Estimates of Spatial and Temporal Patterns of Northern Quahog (*Mercenaria mercenaria*) Larvae in Narragansett Bay using qPCR Technologies

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Abstract

Benthic marine invertebrates have complex life cycles, with each life stage requiring specific habitats for survival. The larval stage for such sessile organisms is typically planktonic, representing the largest mechanism for population movement and dispersal. Thus, identifying larval spatial and temporal patterns is critical in understanding the population connectivity and dynamics allowing for these species to complete their life cycle. This study analyzes the larval abundances for Northern quahog (*Mercenaria mercenaria*), a commercially, economically, culturally significant shellfish of Narragansett Bay, RI. Plankton samples were taken in June and July (spawning season) of 2011 to enumerate larval abundances. Using quantitative polymerase chain reaction (qPCR) methods, samples' larval quahog DNA concentrations were estimated and converted to age-equivalent larval abundances (number m^{-3}). This work provides the first example of constructing a quahog-specific primer for larval enumeration. Age-equivalent abundances from samples varied over the Bay, with two spawning pulses identified during the 2011 spawning season. Over this time frame, larval abundances varied spatially based on management area type. Closed regions had higher densities than and the less restrictive management areas. These results highlight the effect of reduced harvest pressure on the quahog population's larval production, and the value of *de facto* or designated marine reserves in sustaining the stock's larval production. Larval densities and regressions presented are valuable for future validation of biophysical models predicting larval quahog densities from physical transport.

Key words: Northern quahog, larvae, qPCR, Narragansett Bay

Citation: Mercer, J.M., Oviatt, C.A., and McManus, M.C. 2016. Estimates of spatial and temporal patterns of Northern quahog (*Mercenaria mercenaria*) larvae in Narragansett Bay using qPCR technologies. Rhode Island Department of Environmental Management, Division of Marine Fisheries Technical Report Series. 32pp.

Introduction

Many marine species exhibit life cycles with stages requiring different habitats. Such requirements often result in life stages spatially segregated. For marine benthic invertebrates, life cycles usually consist of a demersal spawning stock releasing small pelagic eggs to be transported by tidal and ocean currents. Given that many of these species are sessile in their benthic phases, the early planktonic stage serves as the sole dispersal mechanism. Therefore, the dispersal of planktonic the larval stage is fundamental in structuring local and meta-population dynamics, genetic diversity, and the resiliency of populations to human exploitation (Cowen et al., 2007). Additionally, larval dispersal and transport become critical components of the life cycle for ensuring population connectivity between successive life stages, maintaining recruitment, and sustaining a harvestable population (Llopiz et al., 2014)

Larval dispersal has traditionally been assessed by modeling larvae as passive particles, with prevailing currents dictating larval trajectories. While some studies support show evidence for passive behaviors during larval transport (Scheltema 1995, Miller and Shanks, 2004), a mounting body of evidence suggests this view is inaccurate for many species that exhibit active behavior at specific larval ages or in response to environmental cues (Pawlik and Butman, 1993; Welch and Forward, 2001; Baker and Mann, 2003; Reyns et al., 2007). Accounting for larval behavior in dispersal modeling has shown to increase local planktonic retention, whereas passive dispersal via currents suggests longer distance transport (Cowen et al., 2006). Studies quantitatively tracking larval transport, have also found that the transport larvae do not necessarily match that of passive surface drifters

(Arnold et al., 2005). With evidence for both passive long-distance planktonic transport and local planktonic retention, there is a critical need to quantify true planktonic transport and local densities, and incorporate this knowledge into spatial management strategies (Hare and Walsh, 2007).

Understanding larval behavior, seasonality, and spatial distribution is imperative for properly managing economically significant shellfish species. Northern quahogs (*Mercenaria mercenaria*, or hard clams) are an important cultural and economic species found throughout the coastal northwest Atlantic, spanning from the Gulf of St. Lawrence through the Gulf of Mexico (Henry and Nixon, 2008). The Narragansett Bay, Rhode Island (Figure 1) quahog commercial industry also supports the largest fishery solely harvested in the Bay, with an ex-vessel value of over $5.4 million and supporting over 500 active shellfishers (ACCSP, 2016).

Northern quahogs also provide a significant ecological function in estuaries by filter feeding suspended organic matter and phytoplankton from the water column (Doering et al. 1986). The quahog is unique because it is one of the few species that has likely benefited from the anthropogenic impact of Narragansett Bay. Stable isotope analyses have shown that a large portion of the diet of quahogs obtained in the upper Bay region is derived from nutrients entering the bay as sewage (Oczkowski et al, 2008). Field studies also indicate that quahog predation is reduced by intermittent hypoxia induced by eutrophication and stratification, presumably because sedentary quahogs are more tolerant to low oxygen levels compared to their mobile predators that migrate out of areas during hypoxic conditions (Altieri, 2008).

Rhode Island management strategies involving closed areas for shellfishing due to pollution and runoff have been advantageous for sustaining quahog spawning stock and larval production. Approximately 27% of the Narragansett Bay is permanently closed to harvesting shellfish due to the human-health hazards associated with the consuming polluted shellfish (RI DEM Office of Water Resources, 2014). These non-harvested polluted areas therefore serve as *de facto* marine reserves where quahog biomass tends to accumulate and individuals can extend their life spans (Rice et al., 1989). Analyzing the quantity and distribution of larvae from areas closed to harvest and the reproductive contribution of these areas to the Narragansett Bay egg production is critical in developing appropriate management strategies for the quahog resource and fishery.

Biophysical models have been a common tool quantify benthic invertebrate larval abundances spatially, including the quahog (Arnold et al., 2005). However, most of these models are purely theoretical and have not been validated with quantitative measurements of larval densities spatially (Metaxas and Saunders, 2009). Observed invertebrate larval densities are often not used in these models due to the time intensive methods (i.e. sampling, species identification, enumeration). Microscopic identification of early stage bivalve larvae is particularly difficult (Loosanoff et al 1966; Le Pennec 1980); later stage larvae may be identifiable, but still require considerable expertise, effort, and time (Chanley and Andrews 1971). In the case of quahogs, a molecular testing has revealed that morphological identification of northern quahog larvae can result in a false positive rate of nearly 100% in natural plankton assemblages (Perino, et al.2008).

