IDENTIFICATION OF GEOCHEMICAL TRACERS WITHIN BIOMINERALS OF JUVENILE AND LARVAL *MERCENARIA MERCENARIA*: IMPLICATIONS FOR MODELING LARVAL DISPERSAL

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This study was designed to investigate the application of geochemical signals within biominerals to identify site fidelity and natal origin of the commercially valuable bivalve *Mercenaria mercenaria*. My first study investigated the spatiotemporal stability of elemental signals within newly recruited bivalve shell as a means to identify site fidelity within a well-mixed estuarine-lagoonal system. My results demonstrate for the first time the existence of seasonally reproducible small-scale spatial differences (~12-40 km) in the elemental chemistry of juvenile bivalve shell exclusively within an estuarine-lagoonal system. My second study built upon these results and investigated spatiotemporal variability of potential elemental signals within the early larval shell of *M. mercenaria* as a method to identify natal origin. The results of this investigation demonstrate for the first time the existence of small spatial (~1-50 km) and temporal (tri-weekly) differences in the elemental chemistry of hard clam larval shell. These naturally occurring geochemical tags are indicative of natal origin and may be used trace the dispersal trajectory of this economically important species. My third study empirically investigated the efficacy of the microprobe assay laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) to only target specific areas of interest within *M. mercenaria* recruited shell. The results of this study suggest that at this time LA-ICP-MS is not a tractable methodology to sample only the retained larval shell of the hard clam without introducing substantial contamination from underlying carbonate.

IDENTIFICATION OF GEOCHEMICAL TRACERS WITHIN BIOMINERALS OF JUVENILE AND LARVAL *MERCENARIA MERCENARIA*: IMPLICATIONS FOR MODELING LARVAL DISPERSAL

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CHAPTER I: INTRODUCTION

Patterns of larval dispersal have great potential to impact the population dynamics of marine invertebrates by influencing the number of potential recruits in a given area (Thorson 1950). The recognition that planktonic larvae represented a life history stage of adult invertebrates has stimulated a debate regarding the connectivity of marine populations. Initial assumptions perceived marine populations as highly open where larvae were commonly exchanged between populations of adults. However, several lines of evidence have suggested that populations may be less open than originally suspected (as reviewed by Levin 2006). In light of these recent developments, data are badly needed to help understand the degree of connectivity among marine invertebrate populations. Attempts to understand what mechanisms structure benthic marine invertebrate populations have highlighted recruitment events as critical periods in the life history multiple bivalve species including the hard clam (Peterson 2002, Kraeuter 2005). The importance of this life history stage in structuring the dispersion and sustainability of bivalve populations highlights the need to develop methods to more fully understand factors that influence patterns of larval dispersal and population connectivity. This information is critical to provide insight regarding the influence of larval dispersal on bivalve metapopulation dynamics.

The analysis of the elemental composition of biominerals is gaining attention as a methodology to investigate the larval dispersal of invertebrates and has successfully been used to identify the natal origin and/or site of collection for

multiple invertebrate larvae including: crab zoea, encapsulated gastropod veligers, and more recently pelagic bivalve veligers (as reviewed by Thorrold et al. 2007). I investigated the applicability of this technique to the hard clam *Mercenaria mercenaria* by 1) Investigating the spatial distinction and temporal stability of elemental signals within juvenile and larval shell and 2) Determining if current analytical techniques can precisely quantify the elemental content of retained larval shell of recruited individuals. To date no study has investigated the application of elemental fingerprinting to identify patterns of invertebrate larval dispersal within an estuarine system.

Estuaries support many commercially viable invertebrates for all or a portion of their ontogeny and due to their natural variability in environmental factors such as temperature and salinity, these systems are well suited to investigations examining the incorporation of trace elements into biominerals (Swearer et al. 2003). This work may facilitate a more complete understanding of hard clam population dynamics and could have broad implications for the management, conservation, and restoration of populations throughout its entire range. It is hoped that this work will help to promote sustainability of a resource that supports, as well as defines, entire coastal communities.

Reductions in commercial landings of hard clams have occurred in many areas and are believed to be driven by recruitment limitation stemming from reductions in adult standing stock biomass (Peterson 2002). The implementation of conservation, management, and restoration strategies will be critical to facilitate sustainable management of hard clam stocks. The stock status of the

hard clam is currently unknown in many states and little data is available concerning the relationship between biology, ecology, and the population dynamics of this species. To date no study has examined patterns of hard clam larval dispersal using trace element microchemistry. If patterns of hard clam larval dispersal and population connectivity can be elucidated, the potential exists to identify subpopulations that may differentially contribute to overall population dynamics by disproportionately supplying larvae. Validation of this methodology will provide an extremely powerful management tool that can be used to identify populations that should be conserved (source) vs. those that should be open to commercial harvest (sink) throughout this species entire range.

***Reproduction and Larval Ecology of* Mercenaria mercenaria**

Mercenaria mercenaria (hereafter the hard clam) of the family Veneridae is a commercially important shellfish species whose range extends along the western coast of the Atlantic Ocean from the Gulf of St. Lawrence south to Texas (Porter and Tyler 1976, Ansell 1968). Since the late 1970s these shellfish have historically supported a robust fishery in North Carolina waters ranging from 1st to 4th in dollar value between 1980-2000, with maximum market value of $8,388,000 (Peterson 2002). In Maryland and Virginia, extraction peaked during 1969 and 1981 at 800,000 and 1,000,000 lbs of meat, respectively (McHugh 2001). The exhaustion of stocks within the Chesapeake Bay and other areas of New England coupled with a consistent market demand promoted increased harvest from North Carolina waters during the 1980s and 1990s (Peterson 2002).

The hard clam is an estuarine dependent species that can be found

occupying both subtidal as well as intertidal habitats. These bivalves possess the ability to withstand a wide range of physiological tolerances including temperature ranges from 9-31°C and salinity ranges from 14-40, though growth is optimal at temperatures around 20°C and salinities between 24-28 (Grizzle et al. 2001). Hard clams form dense assemblages or beds within a wide spectrum of habitat types ranging from seagrass and shell bottom to bare substrata. However, the abundance of hard clam adults and juveniles, as well as growth of hard tissues (i.e., shell), is significantly enhanced in spatially heterogeneous habitats such as shell bottom and seagrass beds (Peterson 1986, Wells 1957).

Due to its commercial importance, the life history of the hard clam has been the subject of extensive investigation throughout its geographic range. The historical harvest of wild populations has necessitated numerous investigations concerning multiple aspects of this species' reproductive ecology. This body of work suggests that the production of reproductive tissues can be influenced by a variety of exogenous factors including: food quality, temperature, tidal level, adult density, disease and parasites, and salinity (Newell et al. 2009, Ansell 1964, Walker and Heffman 1994, Kraeuter et al. 2005, Marroquin-Mora and Rice 2008, Pline 1984, Eversole et al. 1980). Additionally, endogenous factors such as sex, age, size, and genetic composition may also influence the timing and duration of gametogenesis and reproductive output (Eversole et al. 1980, Knaub and Eversole 1988).

The reproductive periodicity of the hard clam has a distinct seasonality that is a function of latitude. Populations located at lower latitudes experience

earlier development of reproductive tissues as well as an earlier induction of spawning events (Eversole 2001). For populations located at more southern latitudes, an earlier onset of gametogenesis and spawning facilitates the ability to reconstitute reproductive tissues and experience a protracted spawning period (Eversole 2001). Hard clams are a protandrous consecutively hermaphroditic species with a juvenile male phase occurring in 98% of individuals (Loosanoff 1936, 1937a). Additionally, throughout their geographic range the sex ratio of hard clam populations is consistently 1:1 for individual's ≥ 1 year of age (Eversole 2001). As with many sessile invertebrates, hard clams have a biphasic life cycle where the immobile adult stage is complemented by a pelagic larval period that lasts between 11-20 d. At the initiation of spawning, gametes are released into the water column via the hard clam's exhalent siphon. Ova range from 60-85 μm and an increase in larval survival is significantly correlated with egg diameter (Kraeuter et al. 1982).

