augment fishery resources (Grabowski and Peterson 2007, Scyphers et al. 2011), and help to regulate ecosystem processes through top-down control of phytoplankton (Coen et al. 2007). Additionally, healthy *C. virginica* populations influence ecological processes across gradients, driving food-web dynamics through direct and indirect effects on species interactions and changes to biomass across trophic levels (Grabowski et al. 2020). Consequently, the continued decline of oyster populations is a cause for concern and could prove to be catastrophic for some estuarine ecosystems (Coen et al. 2007, zu Ermgassen et al. 2013, Kaplan et al. 2016), particularly estuaries in northern New England. For example, the Gulf of Maine is currently one of the fastest warming regions on Earth, where sea surface temperatures increased at a rate of 0.26°C yr^{-1} between 2004–2012 (Mills et al. 2013). Such rapid change can facilitate both biotic and abiotic changes that may be contributing to the decline in oyster abundance in the Great Bay Estuary (GBE) of New Hampshire.

In the face of the declining New England and specifically in New Hampshire (NH) oyster populations, efforts toward oyster reef restoration have intensified (Grizzle and Ward 2016).

Oyster reefs previously existed throughout much of New Hampshire's estuaries, which include GBE, but current oyster populations in NH are <10% of their numbers in the 1980s (Grizzle and Ward 2016). These declines have been attributed to disease, anthropogenic impacts, lack of natural shell for larval setting, and sedimentation leading to the burial of shell (Grizzle and Ward 2016). Current shellfish restoration efforts in GBE have demonstrated some successes. Spat on shell have been reared at the University of New Hampshire and deployed onto GBE restoration sites as part of The Nature Conservancy oyster restoration efforts after which it was observed that ~5.8×10^4 oyster spat recruited to a 1 ha reef constructed in the mouth of the Lamprey River (Konisky et al. 2011). Another restored site in GBE near Lamprey River has shown indications of natural recruitment (Grizzle and Ward 2016). A recent study has demonstrated the potential for larval recruitment of oysters in the GBE, suggesting that recruitment is more favorable at sites that are in proximity to existing natural reefs with established adult oysters (Atwood and Grizzle 2020).

Due to increasing carbon dioxide into the atmosphere, which the ocean absorbs, pH is declining and causing acidic water conditions (Cai et al. 2011). This process known as ocean acidification negatively affects early developmental stages and metamorphosis in shellfish (Miller et al. 2009, 2020, Mabardy et al. 2015). Larval recruitment to wild populations is critical to sustainable restoration of eastern oysters, and this recruitment ultimately depends on production of larvae that survive to metamorphosis and settle on reef surfaces. But currently, spatial and temporal preferences of larval dispersal in GBE remain largely unknown and understanding larval dispersal will help streamline possible strategies to successfully restore healthy oyster populations in GBE (Brumbaugh et al. 2005, Rodriguez-Perez et al. 2020). There are a variety of threats that could affect the successes of oyster larval reproduction and settlement (NOAA 2007) including suitable settling habitat, temperature, acidification, disease, predation, overharvesting, and extreme

fluctuations in physicochemical variables (temperature, salinity, pH, chlorophyll). As a result, it is imperative to discuss and view oyster restoration efforts as hierarchies depending on the quantities of larvae and the source(s) of stress. This present study aims to 1) reveal the dynamics of abundance of *C. virginica* larvae in GBE by examining changes in larval abundance and 2) ascertain whether there are differences in settlement across representative sites where oysters previously were abundant. These data will provide a baseline understanding of the reproductive output of eastern oysters in the GBE, help ascertain possible differences in oyster larval abundance among sites, and provide insight that will aid oyster restoration efforts in the GBE by optimizing the larval season, deployment of clutch at the best sites for recruitment.

METHODS

Study area

In 2020, four sites were sampled throughout the study: Woodman's Point (WP), Nannie Island (NI), Adams Point (AP), and an oyster farm (OF). These were selected to examine the relationship of oyster larval abundance and settlement at naturally occurring reefs and an oyster farm and encompass a broad area of the GBE (13 km^2, Fig. 1), reflecting conditions throughout the entirety of the estuary during the sampling period.

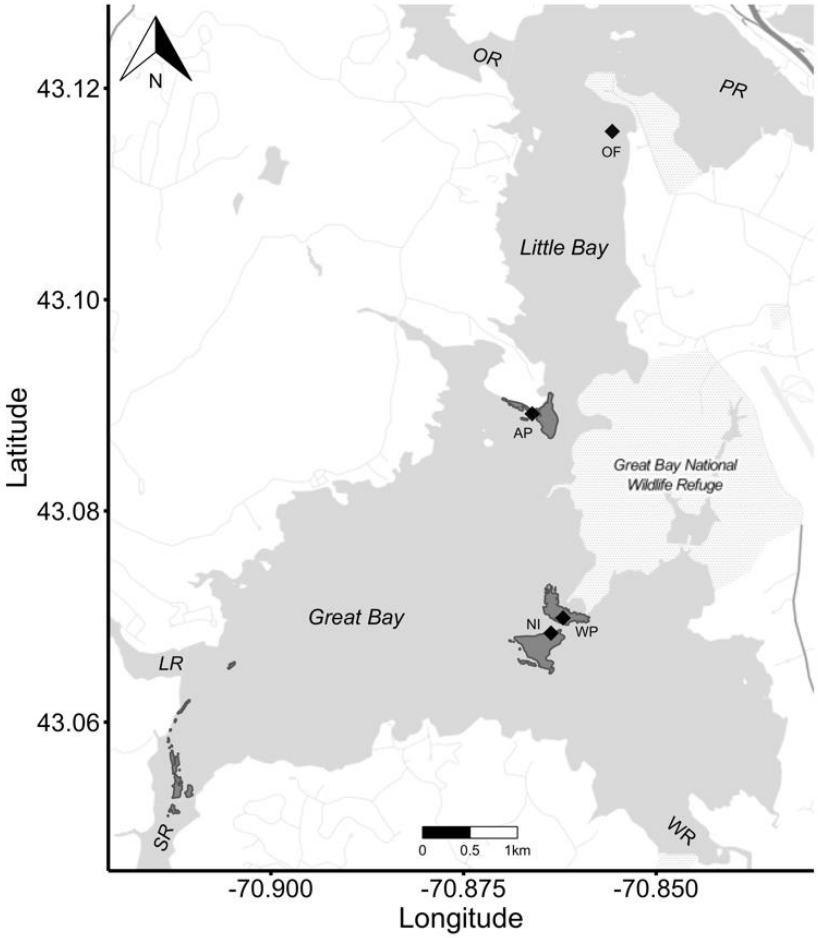

Figure 1.1. Map of Great Bay Estuary showing four sites (black diamonds) where larval tows were conducted and spat collectors were deployed. Natural reefs are shown as dark gray areas. Those sampled in 2020 were NI, WP, and AP, located at natural oyster reefs, and OF located at an oyster farm. SR: Squamscott River, LR: Lamprey River mouth, OR: Oyster River, PR: Piscataqua River, WR: Winnicut River are shown on the map for reference. Not shown are Bellamy River, and Salmon Falls and Cocheco Rivers that feed into the Piscataqua River.

Experimental Design

Each year, horizontal surface larval tows using a 64-µm mesh net were conducted at 4 sites within GBE approximately once per week with 2 larval tows. Tows were conducted ~0.3 m below the surface for approximately 2 min at 0.5-1.0 m sec^{-1}, resulting in an average of 38 m^3 of water

sampled per tow. Samples collected in 2020 from larval tows were preserved with formalin sucrose (Haney and Hall 1972).