Given the complexities and difficulties with manual identification of marine fish and invertebrate larvae, nucleic-acid–based technology has emerged as an appealing

technique because of its potential to resolve two of the major constraints on larval transport studies: identifying and quantifying invertebrate larvae. Polymerase chain reaction (PCR) is a technique used to exponentially amplify a target sequence of DNA (Saiki et al., 1988). This technique has aided in accurately identifying the presence of microorganisms in samples. Quantitative PCR (qPCR) further allows to enumerate the amount of DNA by measuring fluorescence of double stranded DNA, providing estimates of total DNA and proxies for abundances. qPCR techniques have proven effective in quantifying specific phytoplankton species in mixed samples of marine plankton (Hosoi-Tanabe and Sako, 2005; Zhu et al., 2005; Countway and Caron, 2006). Vadopolas et al. (2006) showed that qPCR could be used to quantify mollusk larvae accurately down to a fraction of an individual larva when examining monocultures of pinto abalone larvae. In addition, Wight et al (2009) determined that Olympia oysters could be accurately enumerated using qPCR when combined with a mixture of larvae from 11 other common pelagic microorganisms.

This study aims to quantify quahog larvae from plankton samples during the spawning season in Narragansett Bay using qPCR. Quantifying larval densities will allow for describing the spawning period for quahog and areas of critical important for supporting larval production. Larval densities are also compared by region's management strategies to understand the importance of different management strategies in supporting one of Rhode Island's largest fisheries.

Methods

Primer Development, Larval Production and Standard Curves

A quahog specific primer for the mitochondrial DNA (mtDNA) locus encoding the cytochrome oxidase I gene (COI) was developed for this study. The COI gene has

been used for species identification due to its relatively rapid rate of evolution (Hebert et al., 2003). Hare (2000) found that the primer could be used to differentiate commercially important bivalve species; however, this quahog specific primer of the COI gene was found to produce some false positives, principally the amplification of the amethyst gem clam *Gemma gemma*, a highly abundant bivalve in New England coastal waters and a close relative of the quahog. Thus, a new quahog specific primer was developed in this study. A melt curve analysis, or dissociation curve, was used to detect spurious amplifications and the formation of primer-dimers or amplification of other regions of the genome. The reactions in this study used SYBR green as the reporter dye, which fluoresces when it binds to any double stranded DNA (Zipper et al., 2004). PCR products melt as they heat to a characteristic temperature, becoming single stranded and releasing the SYBR dye and decreasing fluorescence. The melting temperature is based on the length and base content of the PCR product.

A pure culture of quahog larvae was needed in order to test specificity of the quahog specific primer and the effectiveness of the qPCR method in unfiltered sea water samples. The pure culture was also required to obtain estimate mitochondrial DNA (mtDNA) concentrations in individual larvae to convert mtDNA quantity to number of larvae. Indoor phytoplankton production and heated seawater systems were used to condition and spawn quahogs and obtain larvae of known ages. After initiating adult quahogs spawning, sub-samples of larvae were preserved daily over a ten-day period to sample known aged larvae. A 10-day period was chosen to match the reported average larval duration of quahogs (Carriker, 1961). Obtaining age-specific larvae allowed for understanding how the quantity of mtDNA differed with age.

A light microscope was used to pipette known quantities of larvae (from 2-1000 individual larvae) into a series of microcentrifuge tubes qPCR. This process was performed for 3, 5, 8, and 10-day old larvae. Standard curves were developed for different quahog larval ages relating the age and quantity of larvae to the PCR cycle. An analysis of covariance was completed on the standard curves obtained from 3, 5, 8, and 10-day-old larvae.

When aged larvae from 3 to 10 days old were tested, they produced significantly different Ct levels, indicating different starting DNA template quantity (Figure 3). This result indicates that larger, older quahog larvae have more DNA than younger, smaller larvae, contrary previous research on other mollusks (Vadopolas et al. 2006, Wight et al. 2009). As such, without further calculation, only total quahog DNA in the samples could be used as a proxy for quahog densities. The amount of DNA in an individual larvae increased exponentially with age (Figure 2):

$$DNA_{larva} = DNA_{egg}e^{0.3(age)}$$

Assuming that total larval mortality is 95% over the average 10-day larval period with the same exponential rate, but of decay and not growth, a mortality model was derived to estimate the number of larvae at any age in the samples:

$$N_{larvae} = N_{egg}e^{-0.3(age)}$$

These relationships allowed for converting total DNA to an age-equivalent abundance. Similar approaches have been previously used in estimating instantaneous mortality for larval fish (Houde, 1989), with the estimated 95% mortality from egg to settlement stages derived in this study (Z=0.3) also falling within the range of the only published estimates of quahog larval mortality (Butet, 1997).

Field Sampling

In 2011, larval sampling was conducted over a six-week period beginning June 15 and ending on July 20th. This time period encompasses the majority of the typical spawning season of quahogs, usually beginning when the seawater temperature reaches 20°C (Loosanoff, 1937). Samples were taken at sixty sites throughout Narragansett Bay on a weekly basis to ensure high and feasible spatial and temporal coverage (Figure 1). The sampling sites were chosen based on physical characteristics, such as prominent geological features and known current patterns (Chen et al. 2008; Pfeiffer-Herbert et al., 2015). However, existing features, such as defined closed and managed shellfishing areas (Figure 1, Table 1) or navigation buoys, were also used in site selection in recognition of the long-term goals of a spatial management plan being developed and evaluate larval densities of these areas.

At each site, fifty liters of surface water were filtered through a 40 µm mesh sieve. The plankton collected on the mesh sieve were rinsed into a small glass jar and preserved in 50 ml of ethanol, thus 1 ml of preserved sample was equal to 1 liter of seawater. Time of day was also recorded assess tidal state during sampling.

qPCR Analysis

DNA was extracted from 1 ml subsamples of the field samples for quantification using qPCR. Initial DNA extractions of field samples utilizing methods from prior studies of bivalve larvae genetics (Hare et al., 2000; Vadopolas et al., 2006; Wight et al., 2009) resulted in no amplification in the field samples, likely because the DNA extraction techniques did not effectively remove PCR inhibitors. To reduce inhibitors,

DNA extraction DNeasy spin columns were used to extract and purify the DNA (Qiagen, 2006).

Quahog DNA enumeration in plankton samples was performed utilizing the Agilent Mx3005P qPCR system and Brilliant II SYBR green qPCR Master Mix following the manufacturers protocol (Agilent Technologies, Santa Clara, CA). Sixty samples from each sampling date (360 total samples) were run in duplicate. Eight standards of 10-day old larvae (to produce a standard curve) and a no DNA template control (NTC) were also run in duplicate for each analysis in the qPCR system. The Mx3005P uses 96 well plates so a total of 39 samples could be analyzed at once after accounting for standards and the NTC. Each well contained 12.5 µl of 2X SYBR Master Mix, 1 µl of forward primer, 1 µl of reverse primer, 0.5 µl of bovine serum albumin (BSA, a PCR inhibitor blocker), 0.375 µl of 1:500 ROX reference dye, 7.625 µl of ultrapure water, and 2 µl of sample DNA. NTC wells included an additional 2 µl of ultrapure water in place of the 2 µl of sample DNA. The thermal profile of each run was 10 minutes at 95°C, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 57°C for 33 seconds (during which time fluorescence measurements are made), and extension at 72°C for 20 seconds. This is then followed by a melt curve analysis during which the temperature is slowly ramped from 55°C to 95°C during which fluorescence measurements are made continuously.