Fertilization is external and subsequent embryogenesis is indirect and characterized by spiral cell division resulting in the formation of a ciliated blastula approximately 6 h post fertilization (Loosanoff and Davis 1950). Within 12-14 h of fertilization the trochophore larvae is formed and between 24-48 h the larvae, now termed a veliger, has secreted its first larval shell,the prodissoconch I (PI) and formed a highly ciliated velum (Fig. 1). The velum entrains food particles such as diatoms and flagellates from the surrounding environment and also functions in a locomotive capacity, thus allowing the larvae to manipulate its vertical position within the water column. At a length of approximately 120 μm,

the (PI) larval shell has completely enveloped the larva, which can then be termed a straight hinge or D-stage veliger (Carriker 2001) (Fig. 1). In laboratory experiments, D-stage veligers aggregated at the surface of seawater and were unaffected by light. However, at salinity thresholds between 15-20, they reduced swimming speeds and remained below the isocline (Turner and George 1955). These authors postulate that this may be a mechanism to combat seaward movement due to gravitational circulation in highly stratified estuaries.

At the completion of the D-stage, the veliger begins the secretion of the second larval shell, the prodissoconch II (PDII), which grows to accommodate the development of somatic tissues including the mantle, mantle cavity, alimentary canal, kidney, heart and vascular system, and sensory organs (Carriker 2001) (Fig. 1). During this stage (PDII), veligers remain distributed within the middle of the water column to reduce benthic predation pressure. The (PDII) stage lasts approximately 11-20 d or until the larvae is approximately 230 µm, at which time the veliger undergoes morphological changes to facilitate its transition from the water column to the benthos. At this time, the larva is termed a plantigrade. Specific changes include the loss of the velum and the appearance of a highly ciliated muscular foot, inhalant and exhalent siphon, and byssal gland (Carriker 2001). During the plantigrade stage, the larva will transition from a pelagic existence and alternate between swimming and crawling as it searches for suitable substrate. When suitable substrate is encountered, the plantigrade will secrete an attachment structure termed a byssal thread that

enhances retention. It is at this time that the adult shell, or dissoconch, begins to form and the larva is spatially constrained (Fig. 1).

Many factors have been demonstrated to influence the settlement of bivalve larvae including: hydrodynamics, substrate type, chemical irritants produced by benthic infauna, and benthic predation pressure (Woodin et al. 1997, Turner et al. 1994, Grizzle et al. 1996, Tamburri et al. 1996, Olafsson 1988, Buekema et al. 1998). The settlement of hard clam larvae is chemically stimulated by the presence of conspecifics (Keck et al. 1974) and settlement rate is independent of adult density (Peterson 2002).

Larval Dispersal and Trace Element Microchemistry

The life history of many estuarine and marine bivalves includes an obligate period of planktonic larval dispersal. Given that adult populations are spatially constrained, the planktonic life stage has the potential to exert a tremendous influence on the dispersion and sustainability of adult populations by directly influencing the number of potential recruits that settle in a particular area (Thorson 1950). Investigations concerned with identifying patterns and mechanisms of bivalve larval dispersal have employed methodologies from multiple scientific disciplines including: physical oceanography, larval behavior, larval tagging, and genetics (Luettich et al. 1999, Garland et al. 2002, Becker et al. 2007, Gilg and Hilbish 2003). Each of these methodologies is applicable at various spatial and temporal scales and will vary in response to the research questions being addressed. However, to infer patterns of larval dispersal on temporal and spatial scales that are ecologically significant, the use of artificial or

natural markers is useful. Artificial tags such as tetracycline, calcine, fluorescent dyes, radiolabels, and thermal stress marks can be applied to biominerals such as fish otoliths and larval shells (as reviewed by Thorrold et al. 2002). However, the application of artificial tags has been limited due to the extreme dilution of larvae within the water column (as reviewed by Levin 2006). The identification of distinct elemental signals within biominerals has emerged as a technique to infer patterns of bivalve larval dispersal on ecologically significant temporal and spatial scales (Becker et al. 2007). The usefulness of examining biogenic carbonate is that the composition of biominerals (i.e., otolith, larval shell) may reflect a unique chemical composition associated with the habitat in which they form (Gillanders and Kingsford 1996, Zacherl et al. 2005, Becker et al. 2007).

The process of minor and trace element incorporation is facilitated by substitution reactions between similar sized divalent elements such as Sr^{2+} and Ba^{2+}, among others, with Ca^{2+} within the mineral fraction of the biogenic carbonate matrix with smaller cations such as Mn^{2+} and Mg^{2+} constituting the interstitial organic fraction (Morrison and Brand 1986, Takesue et al. 2008). Physiological processes (i.e, growth, metabolism) are biased in favor of optimal environmental conditions and strongly influence elemental incorporation into molluscan biominerals (Vander Putten et al. 2000, Carré et al. 2006, Stecher et al. 1996). These vital effects alter the chemicals present within the carbonate secreting fluid, and by extension the biomineral (Urey et al. 1951). For example, field and laboratory studies have demonstrated that co-occurring species exposed to the same environmental conditions differentially incorporate trace

elements into their otoliths (Geffen et al. 1998, Swearer et al. 2003). These studies conclude that species-specific metabolic processes strongly influence the availability and incorporation of trace elements into biominerals. Specific food resources, particularly diatoms, may also influence elemental incorporation (Sternberg et al. 2005, Thébault et al. 2009). Additional environmental factors further contribute to this process and evidence suggests that the incorporation of some elements may be predictably influenced by the chemical and physical characteristics of the water in which they form. Laboratory experiments have demonstrated a positive linear relationship between the concentration of Mg:Ca, Pb:Ca, Ce:Ca, and Ba:Ca within molluscan biominerals and the ratio of these elements within culture water (Lorens and Bender 1980, Llyod et al. 2008, Zacherl et al. 2009). Variation in temperature may also influence the incorporation of trace elements within biominerals (Schöne 2008). Specifically, exothermic reactions have a negative enthalpy and are predicted to be preferred at colder temperatures. For example, empirical evidence supports equilibrium thermodynamic predictions by demonstrating an inverse temperature effect regarding Ba incorporation into gastropod and cephalopod statoliths (Zacherl et al. 2003*b*, Zumholz et al. 2007). However, as suggested by Zacherl et al. (2003*b*) and demonstrated conclusively by Lorrain et al. (2005), kinetic effects such as the precipitation rate of cations to the crystal surface may override thermodynamic predictions to further influence elemental incorporation into molluscan biominerals. Finally, interactions between multiple environmental

factors can influence elemental incorporation (Martin et al. 2004, Eldson and Gillanders 2004, Zumholz et al. 2007).

Elemental fingerprinting represents a powerful tool to trace patterns of invertebrate larval dispersal and population connectivity due to the potential for all larvae within a particular area to incorporate a geospatially distinct chemical signal (Thorrold et al. 2007). If habitat specific signals exist and are temporally and spatially stable during seasonal periods of reproduction and larval shell deposition, the potential exists to investigate questions regarding the natal origin and dispersal trajectory of successfully recruited bivalves (Becker et al. 2007). In order to apply this methodology, it is necessary to determine if the chemical composition resident within generations of larval shell is stable through time (Gillanders 2002, Becker et al. 2005, Fodrie et al. 2011, Dunphy et al. 2011). Additionally, the chemical signal of all potential natal origins must be investigated to accurately assess the spatial distinction of trace element incorporation into larval biominerals (Campana et al. 2000). Finally, provided the former assumptions are found to be true, chemical analysis of biominerals formed during larval development and retained through subsequent ontogeny is critical to reconstruct larval origin and subsequent dispersal trajectory (Levin and DiBacco 2000). Such structures are observed in fish (otoliths), gastropods (statolith and protoconch), and bivalves (prodissoconch) among others (Campana 1999, Zacherl et al. 2003*b*, 2005, Becker et al. 2007).