Sampling was conducted from June 2020 – November 2020 with deployment of recruitment devices occurring in the first sampling week. In 2020, a total of 12 recruitment devices were created by placing n=3 ceramic tiles (each 55×3×0.6 cm) enclosed within a 75×50×55 cm coated metal wire cage, with n=3 devices per site situated approximately 15 m apart. Recruitment devices deployed in May were monitored at weekly intervals, field and weather condition dependent. One tile was removed from one of the replicate recruitment cages at each of the four sites weekly and was replaced with a new seasoned ceramic tile. The third cage remained undisturbed until the study concluded to ascertain the total number of spat settled cm^2 over the season. At the end of the 2020 sampling period, all 3 spat collectors were retrieved at sites NI and WP, but due to storms that occurred throughout the season, only 1 recruitment device was recovered from sites AP and OF.

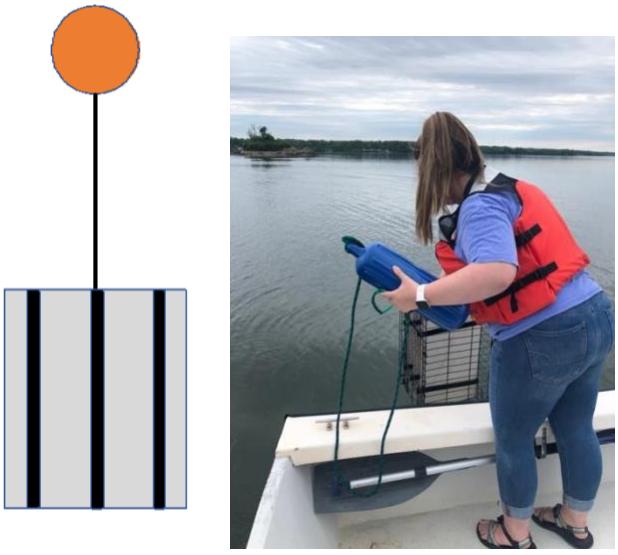

Figure 1.2. Prototype of the spat collector used in 2020 and a picture of the deployment of a spat collector in 2020. A large cage held 3 large ceramic tiles in place. This was attached to a rope that led to a buoy on the surface.

Physicochemical data

To assess potential relationships among abiotic factors and oyster larval abundance, physicochemical data (temperature, salinity, pH, and chlorophyll) were retrieved for each sampling date from the NOAA National Estuarine Research Reserve System (NERRS) data buoy, which monitors water quality, nutrient/pigment, and meteorological data in real-time using a System-Wide Monitoring Program. The real-time data were accessed using the data graphing and export system for the Great Bay station from their Centralized Data Management Office website (Fig. 3). Due to the unique circulation of GBE, data provided by the NERRS buoy have been shown to reflect environmental conditions of GBE (Pennock 2007).

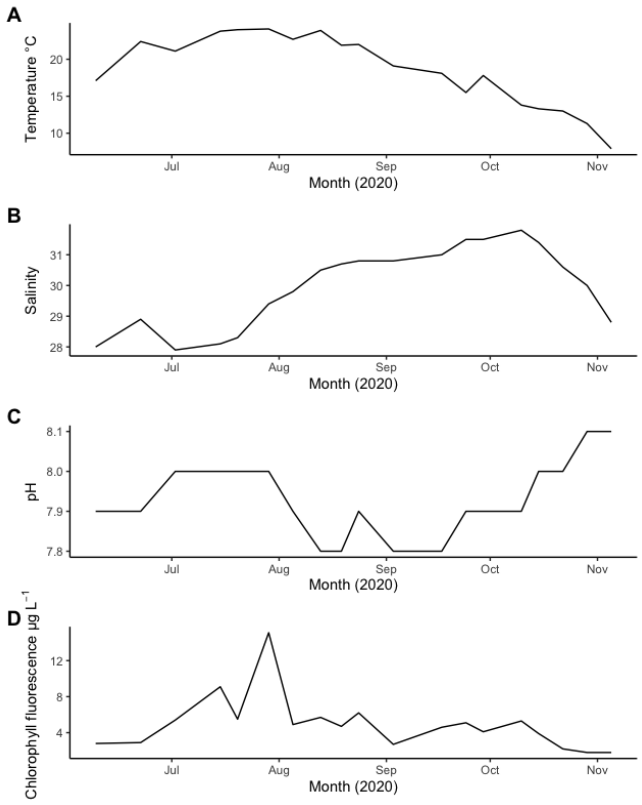

Figure 1.3. Physicochemical data from GBE in 2020. Data include A) temperature, B) salinity, C) pH, and D) chlorophyll (µg L^{-1}).

Enumeration of larvae

Formalin-preserved plankton samples were filtered through a set of brass sieves retaining 45 – 106 μm, then diluted with 200 mL of filtered seawater prior to enumeration via light microscopy. Three well-mixed 1-mL replicate subsamples were applied to Sedgewick Rafter Counting Chambers and then enumerated under 4× magnification with an Olympus CX31 microscope. Oyster larvae were identified based upon shell shape, umbo character, and velum. All size fractions were returned to the original container and reconstituted in formalin-seawater after which samples were filtered through a 177-μm sieve to isolate oyster larvae from other larger biological material then enumerated using an automated FlowCam (Yokogawa Fluid Imaging Technologies). A 600× flow cell was used to process three 5-mL replicates per sample. A capture filter set from 10–300 μm (Baldwin and Newell 1995) allowed capture of images of organisms in the expected size range of oyster larvae. To further optimize detection of oyster larvae, images of putative oyster larvae were filtered using a library created from known oyster larvae. Results of the two enumeration methodologies were compared, allowing for quality assurance. Both Sedgewick Rafter and FlowCam enumeration for all samples were performed by several individual workers and FlowCam counts were validated by having three readers assess saved images of oyster larvae. At least two readers needed to come to agreement to validate the total count of oyster larvae present in each sample.

Analyses

Counts of both D-hinge and veliger oyster larvae m^{-3} determined by microscopy (n=3) and the FlowCam (n=3) were averaged per site and GBE, corresponding with their respective sampling dates. Although veligers can be positively identified as oyster larvae, it is not possible to

distinguish oysters from other bivalves at the D-stage, leading to a probable inflation of oyster counts at the D-stage since those counts also can include mussels and clams. Average counts were log (x+0.05) transformed to meet normality and homogeneity assumptions. Shapiro-Wilks test for normality and Bartlett's test were used to examine normality and homogeneity respectively. A one-way repeated measures analysis of variance (ANOVA) was run between each sampling date for both D-hinge and veliger oyster larvae. A Kruskal-Wallis test was performed to test for differences in the number of spat cm^{-2} settled among dates. Model selection of explanatory variables was performed using the "dredge" function in the "MuMIn" package (Barton 2015). Regression models were performed using temperature, salinity, and pH as the independent variables if they were found to be significant during the model selection process, and D-hinge and veliger counts as the dependent variables. All statistical analyses were done in R (v 3.6.3, R Core Team 2020).

RESULTS

Baselines for oyster larval abundance were observed: there was a mean occurrence of 273 D-hinge m^{-3} (SE 85.9, range 1.18 – 2009), median of 83.7 D-hinge m^{-3}, 0.532 veliger larvae m^{-3} (SE 0.198, range 0.003 – 2.86, Fig. 4), and median of 0.301 veliger larvae m^{-3} per sampling date. ANOVAs demonstrated no significant difference among sites for each corresponding date. One-way repeated measures ANOVA showed each sampling date differed significantly from each other ($p < 0.001$). Model selection indicated that salinity and pH were not significant for both veliger and D-hinge larval abundance; temperature was positively associated with D-hinge oyster larval abundance ($p < 0.001$, adj. $R^2 = 0.5998$) and with veliger oyster larval abundance ($p < 0.001$, adj. $R^2 = 0.2098$, Fig. 5). Chlorophyll fluorescence was positively associated with D-hinge oyster larval abundance

(p <0.001, adj. R^2 = 0.1913) and veliger oyster larval abundance (p =0.008, adj. R^2 = 0.0803). The quantity of spat settled (χ^2= 66.329, df = 3, p<0.001, Fig. 6) differed significantly by date.