The standard well containing only 2 larvae was used as an inter-run calibrator (IRC). MxPro software was used to analyze the data and set the cycle threshold (Ct) of each individual system run to a level where the well containing the IRC has the same Ct value throughout all runs being analyzed. This strategy provides the most comparable and accurate way of comparing between separate runs and reduces the amount of error due to

slight differences in reagents used. ROX reference dye was also utilized by MxPro software to correct for differences due to pipetting error.

Evaluation of Larval Densities

Using the DNA and mortality curves (Figure 2) and measured DNA from samples, quahogs densities were estimated as 10 day-old equivalent abundances (number m^{-3}), representing the amount of larvae that would survive to settlement age. While this assumption does indicate that larvae will settle at the location at which they were sampled or always at 10 days old, given ocean currents and larval behavior will cause them to disperse, it provides a reasonable indicator of potential sources or sinks in terms of quantity of larvae and a reasonable metric allowing for comparison of abundances representing mixed age classes.

Larval quahog densities were examined over space and time to understand temporal and stock-management influences on larval production. Kruskal-Walis tests were run to determine if mean densities differed significantly over different management areas within a given sampling period and over the entire sampling spawning period sampled. When significant differences were detected, post-hoc pairwise comparisons were made using Tukey and Kramer (Nemenyi) tests. For the statistical analyses, Closed/Polluted areas south of the Potowomut River and Mount Hope Bay were not included. Additionally, Managed areas included those within Greenwich Bay only.

Larval densities were also compared to the tidal state when sampled to evaluate whether ebbing or flooding may influence surface water larval densities based on more oceanic or river-derived water masses. Daily high and low tides were obtained from the Conimicut Light Station

(https://tidesandcurrents.noaa.gov/stationhome.html?id=8452944). Time from the nearest high and low tides (decimal hours) were calculated using the high and low tide times and time of sampling. For nearest high tide calculations, ebb (negative) and flood (positive) tides were represented in the decimal hours, with relative ebb and flood tides low tide calculations vice versa. Relationships between time to the nearest high and low tides of samples and larval densities (natural log transformed) were evaluated with linear models to assess correlation between tidal state and surface densities.

Results

Primer Development, Larval Production and Standard Curves

The dissociation curve from the quahog larval standards indicated that only the specific target sequence in the mtDNA was amplified to any appreciable extent, evident from the major peak (Appendix 1). No spurious amplifications were detected, confirming that the quahog specific primer is specific to the COI gene and is amplifying the appropriate region of the genome, accurately identifying quahog DNA.

The standard curves using known quantities and ages of larvae produced a strong correlation ($R^2 = 0.996$) between the number of larvae and the Ct value obtained from qPCR. The range of larvae represented in this relationship (2 – 1000 larvae) was much larger than what was found in field samples (Appendix 2). All of the standard curves for each age-specific larval DNA quantity were greater than 95% efficient (Figure 3), indicating no inhibition or insufficient reagents in concentrated samples.

Spatial and Temporal Trends in Larval Abundance

Abundances for 10 day-old equivalent larvae were estimated over all sites in each of the six weeks using qPCR results (Figure 4). The sampling appeared to begin

before any significant spawning had occurred in the Bay, as the June 15-16 sampling event showed very few quahog larvae throughout most of the Bay (Fig. 4a). During this time frame, larvae (<100 m^{-3}) were found in areas in surface waters near Warwick, Apponaug, and Greenwich Coves in Greenwich Bay, in the East Passage north of Prudence Island, and the Warren River (Fig. 4a). All other stations had densities far less.

Nearly all of the samples from June 21-22 were taken on a flooding tide or within a half hour of high tides. A major spawning event appeared to have occurred after June 15-16, as larvae densities from June 21-22 were much greater in closed shellfishing areas of the Providence River, Apponaug Cove and in and around the Warren River (Fig 4b). Conditionally closed areas south of the closed Providence and Warren Rivers also had relatively high densities. The areas just south of Greenwich Bay and east of Potowomut cove also showed moderate densities of larvae and were the only samples taken at the end of an ebb tide or at low tide.

During the next sampling event from June 28-29 most of the samples were taken on an ebb tide (Fig. 4c). The highest concentrations were observed south of the Warren River in the conditionally closed areas, in Apponaug and Warwick coves, and in the area surrounding Warwick Neck and Patience Island. The Providence River was sampled at low tide and the amount of larvae was dramatically lower than the previous sampling event. In general, the larvae appeared to be more evenly dispersed throughout the Bay during this sampling event. July 5-6 samples were taken mostly on a flooding tide on July 5-6 (Fig. 4d). Larval densities were dramatically reduced throughout the Bay, possibly having settled to the benthos after having been in the water column for at least 14

days following the spawning event that occurred around the second sampling week (Fig 5b).

The samples taken from July12-14 were taken on an ebb tide or within a half hour of low tide with the exception of the eastern half of the upper Bay which was sampled on a flood tide (Fig. 4e). A second spawning even appears to have occurred since the last spawning date and very high densities were found in and around Greenwich cove. The densities in the Providence River and Upper Bay conditional areas were moderate and fairly even throughout this area. The final sampling date occurred on July 19-20 with the majority of sites being sampled on a flooding tide with the exception of the Providence River (Fig 4f). Moderate densities were found in the Providence River, the middle of the Upper Bay, and in Potowomut Cove. The rest of the Bay had very low densities, including all of Greenwich Bay.

The Narragansett Bay quahog spawning season was highlighted by two major peaks: June 21-29 and July 12-14 (Figure 5). Sampling periods June 21-22 and June 28-29 represented 32.73% and 33.32% of the larvae observed. Roughly 28.67% of the larvae observed in the spawning season were observed from July 12-14. The first week (June 15-16), fourth week (July 5-6) and sixth week (July 19-20) of the survey represented 1.99%, 3.27, and 6.76% of the total larvae sampled during the study period (Figure 5).

Larval densities also varied based on the management strategy of the area (Table 2). Densities were typically greatest in the conditional and closed areas, with the spawner sanctuary containing consistently low densities. When evaluating the total spawning period (June 15-July20), significant differences in densities by management area emerged. Conditional area densities were significantly different from open areas and those

more strictly managed, while closed area densities were significantly different than open areas. Sampling periods June 21-22 and July 19-20 also significant differences by area type. For June 21-22, closed area densities were significantly different from open and more strictly managed areas, while from July 19-20, closed area densities were significantly different than those from open areas (Table 2).