The first application of trace element fingerprinting to investigate larval dispersal using an invertebrate model employed crab zoea (DiBacco and Levin

2000). These authors were able to identify the origin of larvae from either bay or adjacent open shore habitat by clustering multiple trace element signals. While this study represented a tremendous advance in the application of natural biogeochemical markers to identify patterns of invertebrate larval dispersal, it was limited due to the absence of an anatomic structure that was retained post settlement. The first study to employ an invertebrate model with a retained larval biomineral that was formed pelagically used bivalves as a model organism (Becker et al. 2005). These authors investigated the temporal stability and spatial distinction of potential elemental signals by collecting shells of recently settled mussels *Mytilus* spp. Their results support a growing body of literature suggesting that trace element signals can be stable on both weekly and monthly time scales and can be used to assign individuals to specific areas at spatial scales on the order of ~10-30 km (Dorval et al. 2005*b*, Thorrold and Shuttleworth 2000, Ruttenburg et al. 2008, Cathey et al. 2012). Subsequent work by Becker and colleagues (2007) concerned questions involving patterns of mussel connectivity. The primary objective of Becker and colleagues (2007) was to use *in situ* larval culturing to determine if the distinct chemical signal observed in the shell of the post settlement stage could be validated for the larval shell of the pelagic veliger stage. Their research provided the first evidence that trace element analysis can be applied to bivalve larvae to determine the natal origin and dispersal trajectory of recruited individuals.

Despite limited representation in the literature, the application of trace element fingerprinting to understand patterns of invertebrate larval dispersal and

population connectivity shows great promise. Here I examine its potential utility to model the larval dispersal of the obligate, estuarine invertebrate *Mercenaria mercenaria,* within the estuarine-lagoonal system near Cape Lookout North Carolina, USA and estuarine systems associated with the southern Delmarva Peninsula, Virginia, USA. Within my study areas, hard clams experience a protracted reproductive period that is characterized by concentrations of spawning activity during the spring (April-June), with mature gametes being observed through October (Eversole 2001, Peterson and Fegley 1986). Fertilization is external and subsequent larval development is pelagic and typified by the secretion of an initial larval shell, the prodissoconch I (PDI), within ~24-48 h. The second larval shell, the prodissoconch II (PDII), then forms over the remainder of larva's pelagic duration of ~11-20 d. The retention of the PDI and PDII by recruited individuals carries the potential to provide an elemental signal from natal estuaries (Carriker 2001) (Fig. 1).

Importantly, empirical validation of microprobe assays to target only the retained larval biomineral (PDI) is critical for the application of elemental fingerprinting (Strasser et al. 2007, Zacherl et al. 2009). Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has emerged as the methodology of choice to measure small quantities of trace and minor elements within growth zoned biominerals (Campana 1999), but may be limited in its application to sample the retained larval shell of bivalves due to the three dimensional nature of shell growth. Specifically, shell material is thickened through ontogeny such that juvenile shell underlies the retained larval shell (Fritz

2001). Given the ablation of analyte during LA-ICP-MS analysis, the potential exists to transcend the larval shell and introduce elemental contamination from underlying juvenile carbonate. In light of this possibility, the validation of LA-ICP-MS to sample only the retained larval shell is requisite before elemental fingerprints can be used to accurately identify the dispersal trajectory of bivalves (Strasser et al. 2007, Zacherl et al. 2009). To date, work by Strasser et al. 2007 represents the only documented attempt to empirically address the efficacy of LA-ICP-MS to exclusively analyze the larval shell of bivalves. Using *Mya arenaria* as a model organism, these authors manipulated the concentration of Ba within culture water during subsequent stages of larval shell deposition to experimentally determine if LA-ICP-MS could be applied to accurately analyze only the retained larval shell of a recruited individual. Despite the application of various instrument settings, the results of their investigation conclude that LA-ICP-MS could not accurately sample the PDI due to the laser ablation completely transcending the larval shell and introducing contamination from underlying juvenile shell.

No published study to date has investigated the application of elemental fingerprinting to potentially identify patterns of realized bivalve dispersal exclusively within estuarine systems. The environmental conditions within estuaries are naturally variable due to differential coastal geology, pollution, atmospheric deposition, and inputs from local watersheds making them ideal systems to investigate the application of trace element microchemistry (Swearer et al. 2003, Thorrold et al. 2007). The current investigation explores the

methodological assumptions required for the application of elemental fingerprinting to identify natal origin and potentially model population dynamics of the hard clam. Initially, I investigated the spatial (~12-40 km) and temporal (fall vs. spring) stability of trace and minor elemental signals within early juvenile shells of hard clams collected from the estuarine system associated with Cape Lookout, North Carolina, USA. I then investigated larval shell signatures using samples collected from the Southern Delmarva Peninsula, Virginia, USA. Juvenile and larval shell chemistry was analyzed in solution using inductively coupled plasma mass spectrometry (ICP-MS). Finally, I experimentally addressed the efficacy of laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) to analyze only the retained larval shell of the hard clam. This was accomplished by enriching larvae with the stable isotope ^{138}Ba during subsequent stages of shell deposition (as per. Strasser et al. 2007). If accurately detectable trace elemental signatures within retained larval biominerals are stable both spatially and temporally, then they may be used to track patterns of hard clam larval dispersal. Also, population connectivity can be investigated using larval shell signals, thus the potential exists to identify subpopulations that may differentially contribute to overall population dynamics by disproportionately supplying larvae. This in turn could have significant implications for the management, conservation, and restoration of hard clam populations throughout its entire range.

CHAPTER II: SPATIOTEMPORAL STABILITY OF TRACE AND MINOR ELEMENTAL SIGNALS WITHIN JUVENILE MERCENARIA MERCENARIA SHELL

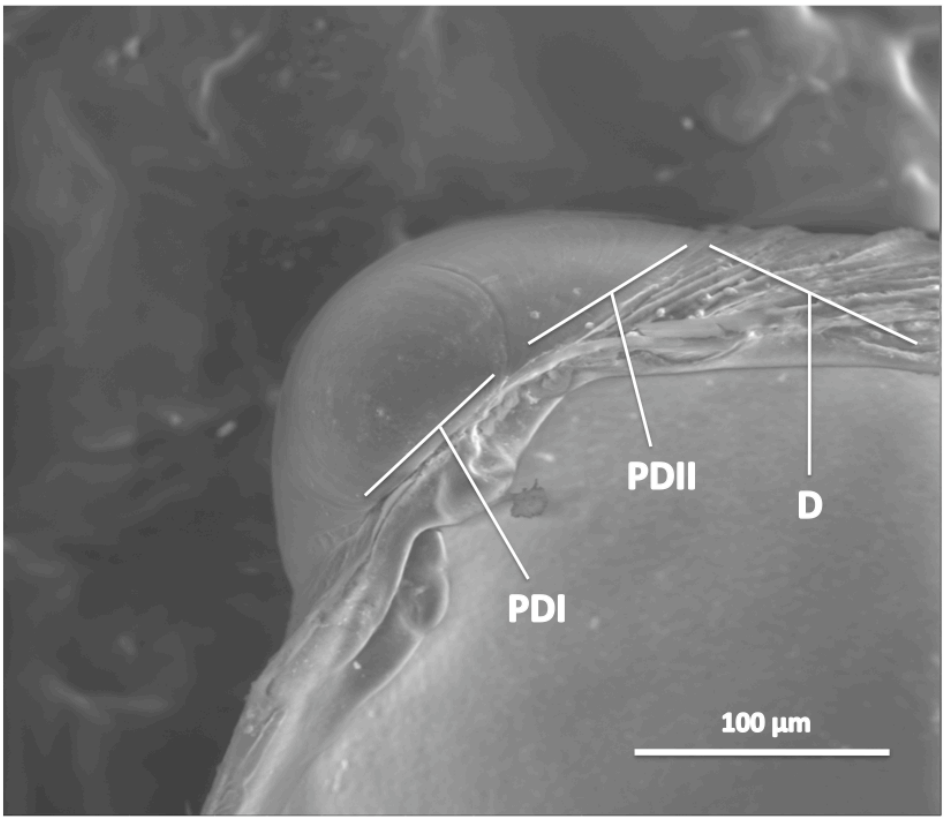
The objective of this chapter was to investigate the spatial distinction and temporal stability of potential elemental signals within hard clam juvenile shell during seasonally elevated periods of larval dispersal. I tested the hypothesis that incorporation of trace elements into hard clam juvenile shell will be insufficient to spatially or temporally discriminate among water masses separated by regional spatial scales (~12-40 km). This was accomplished by collecting newly recruited individuals from 4 water masses separated by at least 12 km during October of 2010 and June of 2011 (Fig 2). Shell microchemistry was analyzed in solution using quadrapole inductively coupled plasma mass spectrometry (ICP-MS). Additionally, between June 2010-October 2010 the trace element composition of estuarine waters were analyzed quasi-monthly using inductively coupled plasma optical emission spectrometry (ICP-OES) to investigate if any observed trends were potentially being facilitated by the chemical composition associated with a particular water mass. My goal was to determine if the trace element composition of juvenile shell was indicative of settlement habitat. If so, this result suggests that trace element signals may exist in the larval shell and may be used to determine natal habitat and subsequent dispersal trajectories.