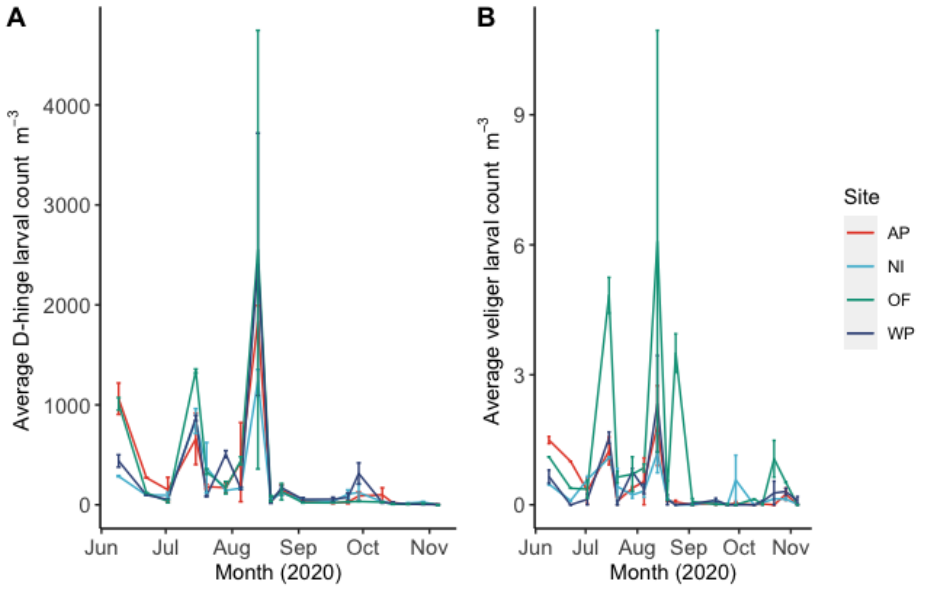

Figure 1.4. *Crassostrea virginica* A) D-hinge and B) veliger larval abundances in 2020. Standard error bars are shown with each data point.

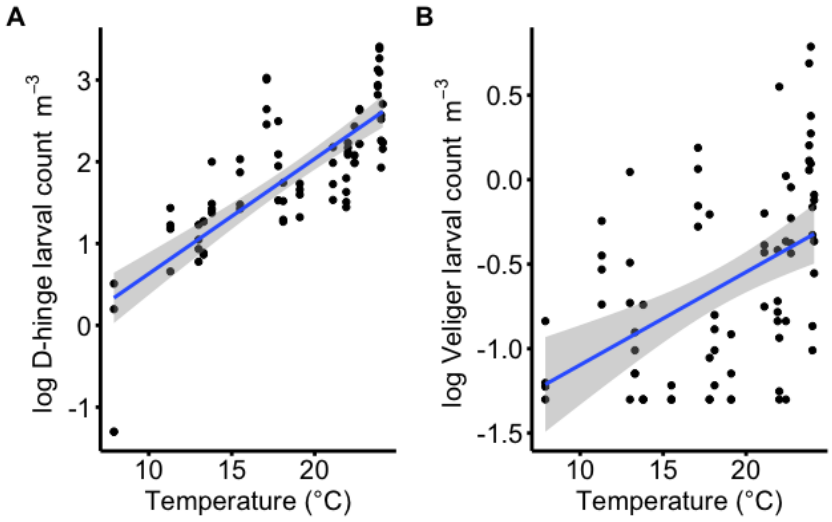

Figure 1.5. Regression model of A) D-hinge oyster larvae and temperature (°C) and B) veliger oyster larvae and temperature (°C) with corresponding trendline, and 95% confidence intervals shown in gray.

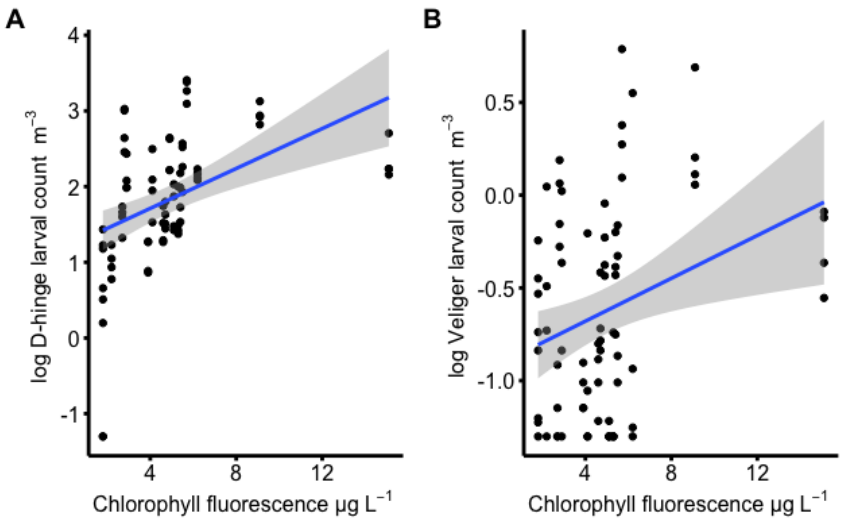

Figure 1.6. Regression model of A) D-hinge oyster larvae and chlorophyll fluorescence (µg L^{-1}) and B) veliger oyster larvae and chlorophyll fluorescence (µg/L) with corresponding trendline, and 95% confidence intervals shown in gray.

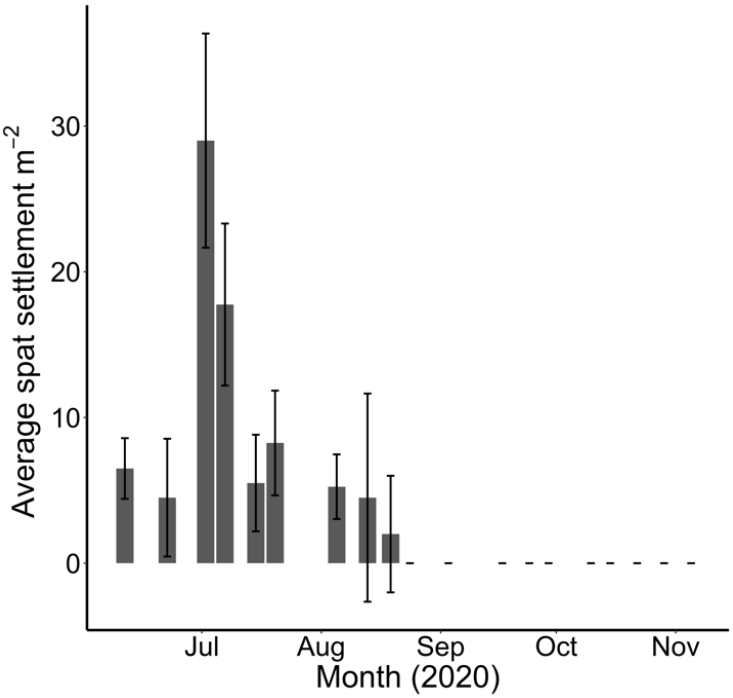

Figure 1.7. *Crassostrea virginica* spat settlement m^{-2} and SE observed in 2020 in GBE.