Larval densities appeared to fluctuate with ebbing and flooding tides. However, correlation between sampling times relation to nearest high and low tides and larval densities did not suggest strong relations. Correlation between time to nearest low tide and natural log-transformed larval densities was not significant (R^2=0.003, p-value=0.32). Natural log-transformed densities significantly correlated to time to nearest high tide, but the correlation was weak (R^2=0.02, p-value=0.014).

Discussion

qPCR Application to Northern Quahog

We have developed molecular-based assays capable of identifying and quantifying larvae of Northern quahog from mixed seawater samples using qPCR. Genetic based assays have become a more time and cost efficient tool to sorting and quantify complex plankton samples than traditional microscopy techniques. Traditional techniques are time intensive and require significant expertise in larval bivalve morphology. High species specificity, high accuracy, short processing time, and low cost (approximately 90 samples in 90 minutes at $2.00 per sample; Vadopolas et al., 2006) have all lend qPCR methods as an ideal tool for examining larval distributions in the marine environment. In addition to molecular identification, the ability of a real-time PCR assay to quantify marine

larvae in seawater samples enables field studies understanding larval abundance and distribution patterns that previously may have logistically been untenable.

Conversely, some limitations exist to using qPCR to detect and quantify marine invertebrate larvae. Bias could arise through variable numbers of cells in target organisms depending on age and/or life history stage. Since variable cell numbers at different larval stages will yield different quantities of DNA per larva, we quantified early and late stage abalone larvae to test the accuracy of the assay. For pinto and red abalone, variable cell numbers did not alter estimates of initial template quantity. This would likely not be the case for larvae of species that undergo more substantial somatic growth during the planktonic phase. Selection of appropriate size-selective sampling gear might mitigate the increased variability associated with variable cell numbers in larvae at different life history stages, requiring multiple size-appropriate standards, but this remains to be investigated.

Northern Quahog Larvae in Narragansett Bay

The sampling period appeared to capture a significant portion of the Northern quahog spawning seasonality in Narragansett Bay (Figure 5). In 2011, bottom temperatures reached 20°C by May 29 in Greenwich Bay and July 12 in northern Conditional Area (RIDEM 2016), corroborating sampling began either shortly after or well before the 20°C trigger in spawning, depending on the station. However, *M. mercenaria* has been found to occur at temperatures below 20ºC (Thompson 2011). As waters continue to warm in Narragansett Bay (Fulweiler et al. 2015) and the northwest Atlantic (Saba et al. 2016), populations off New England may spawn earlier with earlier seasons, especially in estuaries. Earlier spawning that occured in the coves of Greenwich Bay (such as

Greenwich Cove, Apponaug Cove, and Warwick Cove) prior to the June 15-16 sampling event may be due to the shallow waters which warm quicker, thus triggering an earlier spawn than quahogs in deeper waters. Southeastern portions of the Conditional Areas also had rather high larval densities early in the season. These sites were sampled on an ebb tide, suggesting that these larvae may have originated from closed area of the Warren River, upstream from this Conditional Area region.

The spawning pulse on July 12-15 includes a spawning event in the Bay proper, including the Conditional Areas (Figure 5). A slower warming of waters in the Bay proper may also be the reason that larval densities in the deeper waters of the Upper Bay did not increase until later in the season. The large spawning event that occurred around July 12-15 also includes the highest magnitude larvae found Greenwich Cove area seen over the sampling period. The high densities of adult quahogs in this area may cause the adults to spawn latter in the season as their gametes mature slower due to the adverse effects of crowding. Marroquin-Mora and Rice (2008) suggest that populations of quahogs in closed areas may have reduced reproductive capability, either due to crowding or poor environmental conditions. However, future work examining gonadal condition is required to understand prospective density-dependent factors influencing spawning productivity in closed areas.

The lower portions of Narragansett Bay and Mt. Hope Bay had very low densities of quahog larvae throughout the time series and do not appear to be significant sources of larvae. Northern regions of Narragansett Bay where larvae occur or are spawned, such as the Providence River, Warren River, and Greenwich Bay, may provide an opportunity of larvae seeding to the Upper Bay and lower West Passage. However, the

eastern portions of the Lower Bay and Mt. Hope Bay likely do not receive larvae from these areas given the Bay's predominant counterclockwise circulation: oceanic flow through the East Passage and exiting through the West Passage with riverine input (Chen et al. 2008). Both regions have closed areas, yet do not support high densities like other closed areas (e.g. Providence River, Warren River, coves of Greenwich Bay). Low larval densities in these areas likely correspond to low adult densities, perhaps due to other population limiting factors, such as suitable benthic habitats, food availability and/or predator abundances.

Lack of larvae found in the entirety of Greenwich Bay during flood tides on July 5-6 and July 19-20 and very high densities during the ebb tide on July 12-14 may indicate that the larvae had settled out of the water column following spawning events in mid-June and sometime just after the July 5-6 sampling date. Another explanation is that there is some larval behavior in response to the tides, with larvae concentrating in bottom waters during flood tides or the opposite with ebb tides. Future sampling should incorporate tows conducted over the entire water column to capture changes in larval behavior over the larval stage.

While times to nearest high and low tides did not help in describing variability in surface larval quahog abundances, it appears that the tides have a large impact on the location and densities of larvae in the Bay. This could be due to a behavioral response or due simply to changes in location due to tidal currents. Low correlation between tidal state and larval abundances may be in part to a single tidal gauge for comparison to sample locations' times may distort some of the actual relations between the two. Previous work has identified significant impacts of tidal state and intensity on larval abundances.

Thompson (2011) found that during a period when there were sharp tidal signals in temperature and salinity, larval concentrations were higher in bay water compared to coastal waters on incoming tides. Strong currents and a fresh upper layer both prevented larvae from concentrating at the surface (Thompson 2011). A directed study measuring Eularian larval densities may aid in assessing tidal flushing on larval location. Additionally, larval transport modeling using circulation data of Narragansett Bay should be used to evaluate both tidal currents on larval positioning and exchange of larvae between different areas in the Bay.

Larval Densities in Relation to Management Strategies

Highest densities of larvae in closed and conditional areas suggest that these *de facto* sanctuaries and stricter managed regions, respectively, are significant in supporting recruitment for quahogs in Narragansett Bay (Rice et al. 1989). The permanent or intermittent closure of these regions from commercial fishing due to waste-water treatment effluent appears critical in supporting the fishery. Multiple waste-water treatment facilities have upgraded to tertiary treatment, reducing nitrogen inputs to the Bay to improve water quality in regions, such as the Providence River (Oczkowski et al. 2016). If these areas open with improved water quality, commercial harvest has the possibility of reducing larval production for both the Providence River and down Bay areas that receive northern derived larvae. As such, continued closure or strict management of commercial harvesting quahogs in these regions should be considered in the event the pollution closures are lifted due to improved water quality.