Methods

Site Selection

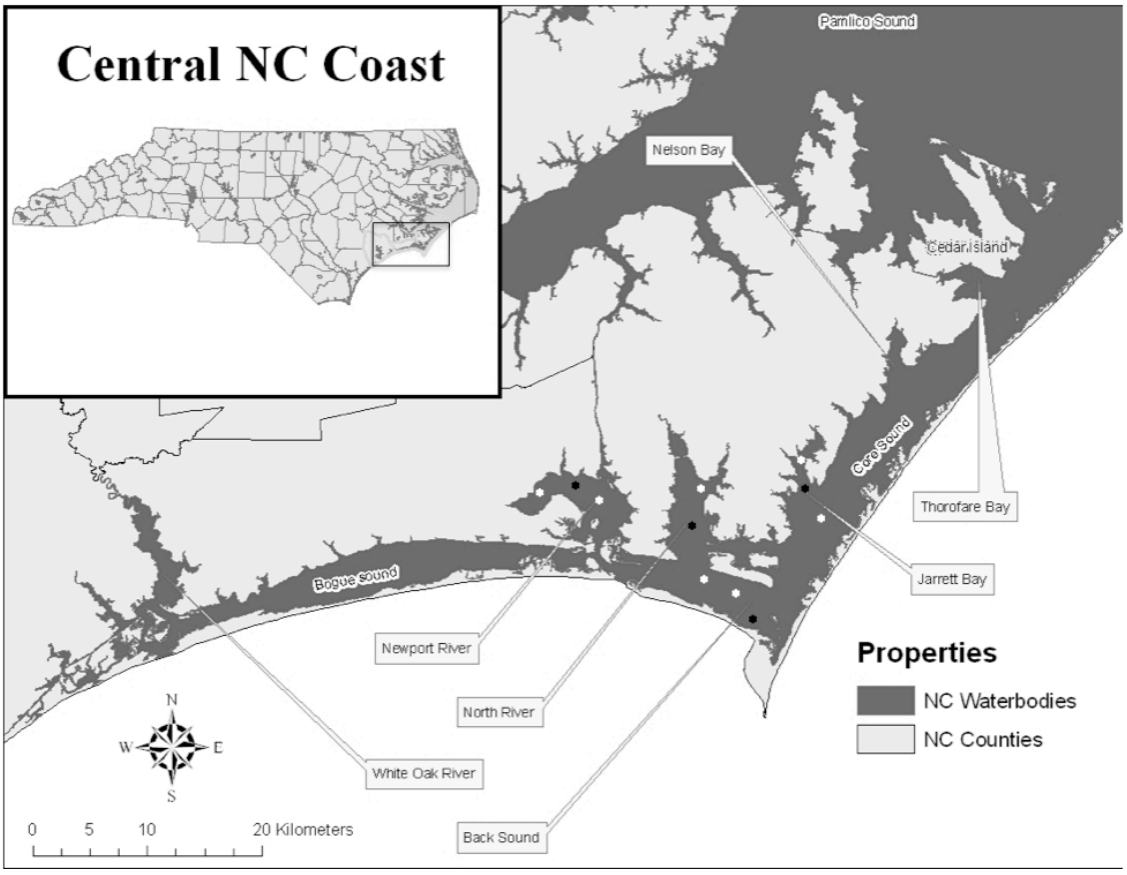
Our study occurred in the Cape Lookout vicinity of the North Carolina Coast and included sites within the Newport River Estuary, North River Estuary, Back Sound, and Jarrett Bay (Fig. 2). These sites were selected because their hydrodynamic properties are such that retention of clam larvae is predicted through the formation of the PDI. The flushing time of the Newport River Estuary has been estimated to be 4-6 d (Evans and Cutshall 1977). Additionally, a particle tracking model developed by Leuttich et al. (1999) to investigate larval transport in the vicinity of Beaufort Inlet demonstrates that a passive particle experiences diminished advection as one moves towards the heads of the associated estuaries due to increased bottom friction associated with shallow water depths, differential geomorphology, and the presence of vegetation. In these areas passive transport is estimated at ≤ 2 km over the course of a semi-diurnal tidal cycle. Based on these investigations and assuming larvae essentially behave as passive particles during the early stages of embryogenesis we expect larvae to be retained within natal estuaries during the first 24-48 h of life from fertilization to the PDI stage (Carriker 2001).

Figure 1



Scanning electron micrograph showing the initial larval shell the prodissoconch I (PDI), second larval shell; prodissoconch II (PDII), and the recruited juvenile shell; dissoconch (D)

Figure 2



Map of the central coast of North Carolina. All asterisks represent areas sampled for water between June-October of 2010. Black asterisks also denote collection sites for Recruited hard clams during October 2010 and June

Sample collection

During October of 2010 and June of 2011 recently settled *M. mercenaria* 2-9.7 mm shell length (approximately 3-19 w of age as interpreted from Carriker 2001) were sampled. Recruits were collected from a single site within each of four water masses that are separated by ~12-40 km: Newport River Estuary, North River Estuary, Jarrett Bay, and Back Sound using a PVC corer of 10 cm diameter and sieved through 1 mm mesh (Fig. 2). After collection samples were immediately frozen in local seawater and stored at -30°C.

Samples were processed in a fashion similar to that used by Zacherl et al. (2005). The isolation of juvenile shell was conducted in a class 100 laminar flow hood. Shell length for each clam was measured to the nearest (mm) using an ocular micrometer and flesh was manually removed from the shell using acid washed plastic forceps (7% HNO_3 (v/v) Optima Grade; Fisherbrand) and remaining organic tissue was dissolved by submersing the shell in an equal parts mixture of 15% H_2O_2 (v/v) (Sigma-Aldridge) buffered in 0.1N NaOH (Fisherbrand) for ~24 h. Shell material was then washed three times with (18.2 Ω) ultrapure water, submersed in 1% HNO_3 (v/v) (Optima Grade; Fisherbrand) for ~10s, rinsed three additional times with (18.2 Ω) ultrapure water, and dried under laminar flow. Once dry, the left valve of each clam was weighed to the nearest (mg). Shells were dry stored in acid-washed polypropylene vials (7% HNO_3 (v/v) Optima Grade; Fisherbrand) until analysis.