DISCUSSION

Temporospatial patterns of oyster spawning and recruitment activity

There was a general lack of spatial variation in larvae and recruitment across the different sites, which is expected as GBE is an extremely well-mixed estuary except at river mouths (Brown and Arellano 1980). This study revealed that oyster larvae likely were present in waters before sampling began in June as oyster larvae already were abundant in early June, which is indicative of prior spawning activity. Furthermore, larvae continued to be observed in November. Prior studies of eastern oyster recruitment in GBE and in other northern estuaries indicate that recruitment activities peak between late July and mid-August (Ayer et al.1970, Kennedy 1996), and that spawning occurs from June to October (Cox and Mann 1992, Haven and Fritz 1985, Mann et al. 2014). Unlike reports from other estuaries (Carriker 1951, Dekshenieks et al. 1996), salinity did not appear to have an impact on larval abundance in this study. Oyster settlement was observed in mid-June, peaked in early July, and ceased around mid-August. Spat settlement in mid-June suggests that eastern oysters in GBE likely spawn by late May, assuming prior data are correct for approximately ~32 days to settlement (Carriker 1951, Dekshenieks et al. 1993). This informs us that restoration efforts should occur earlier in the season than they currently are conducted. Planting oysters earlier in the season will allow for a longer growing season and potential for larger oysters. Despite the continued presence of larvae in the water column, spat were not observed after August on the experimental collection tiles. The lack of set is notable because whereas oyster larvae follow a generally uniform distribution in the water column, more mature larvae are known to inhabit the lower levels of the water column (Carriker 1951) and should therefore be available to set. However, due to unique mixing in GBE (extremely well-mixed estuary), this cannot be

assumed for GBE. As a result, future studies would benefit from incorporation of hydrodynamic modelling to ascertain how oyster larvae move and settle within GBE, especially regarding determining optimal sites for reef restoration.

At all sites, the cages containing oyster settling tiles were noted to have potential predators such as oyster drills, *Urosalpinx*, and green crabs, *Carcinus maenas*. Although there are studies showing predation of oyster drills and green crabs on eastern oysters (Poirier et al. 2017), they are not yet known to be predators of oyster spat/larvae. Lack of appropriate settling area is an additional likely option to explain low numbers of spat. At OF (an oyster farm site), it was noted that from mid-August to the start of November, large quantities of tunicates, *Botryllus schlosseri* (established, Yund et al. 2015) and *Botrylloides violaceus* (invasive), colonized the recruitment devices. Previous studies have shown that colonial ascidians inhibit settlement by other species near or on their tunics (e.g., Dijkstra et al. 2007). Furthermore, colonies can fuse to create large colonies that occupy a greater amount of contiguous space, further limiting the amount of bare space for oyster settlement (Westerman et al. 2009). Ascidians have limited capacity to regulate salt concentrations, which limits their distribution to areas with higher (>20) salinity (Dijkstra et al. 2008, Lambert et al. 2018) and may mitigate their potential effects on settlement at river mouths where salinity is slightly lower. Finally, nutrition has long been acknowledged as a factor in larval period and settlement success (Stanley and Sellers 1986) and it is known that eastern oysters consume phytoplankton selectively based on size and the chemical quality of the food particles (Baldwin 1995, Weissberger and Gilbert 2021). Future studies focused on the composition and size ranges of phytoplankton communities may shed further light on the lack of spat.

Data from previous years (Eckert 2016) and 2020 show a significant decrease in oyster D-hinge larval abundances in GBE and veliger larval counts that are exceedingly low, 10-100 times lower than oyster larval abundances in other estuaries (Carriker 1951, Andrews 1982, Wahyudin and Yamamoto 2020, Cristo et al. 2021). To obtain a measurement of local recruitment, The Nature Conservancy has placed recruitment devices at or adjacent to five native oyster reefs and three restored reefs since 2018. Although spat did recruit to the devices, results were variable, consistently low at all sites within Great Bay proper, and there was relatively more recruitment at the Lamprey and Squamscott sites. The GBE 2020 spat set (generally < 10 spat m^{-2}) is half the magnitude observed at these GBE sites in 2013-2015 by Eckert (2016) and is 2-3 orders of magnitude lower than observations for *Crassostrea* settlement in other estuaries along the North American Atlantic coast (VIMS 1986, Powell et al. 2009, Narváez et al. 2012, Peters et al. 2017, Southworth and Mann 2020). Thus, the dearth of veligers is the most likely cause of recent spatfall failures in GBE.

Effect of physicochemical parameters on larval abundance

Of the physicochemical changes that were recorded, temperature and chlorophyll levels appeared to drive the phenology and densities of D-hinge and veliger larvae in GBE. Increases in salinity were slightly associated with a decline in veligers but were not otherwise informative. These results concur with other studies that found equivocal correlations among spawning activity, settlement, water temperature, and salinity (Carriker 1951, Cox and Mann 1992, Kim et al. 2010, Narváez et al. 2012). This study reveals a lengthening of the reproductive phenology of the eastern oyster in GBE that quite possibly is related to increased temperature as other studies have shown that elevated temperatures have resulted in shorter planktonic development, longer growth, and

altered reproduction of ectothermic species (e.g., Kimmel and Newell 2007, Dijkstra et al. 2017). Consequently, it is important to begin future sampling by April, possibly earlier. This will provide a more complete understanding of the relationships between physicochemical factors and oyster larval abundance in GBE.

Importance of a baseline

Oyster restoration in estuarine ecosystems is necessary for living resources management and is one of many approaches focused on reduction in the negative effects of eutrophication by top-down filter feeding excess phytoplankton (Fulford et al. 2007) but cannot alone alleviate the effects of algal blooms (Pomeroy et al. 2006, 2007). The larval abundance data presented here can help focus oyster restoration efforts for when and how they are most likely to lead to the resurgence of the GBE oyster population. Additional matured oysters are needed to produce a sustainable amount of larvae for the oyster population in GBE. This baseline knowledge of the occurrence and distribution of oyster larvae within GBE can be used in concordance with NH Fish and Game Review of Oyster Data published online each year, which tracks adult oyster numbers in GBE. In other estuaries (Chesapeake Bay Virginia, Cedar Point Alabama, Hiroshima Bay Japan), despite a focus on restoration efforts, similar dramatic declines in successful oyster restoration efforts have been observed (Rothschild et al. 1994, Kim et al. 2013, Wahyudin and Yamamoto 2020). It has been shown that optimal spat settlement correlates with a 1–2-week period after peak larval abundance for the season (van den Brink et al. 2020), demonstrating the necessity to be able to determine the peak larval abundance and ensure appropriate setting substrate. Studies have shown that climate change has the potential to cause mismatches between phytoplankton blooms and spawning phenology in certain fishes (Asch et al. 2019, McQueen and Marshall 2017), and it is

likely that these impacts do not apply exclusively to fish taxa. These data have shown that high chlorophyll levels correspond with a spike in D-hinge and veliger larvae, where spikes should occur several days after. This most likely is due to only sampling once per week, as there could have been a higher level of chlorophyll previously. Thus, given the low possibility that physicochemical characteristics are the driver of reduced larval abundance, restoration efforts in GBE should consider factors other than simple physicochemical variation (e.g., toxic phytoplankton blooms, nutritious phytoplankton, quantities of larvae relative to the proximity of oyster reefs) to better comprehend how eastern oysters are responding to these interacting factors in New England.

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CHAPTER 2 – INFECTION ETIOLOGY OF CULTIVATED AND WILD OYSTERS, *CRASSOSTREA VIRGINICA*, OF GREAT BAY ESTUARY

INTRODUCTION

Diseases in eastern oysters affect their ability to act as filter feeders: *H. nelsoni* infects the gill epithelium in primarily young oysters or spat (Ford and Haskin 1982), and *P. marinus* invades the gut causing a "wasting" or emaciated oyster (Ford et al. 1999). In Great Bay Estuary (GBE), both Dermo (caused by *Perkinsus marinus*) and MSX (caused by *Haplosporidium nelsoni*) are widely spread in the oyster population (Odell et al. 2006, Eckert 2016). In 1995, there was a spike in advanced MSX infections in GBE and shortly thereafter, MSX abundance declined to the point where between 2000-2013, infection levels were relatively low and stable. Dermo exhibited an extremely low abundance from 1996-2003, but the prevalence has increased since that time (Grout 2014). Whether there is a relationship between these two oyster diseases currently is unknown and there has not been research focusing on why the diseases display alternate infection patterns. It is reasonable, however, to expect that the switch is due to a combination of tolerance to MSX (aquaculturists generally stock MSX-tolerant oysters) and to physicochemical factors that are known to affect infection rates. Understanding the infection rates between the two types of GBE oysters (wild and cultivated) could provide new knowledge about the infections and could lead to the suggestion of possible methods for reducing infection rates in the eastern oysters of GBE.