Export of larvae from closed areas to management areas, conditional areas, or open areas appear to be critical to supporting these regions. Closed coves of Greenwhich

Bay seem to be larval sources for Greenwich Bay proper (Figure 4), a major commercial fishing area in Narragansett Bay. Seasonal closures and reduced possession limits allow for western Greenwich Cove to continually receive larvae without overfishing the population in this region. Similar analogs may be true for the Conditional Areas, where the combination intermittent closures due to rainfall protecting local quahogs and advection of Providence River and Warren River larvae into this area likely provide the region with high larval densities. The Spawner Sanctuary located outside the mouth of Narragansett Bay has high larval densities at various times during the spawning period, but more often abundances in this area are less than closed, managed or conditional areas (Figure 4, Table 2). Larvae in the area are likely the product of both local quahogs spawning, and larvae transported from Greenwich Bay and its coves, as well as the Conditional Areas.

Conclusion

This work provides the first example of constructing a quahog-specific primer for larval enumeration. Age-equivalent abundances from samples varied over the Bay, with two spawning pulses identified during the 2011 spawning season. Over this time frame, larval abundances varied spatially based on management area type. Larval densities in areas permanently or intermittently closed due to pollution had higher densities than and the less restrictive management areas. These results highlight the effect of reduced harvest pressure on the quahog population's larval production, and the value of *de facto* or designated marine reserves in sustaining the stock's larval production. Larval densities and regressions presented are valuable for future validation of biophysical models predicting larval quahog densities from physical transport.

Acknowledgements

We thank Tatiana Rynearson for advice on qPCR methods and curve construction. We also thank Ed Baker for use of the University of Rhode Island's Blount Aquaculture Laboratory for culturing larval quahogs. JMM was funded by The Nature Conservancy Global Marine Initiative Student Research Award Program.

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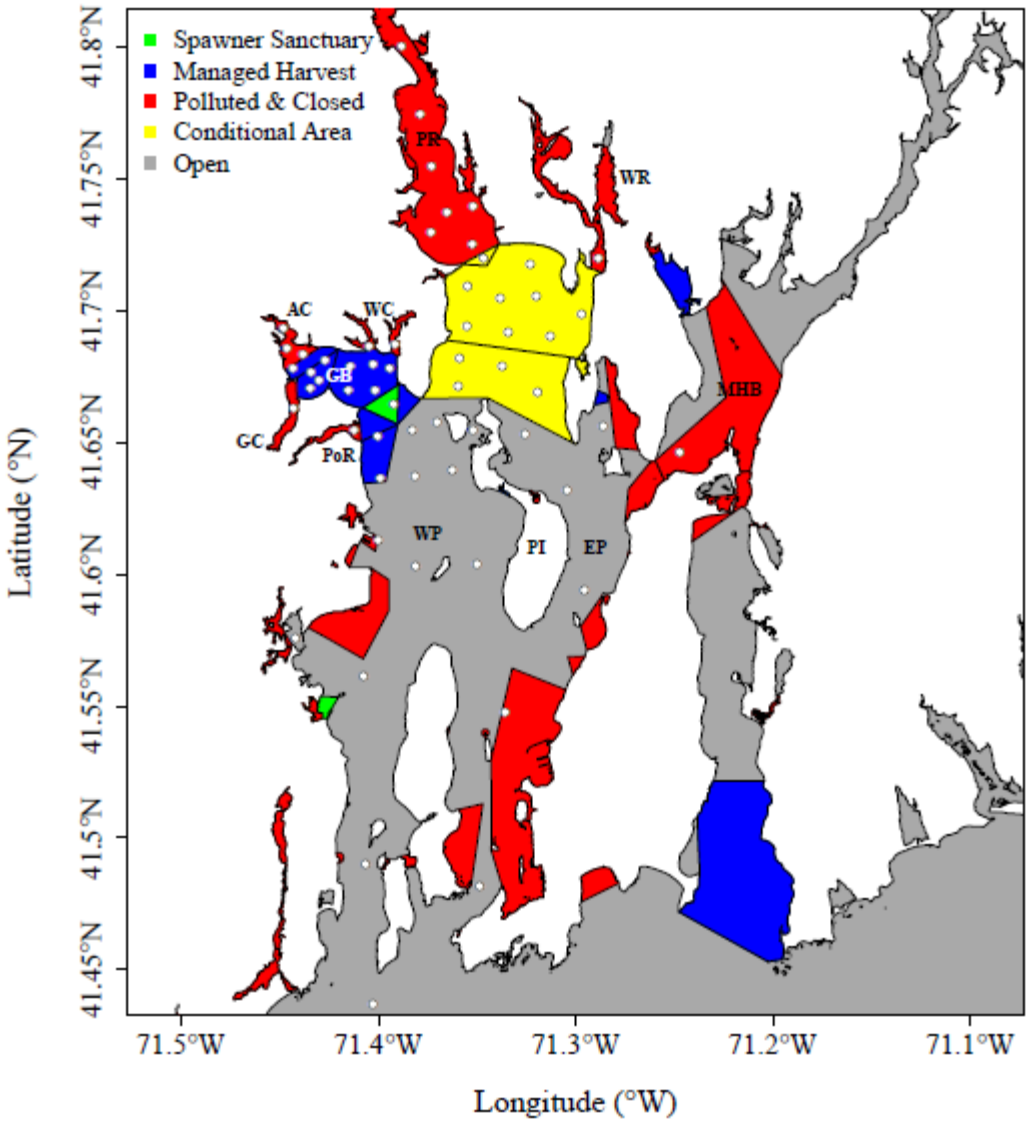


Figure 1. Narragansett Bay sampling stations (white circles) for quahog larvae from June 15 – July 20. Colored regions represent the management style of the area as described in Table 1. Specific areas of the sampling region are indicated on the map with abbreviations: Providence River (PR), Warren River (WR), Greenwich Bay (GB), Greenwich Cove (GC), Apponaug Cove (AC), Warwick Coves (WC), and Potowomut River (PoR). Features of Narragsansett Bay are also labeled for reference: West Passage (WP), East Passage (EP), Prudence Island (PI).