Elemental Analysis of Clam Shells

Juvenile bivalve shell samples (49 single valves, each ranging from 1 to 54.5 mg; average: 5 mg) were placed in acid-cleaned polypropylene microvials and reacted in 1 mL of 2% HNO3 (v/v) or 5% HNO_3 (v/v) over 24 hours until complete dissolution occurred. The acid was made from Fisher trace metal grade HNO_3 (70%; lot 1107120) and Type 1 (18.2 Ω) ultrapure water. Following centrifugation (5 min @ 11,000 rpm), bivalve leachate aliquots were transferred to acid-cleaned polypropylene tubes and further diluted in 2% HNO_3 (v/v) to obtain final dilution factors appropriate for trace-minor (2000-2500x) and major element (Ca: ~40,000x) determinations.

Samples were analyzed for major, minor, and trace elements, in two analytical sessions, on an Agilent 7500ce ICP-MS. The analytical method surveyed 34 elements [B, Na, Mg, Al, Si, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Zr, Mo, Ag, Cd, Sn, Sb, Cs, Ba, Tl, Pb (^{206}Pb + ^{207}Pb + ^{208}Pb), Bi, Th, and U] and 51 isotopes. Operating conditions were 1500 W forward RF power, 1W reflected RF power, 15L/min plasma gas flow, 0.9 L/min auxiliary gas flow, 0.9 L/min carrier gas flow, 0.2 L/min make-up gas flow, spray chamber cooled to 2°C, and peristaltic pump speed of 0.1 rpm. Prior to quantitative analysis, the instrument was tuned to optimize sensitivity across the mass range and normalize linearity for pulse and analog counting modes of the electron multiplier. Oxide production and doubly charged species, respectively monitored from masses 156/140 (CeO/Ce) and 140/70 (Ce^{2+}/Ce^{+}), were < 2% for all runs. Spectral interferences were removed using a collision/reaction cell (Octopol

Reaction System ®) by kinetic energy discrimination, using helium as the collision gas [^{39}K, ^{51}V, ^{52}Cr, ^{53}Cr, ^{75}As], or by mass discrimination, using hydrogen as the reaction gas [^{28}Si, ^{40}Ca, ^{56}Fe, ^{78}Se]. Reported concentrations represent the average of three separate analyte *m/z* scans of the quadrupole. Calibration standards, made gravimetrically from multi- and single-element standards (VHG Labs) certified for concentration and density, typically obtained linear regression correlation coefficients (r^2) greater than 0.9999.

Trace element detection limits (1σ), calculated from the population of blank (2% HNO_3 (v/v)) replicates interspersed throughout the analytical sequence were typically better than 0.260 ppb (median: 0.015 ppb). Accuracy and precision were evaluated from three quality control standards, also interspersed throughout the analytical sequence. QC1 is a lab fortified matrix spike made from the same stocks as used for calibration standards, with analyte concentrations near the middle of the calibration range. QC2 is an equivalent concentration standard, but made from stocks sourced independently from the calibration standards. QC3 is NIST 1643e (trace elements in water). Recoveries among optimal analyte isotopes for these QC samples averaged 99.32 ± 2.9%. Relative standard deviations (1σ) from the mean among QC replicates were better than 1% for QC1 and QC2, and better than 4% for QC3 (certified analytes).

Despite the cleaning of biominerals with H_2O_2 before elemental analysis being widely reported in the literature no studies investigated potential cleaning artifacts associated with this technique (Becker et al. 2005, Zacherl 2005,

Swearer et al. 2003, Lloyd et al. 2008, Dumphy et al. 2011). Importantly, recent work by Krause-Nehring et al. (2011) demonstrates that treatment of biogenic carbonate with (31%) H_2O_2 influences the elemental concentrations present. This is problematic for the interpretation of investigations concerning the chemical composition of biominerals that have been cleaned with H_2O_2. However, given that in the current study all samples were treated with a weaker (15%) H_2O_2 solution and in an identical fashion, we can assume that any potential artifacts will be reduced and equally distributed. Additionally, the high precision of the ICP-MS analysis allows us to assume that even though our measurements likely do not accurately reflect the chemical composition of shell material before it was cleaned the differences observed among sampling locations should not be compromised. Finally, the potential for cleaning artifacts in the present study underscores that the comparison of trace elements within clam shell with those of the ambient waters in which it formed must be taken with caution. Future work will be critical to investigate the extent of artifacts associated with the cleaning of biominerals with dilute H_2O_2.

Collection of Water and Trace Element Analysis

Between June and October of 2010, we examined the spatial distinction and temporal stability of elemental signatures within ambient estuarine waters by designating 2-3 sampling sites in each of the four aforementioned water masses. Sites separated by ~3 km along the length of each body of water were sampled to investigate potential spatial differences at local scales (Fig. 2). Water samples were collected quasi-monthly on a single day using the methodology of Mohan et

al. (2012). At each sampling site two sub-surface samples were collected at ~20 cm depth using a Masterflex peristaltic pump and filtered inline using Whatman glass micofibre filters (GF/D 1.5 µm and 0.7 GF/F µm). Samples were collected in acid washed (7% HNO_3 (v/v) HNO_3 Optima Grade; Fisherbrand) high-density fluorinated Nalgene bottles, rinsed with three sample volumes. Samples were stored on ice and transported to the laboratory on the date of collection and acidified to pH < 2.0 using trace element grade 70% HNO_3 (v/v) (Optima Grade; Fisherbrand). Prior to analysis, samples were filtered through 0.2 µm Supor syringe filters to remove particulate fractions while keeping colloidal and dissolved fractions. All water samples were stored at 6°C until analysis. Environmental characteristics including: temperature, salinity, and pH were collected in concurrence with water using a YSI-85.

The trace element concentration of ambient waters from each sampling site was determined using the methodology of Mohan et al. (2012). Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES model: Perkins/Elmer – Optima 2100 DV) was used to measure elemental concentrations of Ca, Mg, Sr, Ba, and Mn. Samples were diluted with 100 parts of ultrapure water (18.5Ω) to one part of sample. A stock standard solution (10,000 mg/L in 2% HNO_3, Across Organics) for each element was diluted to create an element-specific calibration curve with five standards (lowest low, low, medium, high, and highest high). The combined stock solution was analyzed before sample analysis to produce regression correlation coefficients (r^2) typically greater than 0.9999 for all elements. Trace element detection limits (1σ), calculated from blank (2%

HNO_3 (v/v), Optima Grade; Fisherbrand) replicates interspersed throughout the analytical sequence were typically better than 5.0 ppb. Two replicate field and lab blanks consisting of ultrapure water (18.5Ω) from each sampling date were processed and analyzed in concurrence with environmental samples. Quality control checks consisting of the low, medium, and high calibration standards required greater than 90% recovery and were issued after every twelve samples.

Data Analysis

When assumptions of normality and homoscedacity were met, ANOVA was used to investigate differences in the mean concentration of metal:calcium (hereafter Me:Ca) within juvenile shell among sampling locations for each sampling date. When these assumptions were not met, non-parametric Kruskall-Wallis tests were used. Similarly, *t*-tests and Mann Whitney U-tests were used to investigate differences in the Me:Ca of juvenile shell from individual water masses between sampling dates. Discriminant function analysis (DFA) was employed to investigate the ability to classify juvenile clams to their individual site of collection. DFA is a multivariate statistical test used to produce a predictive model composed of discriminant functions derived from linear combinations of the independent variables (Me:Ca of juvenile shell) that provide the best discrimination among our dependent variables (water mass of collection) (Manly 2005). 2-factor ANOVA was used to test if the concentration of Mg Ca, and Sr in ambient estuarine waters is infuenced by region, time, and their interaction. Region is defined as the Newport River compared to all other water masses (North River, Jarrett Bay, Back Sound).