Disease causing organisms traditionally have been detected and quantified by histology (Ewart and Ford 1993, Dungan and Bushek 2015) and to this day, histology remains a common strategy for identifying oyster disease. To diagnose MSX, where the infectious agent tends to be systemic,

a sample of haemolymph is collected and analyzed. To diagnose Dermo, where the propagules are more localized, the rectum and often a portion of the mantle is excised and analyzed. Stokes et al. (1995) developed a method to identify *H. nelsoni* infection by successful amplification of the DNA of the parasite in eastern oyster tissue samples that were confirmed to be infected with MSX via histology. Although the PCR method was successful, it lacked the ability to quantify the infection. The assay developed by Stokes et al. (1995) was modified to enable the quantification of infection using quantitative competitive PCR (QCPCR) (Day et al. 2000) using known quantities of "competitor" DNA to determine the amount of infection present in the tissue samples. A PCR-based assay to detect *P. marinus* was developed by Marsh et al. (1995) and termed "dilution endpoint" PCR, which was a semiquantitative assay. Soon thereafter, a highly specific assay was developed to detect a single *P. marinus* cell in 30 mg of oyster tissue (Robledo et al. 1998). Later, an assay was developed to detect *P. marinus* that supported the specificity of using QCPCR, making it the preferred method of infection detection (Yarnall et al. 2000). Rather than using QCPCR, Penna et al. (2001) developed a multiplex PCR (MPCR) using three sets of primers to detect *P. marinus, H. nelsoni,* and *H. costale* in a single PCR reaction. This combined MPCR was modified by Russell et al. (2004) to detect (but not quantify) the parasites more rapidly. To detect and quantify *H. nelsoni* and *P. marinus*, Brooks (2004) built upon the QCPCR technique and created a single PCR reaction that used one set of primers that generated different sizes of amplicons for both parasites. A duplex-quantitative real-time PCR (dq-PCR) using two sets of primers were constructed for both pathogens, using the 18S ribosomal RNA region in *H. nelsoni* and the internal transcribed spacer in *P. marinus* (Xie et al. 2013). The current study employed the Brooks (2004) method for simultaneous detection and quantification at an efficient cost because it does not require labeled primers or probes and can be performed in a standard thermal cycler.

Section A of this chapter recounts a study of *H. nelsoni* and *P. marinus* in the water column of both farmed and wild oysters in GBE. This will provide insight into how the two infectious agents are changing throughout the sampling season (June 2020 – November 2020) as well as if there are any differences between farms and natural reefs. Section B will cover results of a pilot study to test a small sample of wild and aquacultured oysters for *H. nelsoni* and *P. marinus* levels to gain additional knowledge and facilitate future studies.

CHAPTER 2A – ASSESSMENT OF *H. NELSONI* AND *P. MARINUS* IN GBE WATERS

METHODS

Collection of samples

Water samples were collected each week from June – November 2020. Sites were selected based on known locations of oyster reefs or farms in the Bay. Collections were conducted at Adams Point, Woodman Point, Nannie Island, and an oyster farm in Little Bay in 2020. Replicate horizontal surface tows using a 64-µm mesh net were conducted once per week at the 4 sites. Tows were conducted ~0.3 m below the surface for approximately 2 min at 0.5-1.0 m sec^{-1}, resulting in an average of 38 m^3 of water sampled per tow. Samples collected in 2020 were preserved with formalin sucrose (Haney and Hall 1972).

DNA extraction and quantification

Water samples were filtered through a 2-µm filter (PALL) using vacuum filtration and were rinsed with 50-mL of the same sample. Filters were carefully rolled and placed into a bead beating tube of the QIAGEN DNeasy PowerWater kit. Protocols from the QIAGEN DNeasy PowerWater kit (GEN, Inc., Valencia, California) were then followed to yield approximately 100µL of extracted DNA at a concentration of ~ 7 ng μL^{-1}. Quantity and quality of the extracted DNA was determined using the TapeStation™ Genomic DNA ScreenTape analysis protocol (Agilent). The concentration of each sample was standardized to 5 ng μL^{-1} and used in PCR to determine the relative amounts of infectious organisms.

PCR to detect presence of oyster larvae, H. nelsoni, *and* P. marinus *in the water column*

The presence of oyster larvae in water samples was determined by PCR using the Cvi11 primer (Table 1) that amplifies a 153 bp microsatellite segment specific to eastern oysters. Each reaction mixture contained 0.5 volume of master mix (Promega GoTaq Green), 0.5 µM Cvi11-F, 0.5 µM Cvi11-R, 0.03 volume of 4mM spermidine, 0.05 volume of 10X BSA, 25 ng of DNA, and sufficient water to bring the volume to the desired total reaction volume. Reactions were cycled in a PTC-100 programmable thermal cycler with a heated lid (MJ Research, Inc., Waltham, Massachusetts) programmed to the following conditions: initial 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 15 sec. Once PCR was completed, amplicons were electrophoresed in a 4% agarose E-Gel (Invitrogen ThermoFisher Scientific) for 23 min and visualized under a blue light transilluminator (ThermoFischer Scientific). Water column samples were tested for both *H. nelsoni* and *P. marinus* using the primers HnPm-A and HnePsp-B (Table 1) that target fragments of 335 bp for *H. nelsoni* and 368 bp for *P. marinus*. Reactions were generated using 0.5 volume of OneTaq™ colorless master mix with standard buffer (New England BioLabs), 0.5 µM HnPm-A, 0.5 µM HnePsp-B, 0.03 volume 4mM spermidine, 0.05 volume of 10X BSA, 25 ng of DNA, and sufficient water to bring the total reaction volume to 50 µL. Reactions were cycled in a PTC-100 programmable thermal cycler with a heated lid (MJ Research, Inc., Waltham, Massachusetts) programmed to the following conditions: initial 95°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec. Amplicons were separated in a 4% agarose E-gel for 23 min and visualized under a blue light transilluminator (ThermoFischer Scientific). Samples that tested positive for either *H. nelsoni* or *P. marinus* were subjected to QCPCR to quantify infection levels.

QCPCR for detection and quantification of H. nelsoni and P. marinus

A competitive sequence of similar size (281 bp) to the *H. nelsoni* and *P. marnius* amplicons was generated using the HnPm-A and HnePsp-BC primers (Table 1), using known infectious DNA as template in a PTC-100 programmable thermal cycler with a heated lid (MJ Research, Inc., Waltham, Massachusetts). The protocol was initiated at 95°C for 2 min and was followed by 23 cycles of a 30 sec at 94°C, 30 sec at 57°C, and 30 sec at 72°C. After PCR, the competitive sequence was purified using a QIAquick PCR purification kit (QIAGEN, Inc., Valencia, California). The resulting competitor amplicon was purified and subsequently quantified using TapeStation™ with the same protocols used for extracted DNA. The competitor was serially diluted to yield the following concentrations: 0.250 pg μL^{-1}, 0.125 pg μL^{-1}, 0.025 pg μL^{-1}, 0.012 pg μL^{-1} and 0.002 pg μL^{-1}.