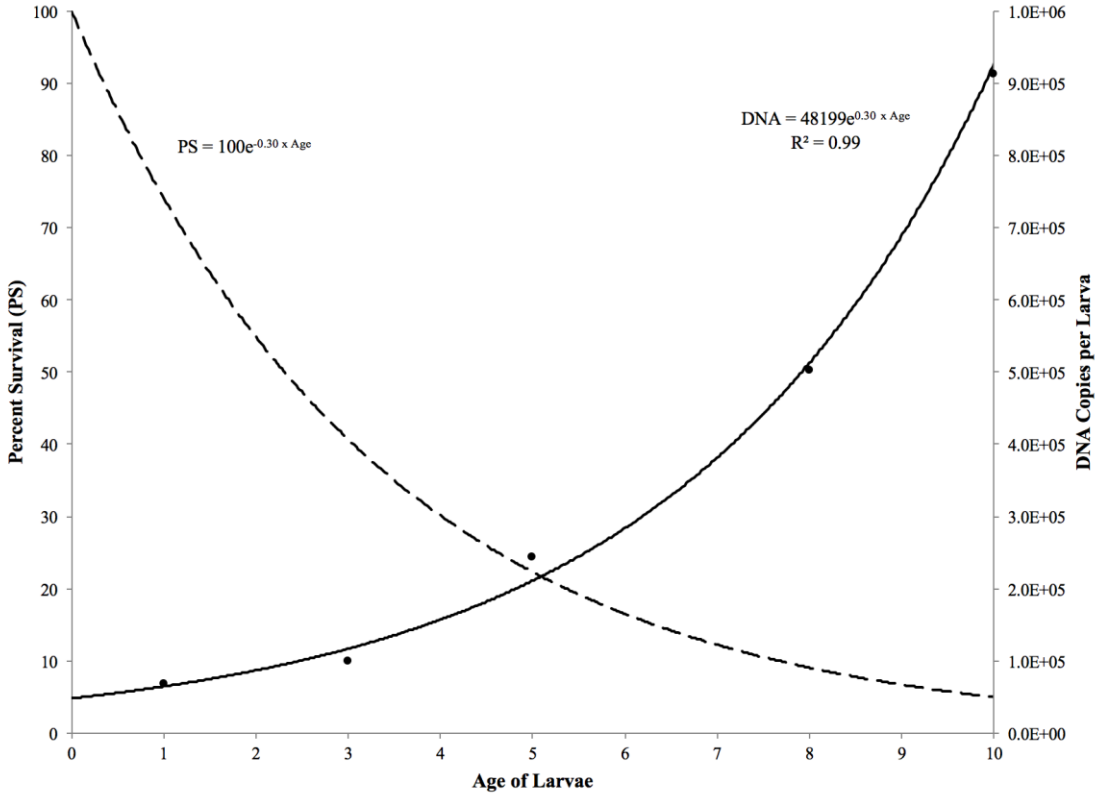


Figure 2. The number of DNA copies per larvae of a given age are plotted on the right hand axis (black dots). The black line is the line of best fit and corresponds to the equation in the upper right-hand corner. The red line corresponds to the percent survival of different aged larvae assuming 95% mortality over the 10-day larval period. The increase in DNA per individual is exactly balanced by individual mortality.

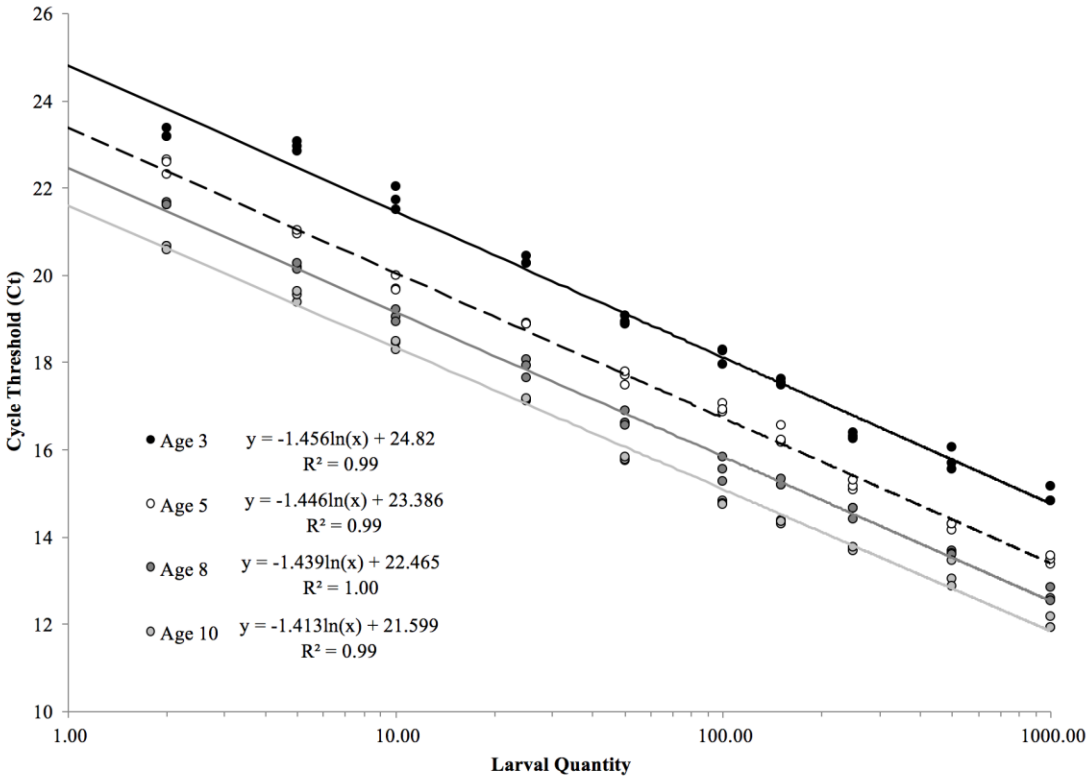


Figure 3. Standard Curve for larvae of 4 different ages. Cycle threshold is inversely proportional to the original amount of DNA in the sample. Figure 4. Maps depicting the number of larvae at each sampling site for individual sampling events (a-f). The size of the circle surrounding a site is directly proportional to the number of larvae estimated in the sample.