Results

Clam Shell Length

No significant difference was detected in the mean shell length of recruited clams among water bodies for samples from 10/12/2011, ANOVA ($F_{3,21} = 0.320$, $p = 0.811$). Similarly, no significant differences were observed in the mean shell length among water bodies for samples from 6/23/2011, Kruskall-Wallis ($p > 0.05$). Significant differences were detected in shell length between sampling dates *t*-test ($t_{47} = -3.62$, $p = 0.001$) with larger clams being observed in the spring.

Spatial Distinction of Elemental Fingerprints

Of 34 elements investigated, 12 were present in juvenile shell carbonate at detectable levels on each sampling date (Spring and Fall) (Table 1). For juvenile hard clams Sampled from 10/12/2010, ANOVA ($p < 0.01$) revealed significant differences in Mg:Ca among sampling locations and Kruskall-Wallis tests ($p < 0.05$) revealed significant differences in the means of Ba:Ca and Mn:Ca among sampling locations. Using Discriminant Function analysis (DFA) with Mn:Ca, Al:Ca, Ti:Ca, Co:Ca, Cu:Ca, Ba:Ca, Mg:Ca, Zn:Ca, Pb:Ca, Ni:Ca, Sr:Ca serving as independent variables juvenile clams were assigned to their water mass of collection with 100% success (Fig. 3A and Table 2). Mn was the primary driver, responsible for 90.9% of the observed variance among water masses. For juvenile hard clams sampled from 6/23/2011, ANOVA revealed significant differences in Mg:Ca, Ti:Ca, Mn:Ca, and Co:Ca among sampling locations ($p < 0.05$) and Kruskall-Wallis ($p < 0.05$) tests revealed significant

differences in the means of Ba:Ca, Pb:Ca, Zn:Ca, Cu:Ca, and Al:Ca among sampling locations. DFA with Mn:Ca, Al:Ca, Ti:Ca, Co:Ca, Cu:Ca, Ba:Ca, Mg:Ca, Zn:Ca, Pb:Ca, Ni:Ca, Sr:Ca serving as independent variables once again assigned juvenile clams to their water mass of collection with 100% success (Fig. 3B and Table 3). Similar to the trends observed in October, Mn:Ca provided the bulk of the discriminating power and was responsible for 71.9% of the variance among water masses. The reduced contribution of Mn:Ca is influenced by the increase in the number of statistically different elements among sampling sites during June sampling.

Table 1

6/23/11	6/23/11	6/23/11	6/23/11	10/12/10	10/12/10	10/12/10	10/12/10	Date
BS	JB	NoR	NeR	BS	JB	NoR	NeR	Site
0.443±0.044	0.474±0.113	0.713±0.109	0.478±0.038	0.472±0.064	0.493±0.788	0.048±0.067	0.318±0.112	Mg:Ca (mmol mol^{-1})
0.119±0.007	0.327±0.005	0.049±0.030	0.019±0.010	0.154±0.268	0.043±0.018	0.070±0.055	0.030±0.021	Al:Ca (mmol mol^{-1})
0.227±0.087	0.507±0.194	0.505±0.117	0.186±0.038	0.731±0.358	0.631±0.179	0.957±0.540	0.385±0.256	Ti:Ca (μmol mol^{-1})
2.563±1.072	4.851±1.781	11.42±1.684	19.10±2.885	3.495±1.072	5.147±1.464	6.843±2.425	16.68±5.629	Mn:Ca (μmol mol^{-1})
0.765±0.324	1.971±1.461	15.94±19.05	0.692±0.083	4.911±3.681	3.178±1.366	2.315±1.262	2.308±2.876	Cu:Ca (μmol mol^{-1})
2.320±0.067	2.341±0.061	2.419±0.057	2.304±0.049	2.076±0.290	2.132±0.303	2.125±0.193	1.862±0.554	Co:Ca (μmol mol^{-1})
0.004±0.003	0.011±0.009	0.034±0.030	0.002±0.001	0.024±0.012	0.018±0.011	0.011±0.008	0.018±0.029	Zn:Ca (μmol mol^{-1})
6.451±1.289	4.716±3.776	3.668±0.656	2.921±1.035	7.488±6.041	2.156±0.275	2.817±0.865	2.108±0.783	Ba:Ca (μmol mol^{-1})
0.302±0.298	0.394±0.289	0.474±0.107	0.068±0.023	0.641±0.395	0.356±0.157	0.349±0.259	0.293±0.212	Pb:Ca (μmol mol^{-1})
1.549±0.048	1.495±0.037	1.546±0.032	1.524±0.043	1.621±0.062	1.565±0.078	1.572±0.059	1.486±0.526	Sr:Ca (mmol mol^{-1})
19.70±1.034	20.20±0.540	22.69±4.309	19.41±0.239	19.63±2.325	19.06±0.963	20.15±1.981	16.96±6.013	Ni:Ca (μmol mol^{-1})

Metal:Ca (Me:Ca) Within Recruited Hard Clam Juvenile shells

Table 2

			Predicted Group Membership				
		Water Mass	Back Sound	Jarrett Bay	North River	Newport River	Total
Original	Count	Back Sound	8	0	0	0	8
		Jarrett Bay	0	8	0	0	8
		North River	0	0	5	0	5
		Newport River	0	0	0	4	4
	%	Back Sound	100.0	.0	.0	.0	100.0
		Jarrett Bay	.0	100.0	.0	.0	100.0
		North River	.0	.0	100.0	.0	100.0
		Newport River	.0	.0	.0	100.0	100.0

a. Date Sampled = 10/12/10

b. 100.0% of original grouped cases correctly classified.

Classification Success of DFA Model to Identify Site Of Collection for Juvenile Hard Clams From Fall 2010

Table 3

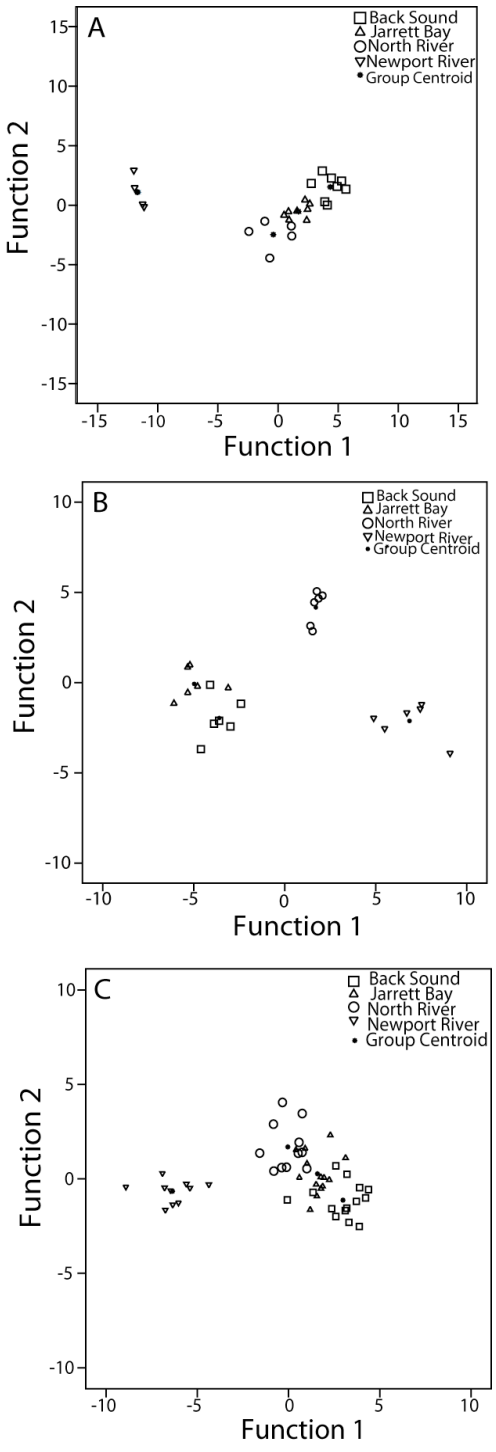
			Predicted Group Membership				
		Water Mass	Back Sound	Jarrett Bay	North River	Newport River	Total
Original	Count	Back Sound	6	0	0	0	6
		Jarrett Bay	0	6	0	0	6
		North River	0	0	6	0	6
		Newport River	0	0	0	6	6
	%	Back Sound	100.0	.0	.0	.0	100.0
		Jarrett Bay	.0	100.0	.0	.0	100.0
		North River	.0	.0	100.0	.0	100.0
		Newport River	.0	.0	.0	100.0	100.0

a. Date Sampled = 6/23/11

b. 100.0% of original grouped cases correctly classified.