Detection of *H. nelsoni* and *P. marinus* DNA in water samples was accomplished using a single PCR reaction (HnPm-A and HnePsp-B). For those specimens showing one or both parasites in the initial detection reaction, quantification was performed using replicate arrays of five serial reactions were performed, each containing one of the competitor dilutions. Each 50 µL amplification reaction mixture consisted of 2 µL of the appropriate competitor stock solution, 1.3 µL of 4 µM spermidine, 2.5 µl 10X BSA, 2 µL each of the appropriate primers at 10 µM, 25 µL OneTaq colorless master mix with standard buffer (New England BioLabs), 12.3 µL deionized water, and 3 µL of the DNA mixture isolated from water. Reactions were cycled in a PTC- 100 programmable thermal cycler with a heated lid (MJ Research, Inc., Waltham, Massachusetts) beginning with 2 min at 95°C, followed by 30 cycles consisting of 30 sec at 94°C, 30 sec at 57°C, and 30 sec at 72°C. Products were stored at 4°C until used for gel electrophoresis.

Products of QCPCR were electrophoresed in adjacent wells of 5% Criterion™ TBE polyacrylamide gel (Bio-Rad), stained with GelRed Nucleic Acid Stain (Thomas Scientific), and visualized using a blue light transilluminator (ThermoFischer Scientific). Relative fluorescence was analyzed using Adobe Photoshop to obtain the integrated density of each band. The relative fluorescence intensities of fragments were compared to each other and densitometric analysis was used to quantify the level of infection in each of the sampled oysters (Brooks 2004). The lane(s) in which the intensity of the competitor (281 bp) and the targets (335 bp and 368 bp) were approximately equal represented the "zone of band equivalence" (originally described by Reiner *et al*. (1993) and implemented by Brooks (2004) for oysters) for the DNA of each parasite. Where the zone of equivalence was intermediate between two adjacent lanes, interpolation was employed to determine the "competition equivalence point" for each parasite.

Primer	**Sequence**	**Reference**
Cvi11	F: 5'–ATCGGCCAGTGACTACCTTGTAAAAG–3' R: 5'–GCGATAACACTAAATACTTTGTTTCGGCCC–3'	Brown et al. 2000
HnPm-A	5'–AGCCATGCATGTCTAAGTATAA–3'	Brooks 2004
HnePsp-B	5'–GATGTGGTAGCCGTTTCTCAGG–3'	Brooks 2004
HnePsp-BC	5'–GATGTGGTAGCCGTTTCTCAGGGCCCATATCCTACCGTCAAGC–3'	Brooks 2004

Table 2.1. Primers used for PCR and QCPCR

Analyses

The concentrations (analogous to infection intensity when used to diagnose infection in oysters) of *H. nelsoni* and *P. marinus* DNA amplified from water samples (both farm and reef) were

corrected for filtration and dilution volume. Non-parametric tests were used to analyze results because data were not normally distributed and did not have equal variances. Kruskal-Wallis tests were run between the two groups (farm and reef) for each infectious agent to determine if there was an overall difference between oyster reefs and oyster farms in terms of detected quantities of *H. nelsoni* and *P. marinus* independently. A Wilcoxon rank test was used to determine differences between *H. nelsoni* and *P. marinus* concentrations throughout the entirety of the sampling season.

RESULTS

The levels of both infectious agents were highly variable throughout the sampling period (Fig. 2.1) and did not differ significantly (*H. nelsoni* $p = 0.840$, Fig. 2.2; *P. marinus* $p = 0.605$, Fig. 2.2). No significant differences were found in the levels of each infectious agent throughout the sampling season ($p = 0.5680$). No statistically significant differences were found between levels of the two disease agents in the water column in the vicinity of farmed and wild oysters: MSX ($p = 0.7666$, Fig. 2.2) and Dermo ($p = 0.5315$, Fig. 2.2).

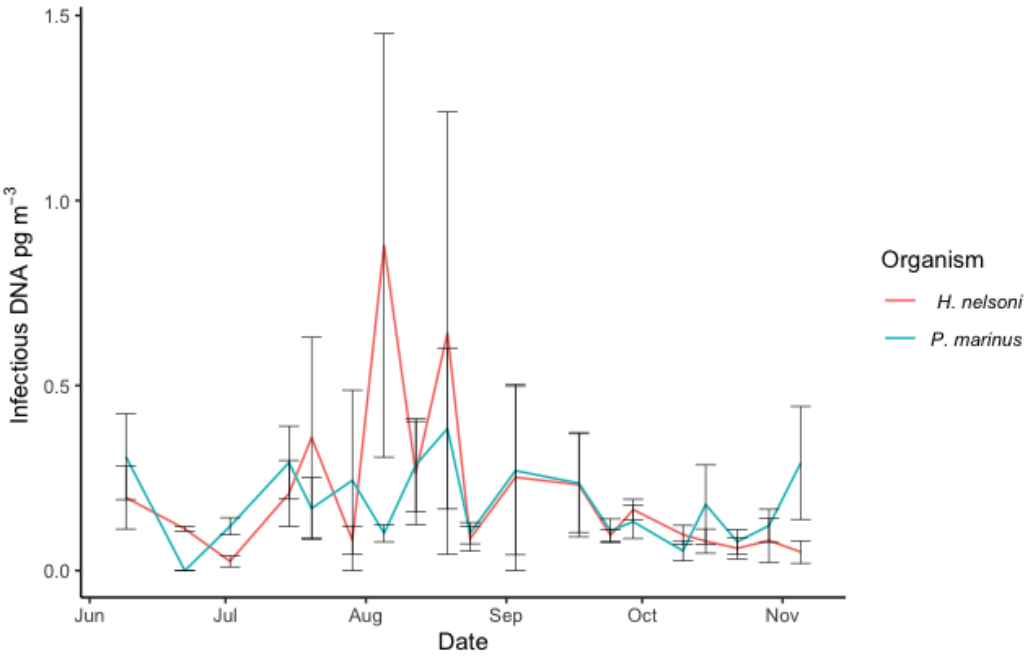

Figure 2.1. Trends in the water column concentration of *H. nelsoni* and *P. marinus* near farms and oyster reefs in GBE for weekly samples collected June - November 2020.

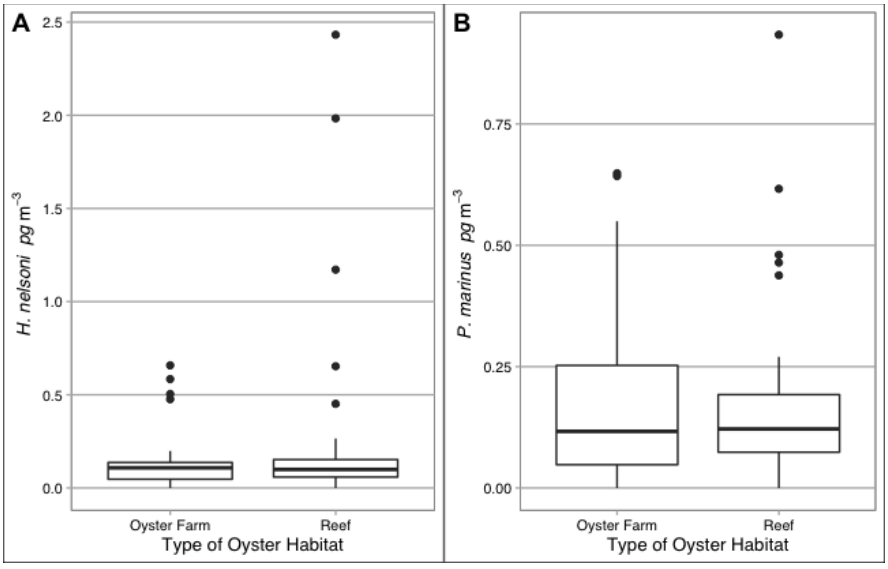

Figure. 2.2. Comparison of A) *H. nelsoni* and B) *P. marinus* levels in the water column of oyster farms and oyster reefs in GBE sampled weekly June - November 2020.