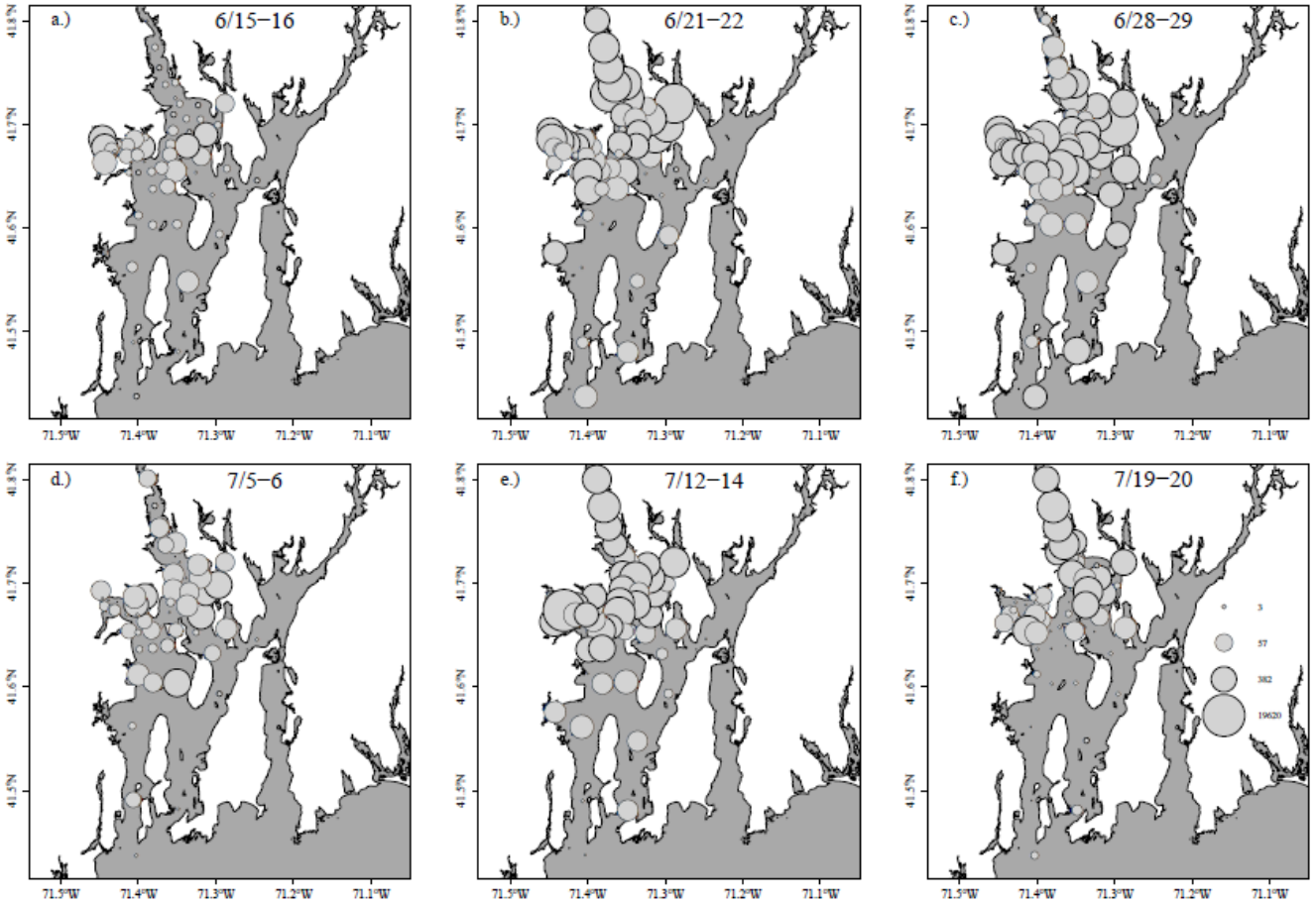


Figure 4. Larval densities (10-day old equivalent, # m^{-3}) spatially through the sampling period.

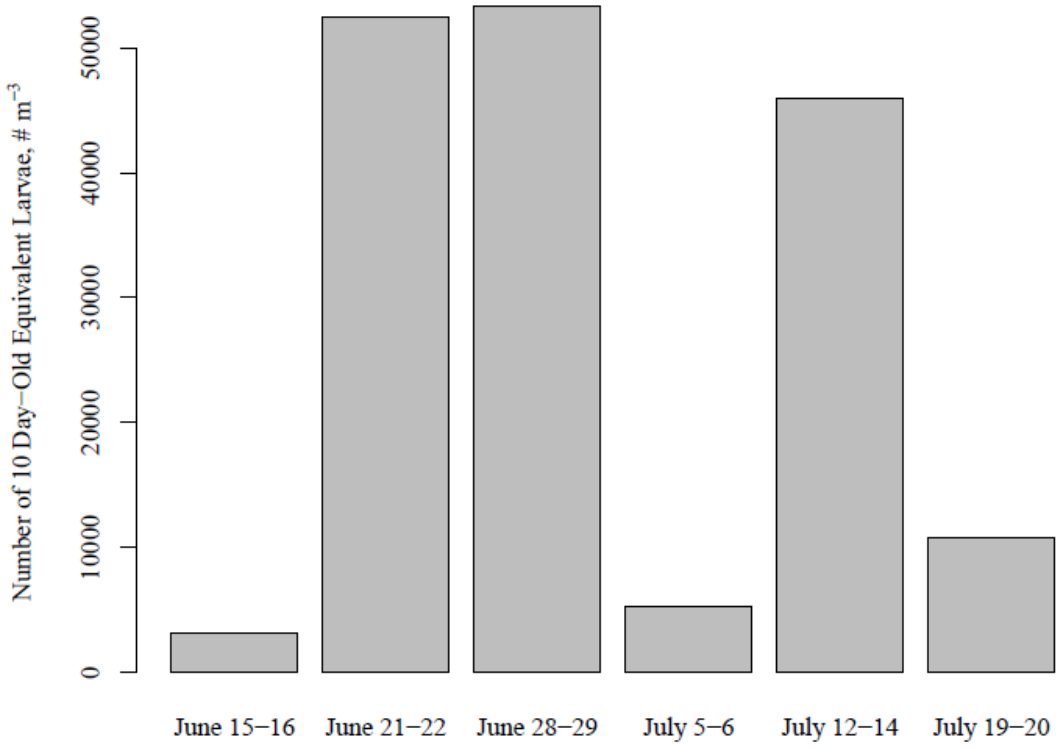
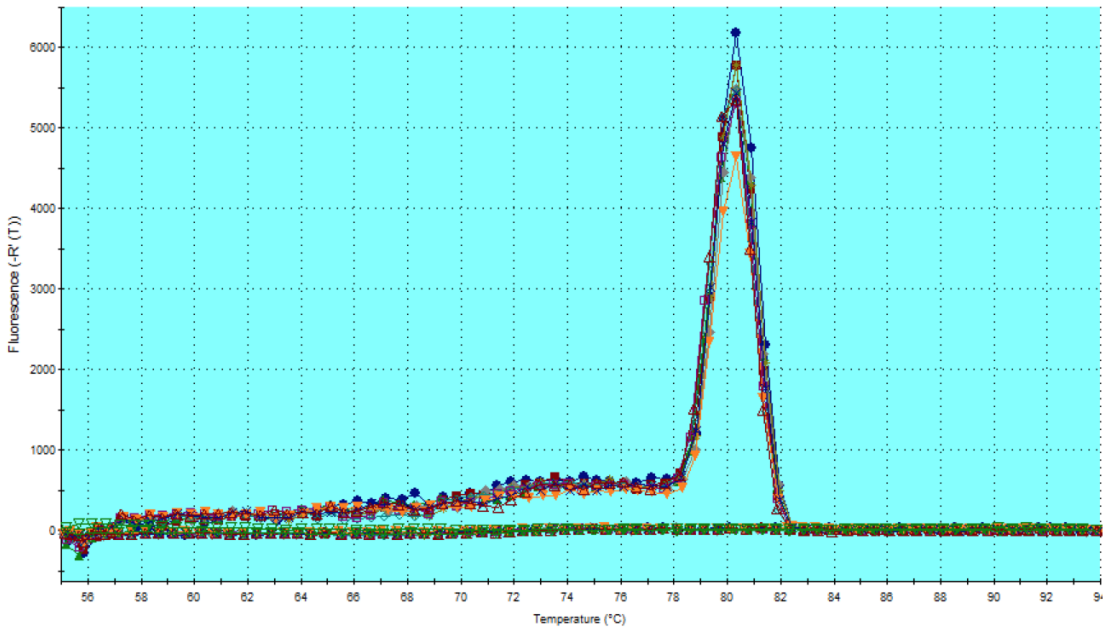
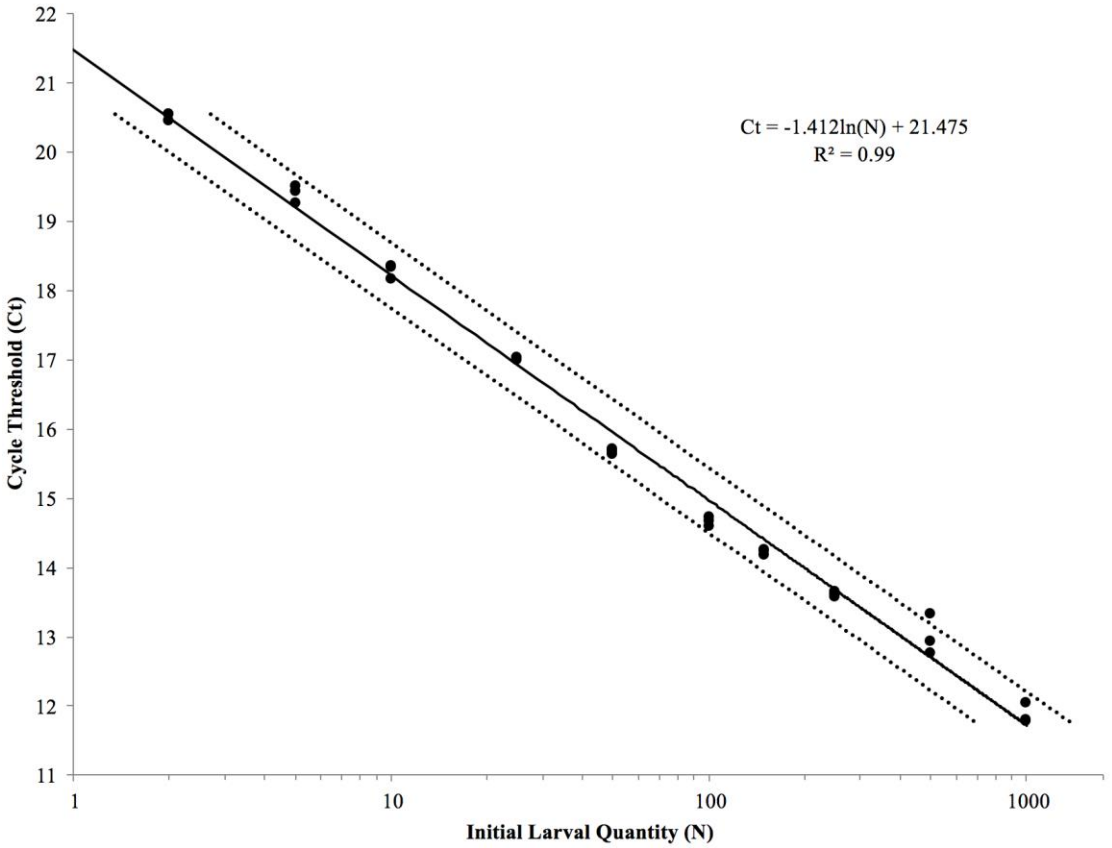