Classification Success of DFA Model to Identify Site Of Collection for Juvenile Hard Clams From June 2010

Figure 3


A
Back Sound
Jarrett Bay
North River
Newport River
Group Centroid
Function 2
Function 1
B
Back Sound
Jarrett Bay
North River
Newport River
Group Centroid
Function 2
Function 1
C
Back Sound
Jarrett Bay
North River
Newport River
Group Centroid
Function 2
Function 1

DFA Plots Using (Me:Ca) to assign juvenile clams to their site of collection during (A) October 2010 (B) June 2011, and (C) A combined DFA using Fall and Spring Shell Sample

Temporal Stability of Elemental Fingerprints

Significant temporal differences were detected in the Me:Ca of juvenile clam shells in all water masses of investigation. In Back Sound, significant decreases of Ti:Ca, Cu:Ca, and Zn:Ca (Mann Whitney U, $p < 0.05$) were observed between October of 2010 and June of 2011. Jarrett Bay saw a significant increase in Ni:Ca (ANOVA, $p < 0.05$) during this period. The North River experienced increases in Mg:Ca, Mn:Ca, and Co:Ca (ANOVA, $p < 0.05$) during the same interval. Finally, in the Newport River Mg:Ca was significantly elevated (ANOVA, $p < 0.05$) in the spring compared to the fall, while Pb:Ca experienced a significant reduction (ANOVA, $p < 0.05$).

Despite a larger suite of elements experiencing significant differences among water masses in the spring relative to the fall, DFA was 100% successful in assigning juvenile clams to their water mass of collection on each sampling date. A combined DFA using Me:Ca from both fall and spring sampling revealed a reduction in classification success to 81.9% (Fig. 3C and Table 4). Mn:Ca was responsible for 89.9% of the among group variance and only differed significantly in the North River between sampling dates. These data suggest that in our area of investigation, a reproducible elemental signal exists in hard clam juvenile shell that is driven predominately by Mn:Ca (Fig. 4).

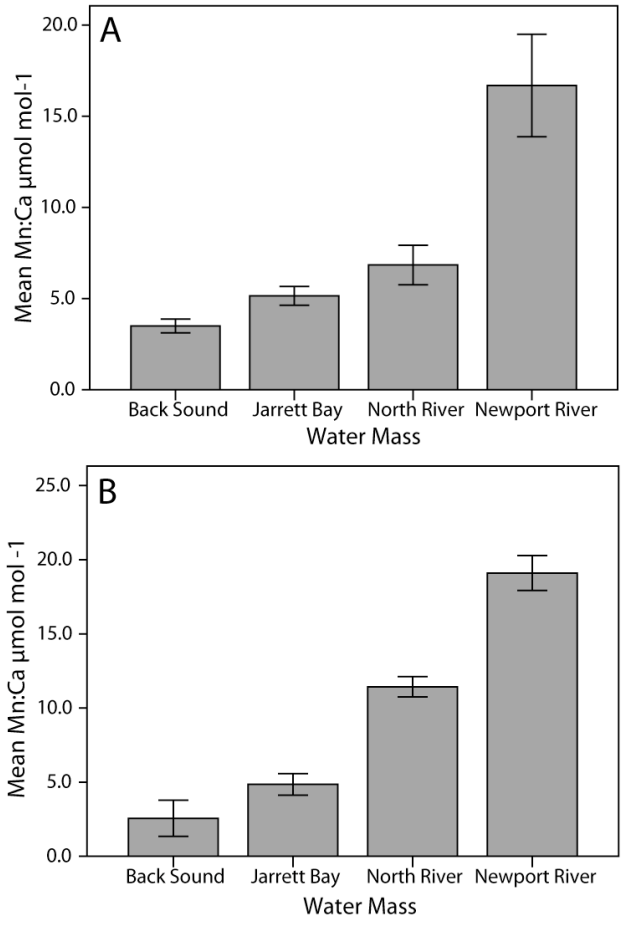
Table 4

			Predicted Group Membership				
		Water Mass	Back Sound	Jarrett Bay	North River	Newport River	Total
Original	Count	Back Sound	10	4	0	0	14
		Jarrett Bay	2	10	2	0	14
		North River	0	1	10	0	11
		Newport River	0	0	0	10	10
	%	Back Sound	71.4	28.6	.0	.0	100.0
		Jarrett Bay	14.3	71.4	14.3	.0	100.0
		North River	.0	9.1	90.9	.0	100.0
		Newport River	.0	.0	.0	100.0	100.0

a. 81.6% of original grouped cases correctly classified.

Classification Success of DFA Model to Identify Site Of Collection for Juvenile Hard Clams From October 2010 and June 2011

Figure 4



Mean concentration of Mn:Ca expressed as µmol mol^{-1} within the shells of recruited hard clams collected during (A) October, 2010 (B) June, 2011. Error bars ±1 standard error