CHAPTER 2B – COMPARISON OF *H. NELSONI* AND *P. MARINUS* IN WILD AND AQUACULTURED EASTERN OYSTERS OF GBE

METHODS

Collection of samples

Oyster samples were collected at the beginning of the season (June 2020) from an oyster reef (Woodman Point) and a farm (Choice Oyster farm) in GBE. In addition, six oyster tissue samples were obtained from Emily McGurk and Dave Bushek at the Rutgers University Haskin Shellfish Research Laboratory. Those six specimens previously were diagnosed using histology and classified as no disease, low disease, medium disease, or high disease. All oyster tissue samples (wild, cultivated, and those previously diagnosed) were homogenized by blending the tissue in 5 volumes of PBS.

DNA extraction and quantification

Approximately 250 µL of oyster homogenate was added to the bead tube and DNA extraction from that point followed similar procedures as described above in Chapter 2A using the Power Water Kit (QIAGEN, Inc., Valencia, California) for extraction and the TapeStation (Agilent) for quantification. The resulting 100 µL of extracted DNA had an average concentration of 100 ng μL^{-1}, which was then diluted to 25 ng μL^{-1}.

QCPCR for detection and quantification of H. nelsoni and P. marinus

QCPCR was run with the extracted oyster tissue DNA using the same methodology as in Chapter 2A.

Analyses

Because of major differences in infection etiology and the methods used for preparation for histology (excise the rectum) *versus* molecular diagnosis (homogenize whole remaining organism), a multiplier was needed to adjust the quantities of Dermo DNA in the six oysters used for validation; preliminarily determined to be a factor of 3.5 (Table 2.2). Results for both infectious organisms from QCPCR were classified as no infectious agent (<0.001 pg μL^{-1}), low (0.001 pg μL^{-1} < 0.015 pg μL^{-1}), medium (0.015 pg μL^{-1} < 0.035 pg/ μL^{-1}), and high (>0.035 pg μL^{-1}) (Table 2.2). Because error terms were not normally distributed and did not have constant variances, non-parametric tests were used to analyze the results. A Kruskal-Wallis test was run between the wild and aquacultured oysters for each infectious agent to determine if there was an overall difference between wild and aquacultured oysters in terms of infection intensity of *H. nelsoni* and *P. marinus* independently.

RESULTS

For *H. nelsoni* samples provided by Haskin Shellfish Research Laboratory, the diagnostic concentration of DNA from QCPCR corresponded precisely with histological classification. For *P. marinus*, correspondence between the DNA and histological diagnoses required use of an adjustment factor to account for the fact that all relevant tissues were not available for molecular diagnosis (Table 2.2, Fig. 2.3). The Kruskal-Wallis tests showed a significant difference between infection rates of *H. nelsoni* in aquacultured and wild oysters ($p < 0.001$) (Fig. 2.4A) but not between infection rates of *P. marinus* in the aquacultured and wild oysters ($p = 0.1277$) (Fig. 2.4B).

Sample	Organism	QCPCR DNA (pg µL^{-1})	Adjusted QCPCR DNA (pg µL^{-1})	QCPCR Class	Histology Classification
1	*H. nelsoni*	0.008	NA	Low	Low
1	*P. marinus*	0.008	0.030	Medium	Medium
2	*H. nelsoni*	0.008	NA	Low	Low
2	*P. marinus*	0.011	0.038	High	High
3	*H. nelsoni*	0.009	NA	Medium	Medium
3	*P. marinus*	0.011	0.038	High	High
4	*H. nelsoni*	0.115	NA	High	High
4	*P. marinus*	0.005	0.019	Medium	Medium
5	*H. nelsoni*	0.224	NA	High	High
5	*P. marinus*	0.260	0.909	High	N/A
6	*H. nelsoni*	0.000	NA	N/A	N/A
6	*P. marinus*	0.002	0.006	Low	Low

Table 2.2. QCPCR DNA concentrations for *H. nelsoni* and *P. marinus* in eastern oysters compared with histological classifications from the Haskin Shellfish Research Laboratory at Rutgers University. Adjustment is needed only for Dermo comparisons because the rectum of those oysters was removed for histological diagnosis.

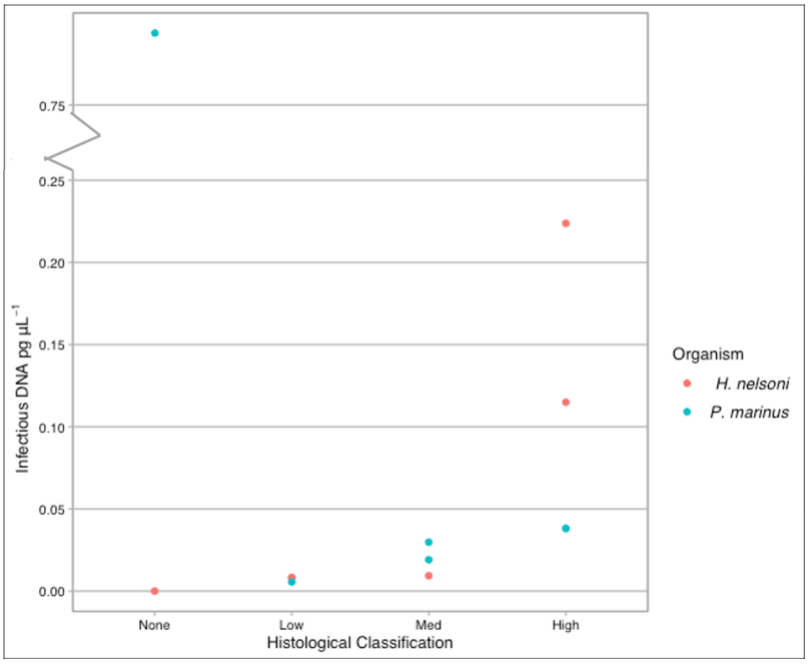

Figure 2.3. Infectious DNA concentration *versus* histological diagnosis of *H. nelsoni* and *P. marinus* in six eastern oysters diagnosed by two different techniques.

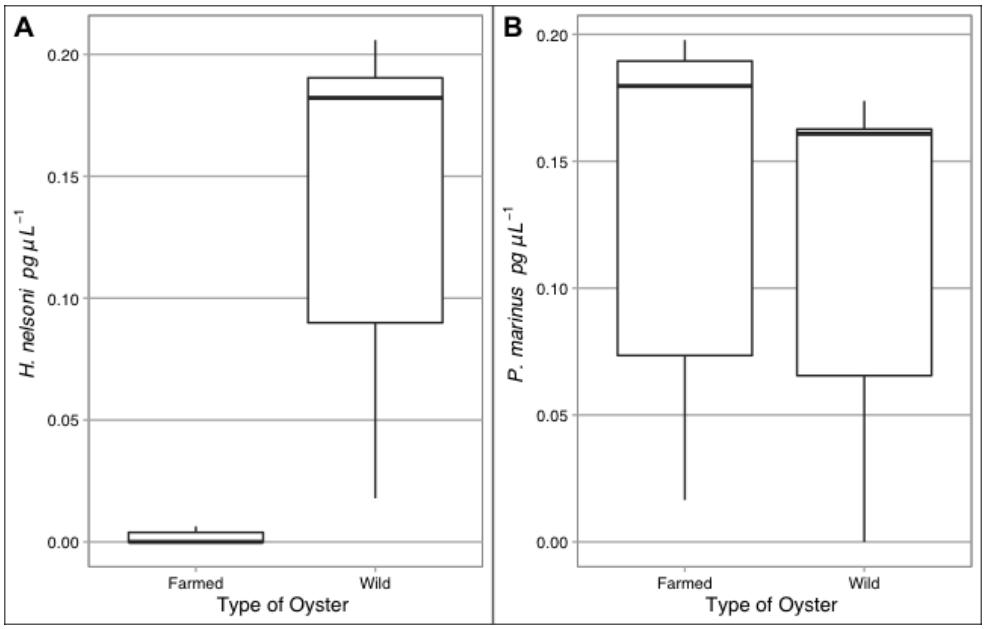

Figure. 2.4. Infectious DNAs in farmed and wild eastern oysters in GBE. A) *H. nelsoni* and B) *P. marinus*.