Figure 5. Total densities (age 10-day equivalent) for quahog larvae over Narragansett Bay by sampling period.



Appendix 1. Dissociation curve for 2-day old larvae. The x-axis shows the melting temperature of dsDNA created during the PCR cycles. Shorter DNA fragments typically have lower melting temperatures. The y-axis is the inverse of fluorescence, which is directly correlated to the quantity of DNA that melts at a specific temperature. The melting temperature of the target PCR product is 80°C The line across the bottom in green represents the ROX reference dye which is not involved in the PCR reactions.



Appendix 2. Standard Curve for 10-day old larvae. The x-axis is the number of larvae on a logarithmic scale. The y- axis is the cycle threshold, or the PCR cycle when fluorescence is detected above a background level. The dotted lines represent a 95% confidence interval. Since the x-axis is on a logarithmic scale, measurements are more precise at the lower end of the spectrum

Area Type	Area Description
Conditional Areas	Harvesting allowed except under conditions caused by rainfall, wastewater discharges, or indicator pathogens
Closed Areas	Harvesting prohibited due to pollution
Open Areas	Harvesting allowed year around with one set catch limit
Shellfish Management Areas	Harvesting allowed, yet seasonal and daily closures, and reduced catch limit
Spawner Sanctuary	Harvesting prohibited to aid in replenishing the stock

Table 1. Description of general shellfishing area types in Narragansett Bay, RI.

Date	X^2	DF	p-value	Mean Densities (# m^{-3})				
				Closed	Conditional	Managed	Open	Spawner Sanctuary
June 15-16	6.6	4	0.1584	70.01	57.06	93.45	17.23	1.67
June 21-22	18.16	4	0.0011	$2362.95^{A,B}$	919.84	129.62^{A}	103.59^{B}	262.3
June 28-29	6.15	4	0.1881	694.45	2282.34	251.39	600.67	8.69
July 5-6	6.16	4	0.1878	76.45	183.11	38.59	75.43	33.21
July 12-14	9.03	4	0.0604	419.36	709.03	2685.7	168.07	264.4
July 19-20	13.46	4	0.0092	463.89^{A}	236.92	22.73	18.45^{A}	40.08
June 15-July20	24.86	4	1.00E-04	681.18^{C}	$731.38^{A,B}$	536.92^{A}	$163.91^{B,C}$	101.72

Table 2. Kruskal-Walis tests for evaluating different densities over area types by date and for the whole spawning season sampled (June 15-July20). Bold dates represent time frames with significant differences (α=0.05) observed within management area types. Mean quahog larval densities by date and management area type are also provided. Significant Kruskal-Walis tests were run with a post-hoc pairwise comparisons using Tukey and Kramer (Nemenyi) test. Significant pairwise differences between management area types are represented using capital letter subscripts (e.g., during the June 21-22 sampling, Open and Closed areas significantly differed in larval densities).