DISCUSSION (CHAPTERS 2A and 2B)

Infectious organism presence in the water column

This study found that although similar amounts of the infectious agents are found throughout GBE waters, the levels of disease in eastern oysters in GBE vary by oyster type (wild and aquacultured oysters). No prior studies have documented the distribution of *H. nelsoni* and *P. marinus* throughout the water column of an estuarine system. This work shows that in GBE, these two infectious agents are not localized to specific areas, but rather the entire estuary system, which is not surprising given that GBE is a highly mixed system (Brown and Arellano 1980). Very high (>0.1 pg μL^{-1}) levels of *H. nelsoni* were found in the water column from late-July through late-August, which coincided precisely with peak eastern oyster larval abundances (Stasse et al. 2021). Currently, the mechanism for infection for *H. nelsoni* is unknown (Ford et al. 2018) and the relationship uncovered in this study could indicate that *H. nelsoni* is dispersed in eastern oyster larvae, demonstrating the need for future studies to explore eastern oysters as a vector for *H. nelsoni*. Investigating individual oyster larvae and looking more closely throughout the sampling season (i.e., water samples every day or every other day during peak times rather than once a week) could provide more in-depth detail on how *H. nelsoni* and eastern oyster larval abundance co-vary, and how the two infectious agents vary throughout peak times.

Comparison of QCPCR vs. histology for identification of H. nelsoni *and* P. marinus

This work demonstrated the ability QCPCR to accurately diagnose *H. nelsoni* and *P. marinus*. Since histology for *P. marinus* looks at the rectum for cells and this QCPCR method homogenized the entire oyster, it was necessary to determine the factor needed to adjust for the whole oyster vs. site-specific studies that might compare molecular and histological diagnoses. Preliminary work

shows a factor of 3.5 accounts for homogenizing oyster tissue minus the rectum. Histology is a low-cost and efficient method for diagnosing infection. Errors can be incurred with counting cells in Ray's fluid thioglycollate medium (RFTM) culture assay (Audemard 2008). This can account for the sole sample where diagnosis did not concur (no disease was detected using histology *versus* high disease via QCPCR). Although QCPCR can precisely quantify the concentration of infectious cells in oyster tissue, it is subject to errors in thermal cycling (especially during thermal cycles 1 and 2 in the protocol) and precision of diluting the QCPCR competitor. A distinct advantage of the QCPCR method for the present study is that it allows investigation of specimen types (individual oyster larvae, plankton in water, putative reservoirs, or other intermediate hosts of the parasites) that cannot be diagnosed using histology.

Diseases in wild and aquacultured eastern oysters

Diseases in eastern oysters affect their ability to act as filter feeders: *H. nelsoni* infects the gill epithelium in primarily young oysters or spat (Ford and Haskin 1982, Fig. 2.5), and *P. marinus* invades the gut causing a "wasting" or emaciated oyster (Ford et al. 1999, Fig. 2.6). Understanding disease levels in reefs or farms of eastern oysters is incredibly important for restoration, oyster consumption, aquaculturists, and the marine ecosystem. For aquaculturists, heavily infected oysters should not be sold as the flesh shows the consequences of infection.

Disease tolerance in eastern oysters is incredibly important for both aquaculture and preserving the health of marine ecosystems. The dichotomous results between farmed and wild oysters is most likely related to the fact that oyster farmers use an MSX-tolerant stock of eastern oysters developed by Rutgers University (Haskin and Ford 1979, Ford and Haskin 1987). Although the selectively

bred oysters can be infected with *H. nelsoni*, the host oysters can restrict the parasites to small, non-lethal lesions in gill or palp epithelia, preventing debilitating infection (Ford and Haskin 1987). Results from this study showed low levels of infection in several aquacultured oysters, but none substantial enough to create a systemic infection (Fig. 2.4). If enough aquacultured oysters successfully reproduce on the farm sending MSX-tolerant offspring throughout the Bay, this eventually could result in a positive effect on MSX tolerance of the wild population. Conversely, the Dermo results of this study underscore the next challenge for oyster farmers and reef restoration biologists in GBE: testing dermo-tolerant oyster strains in this region.

Future implications of diseases in eastern oysters of GBE

This chapter introduced a pilot study of two topics that are relevant to oyster cultivation and restoration: *H. nelsoni* and *P. marinus* in the water column of GBE and infection rates of MSX and Dermo in aquacultured *versus* wild oysters. These findings provide insight that could help mitigate diseases in eastern oysters not only in GBE but elsewhere. Given what was learned from this study, an investigation of more oysters at varying levels of development (larvae, spat, 3 cm, 7.6 cm, and broodstock) should provide important additional information on etiology and how the diseases are changing throughout the life cycle of an eastern oyster.

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CONCLUSIONS AND FUTURE DIRECTIONS

Eastern oyster populations have been on the decline for decades due to factors such as disease, changing climate, and overharvesting (MacKenzie Jr. 2007). This study began by examining eastern oyster larval abundance at two developmental stages (D-hinge and veliger) in GBE throughout the seasons when they were thought previously to be most abundant. Of the available physicochemical data in GBE, only temperature had a significant positive effect on numbers of both D-hinge ($p < 0.001$, adj. $R^2 = 0.5086$) and veliger ($p = 0.021$, adj. $R^2 = 0.279$) larvae, and as the temperature decreased, larval abundance decreased. Salinity, although not significant in this study, previously has been found to influence eastern oyster larval abundance (Carriker 1951, Dekshenieks et al. 1996, Stasse et al. 2021). Finally, despite its implication in numerous other reports, pH showed little to no effect on larval abundance in GBE. The timing and high variability of peaks in larval abundance may be a signal of rapid climate change that is driving how oyster larvae are changing throughout the growing season. Before this, no study has examined how physicochemical factors affected oyster larvae in the GBE water column at both farms and reefs. The current data constitute an important baseline for eastern oyster larval dynamics in GBE. These data will help aquaculturists to know the best times to purchase and remote-set oyster larvae. In addition, because we now know that oyster larvae and disease agents are dispersed uniformly in GBE, we can better appreciate the importance of disease-tolerant oyster stocks. Future studies should help to determine whether the larval trends are a purely demographic phenomenon, as this study indicates, or possibly due to other factors such as predation, competition, or lack of suitable settling habitat.

Trends in abundance of disease-causing *H. nelsoni* and *P. marinus* throughout GBE waters and the general low levels of MSX infection in aquacultured oysters right alongside reefs that exhibited high levels of MSX infection offer a signal to what restoration practices may be best for GBE. Work already is underway to stock reefs with MSX-tolerant aquacultured oysters, sometimes called "uglies" because they are too large to sell commercially and have scars of many biofouling agents on their shells. Assuming these oysters survive Dermo and changing climate conditions and they avoid harvest, they may constitute a critical source of larval input to GBE, helping to enhance natural oyster production. Modeling efforts could provide an indication of how many and where such oysters should be deployed. Knowing that MSX is very low in the aquacultured population whereas, unfortunately, Dermo is very high in both wild and aquacultured oysters of GBE, illustrates the pressing need to breed Dermo disease-tolerant oysters.

This research project brings new insight to the eastern oyster population of Great Bay Estuary and why oyster numbers continue to decline. Currently, there is no known literature that explores the quantity of infectious agents in the water column throughout the sampling season in conjunction with larval abundance. In addition, this study adds to previous larval abundance data, which combined could help to assess the direction of oyster reef restoration efforts. These two components, in conjunction, assess potential threats to eastern oysters and how they impact larval abundance, which in turn affects recruitment and successful growth of adult oysters.

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