Elemental Content of Estuarine Waters

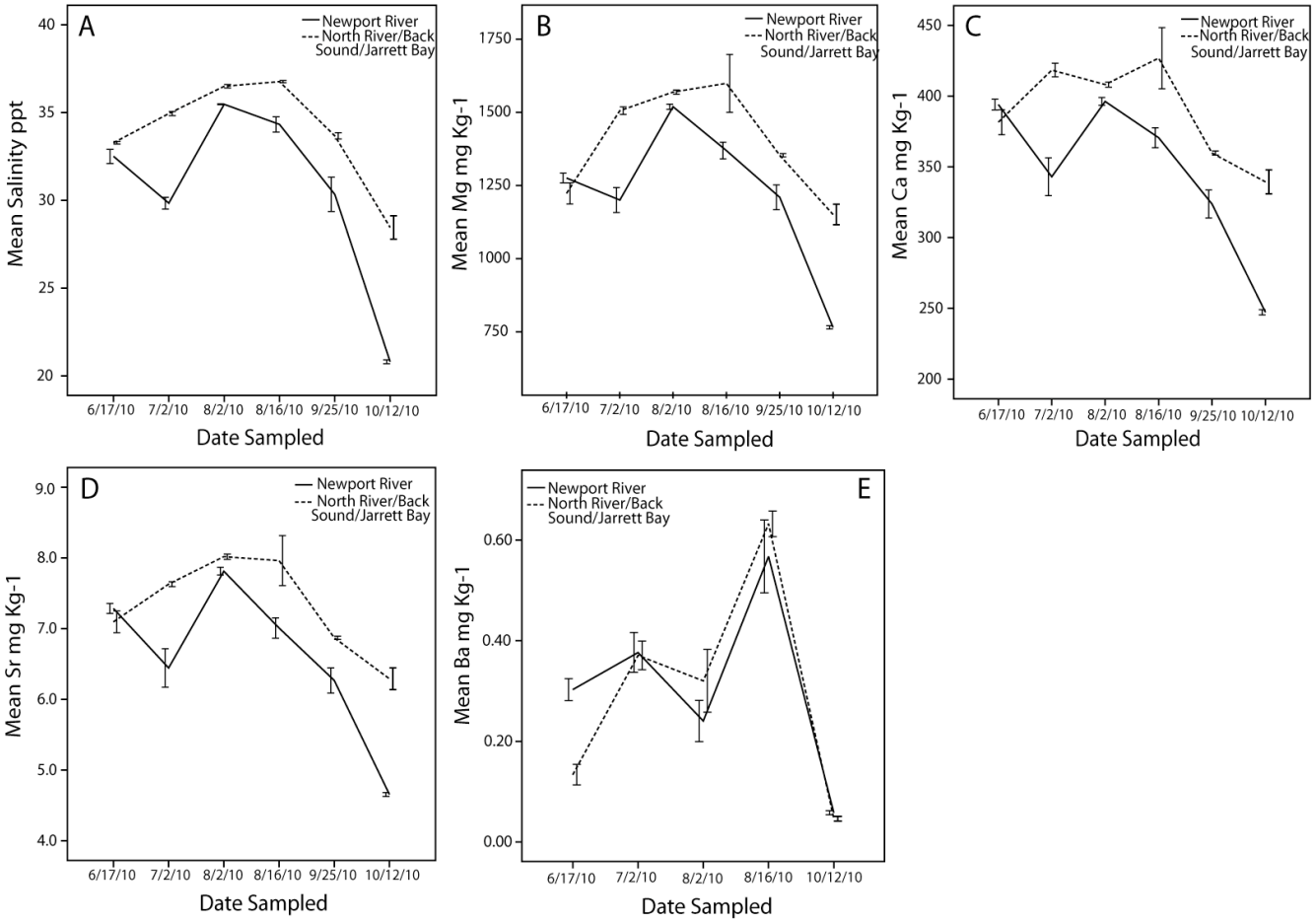
Of five elements investigated Sr, Mg, Ca, and Ba were present at detectable levels. Mn was below limits of detection on all sampling dates. ANOVA revealed significant effects of month, region, and an interaction between month and region regarding the concentration of Sr, Mg, and Ca ($p < 0.001$) (Table 5). The concentration of Sr, Mg, and Ca did not differ significantly between the North River, Back Sound, and Jarrett Bay on all sampling dates. Concentrations of these elements covaried with salinity and were significantly reduced in the Newport River compared to all other water masses on most sampling dates (Figs. 5A & 5B & 5C & 5D). A relationship with salinity is further evidenced by their shared conservative behaviors along the salinity gradient (Fig. 6A & 6B & 6C). Ba varied spatially and was observed to be elevated in the Newport River in June compared to all other areas of investigation. Ba also differed temporally and was observed to increase from June to August in all water masses, be below the limits of detection in September, and exhibit a pronounced reduction October compared to all previous sampling dates (Fig. 5E).

Table 5

Source	Dependent variable	Type III sum of squares	df	MS	*F*	p
Region	Sr	14.1	1	14.1	35.8	0.000
	Mg	824 973.9	1	824 973.9	31.0	0.000
	Ca	49 188.3	1	49 188.3	35.2	0.000
Month	Sr	61.3	4	15.3	38.9	0.000
	Mg	3.7	4	932 004.0	35.1	0.000
	Ca	155 728.4	4	38 932.1	27.9	0.000
Region × Month	Sr	8.5	4	2.1	5.4	0.001
	Mg	509 238.9	4	4.8	4.8	0.001
	Ca	29 443.2	4	5.3	5.3	0.001

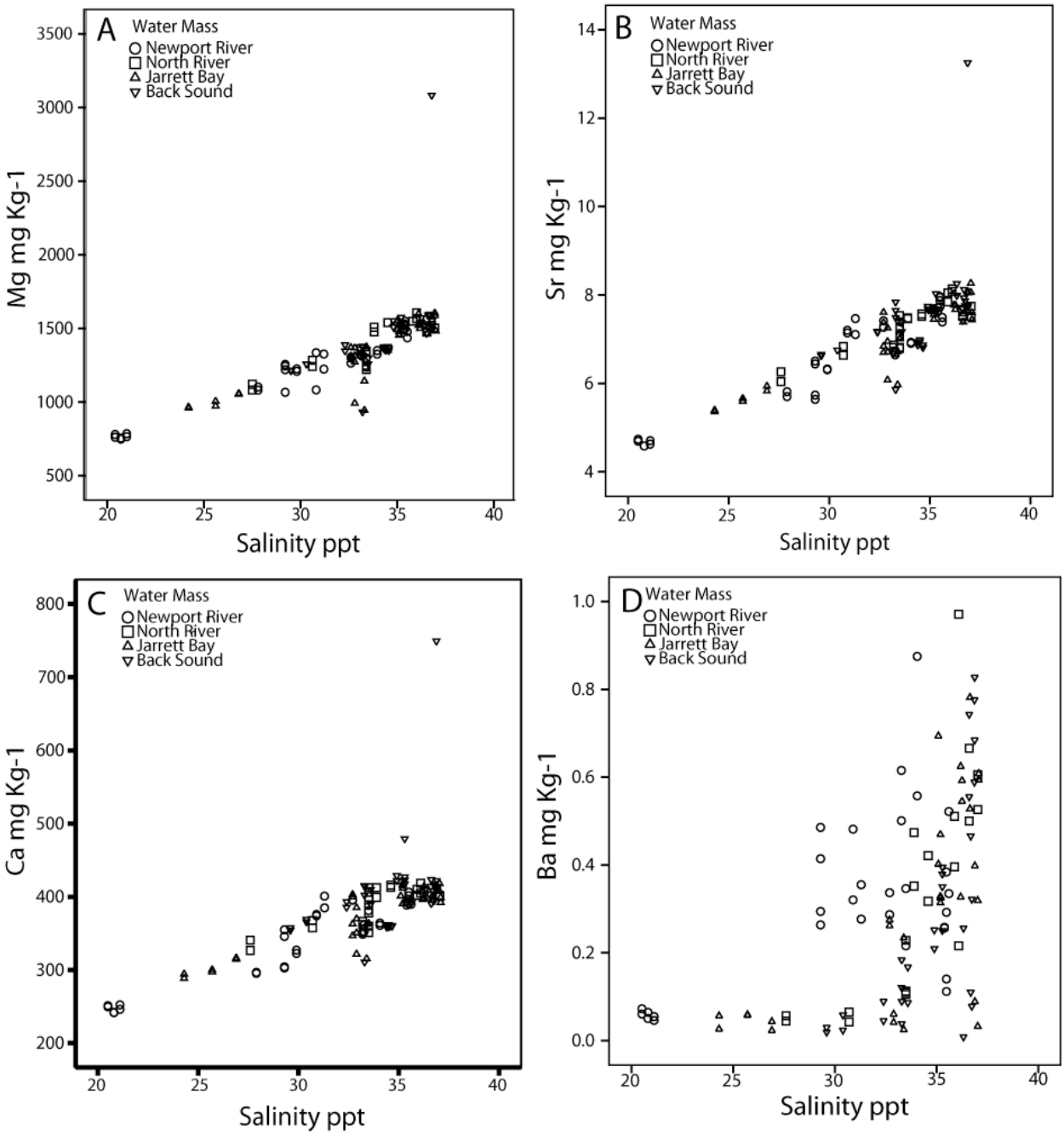
Resluts of 2-factor ANOVA testing if the concentration of Mg Ca, and Sr in ambient estuarine waters is infuence by region, time, and their interaction. Region is defined as the Newport River comapred to all other water masses (North River, Jarrett Bay, Back Sound)

Figure 5



Temporal patterns of salinity and elemental concentrations within ambient estuarine waters collected between June-October of 2010. (A) Salinity, (B) Mg, (C) Ca, (D) Sr (E) Ba. Error bars ±1 standard error

Figure 6



Variation in (A) Mg, (B) Sr, (C) Ca, and (D) Ba and salinity, grouped as Newport River, North River, Jarrett Bay, and Back Sound

Discussion

Spatial Variation of Elemental Fingerprints

Our results demonstrate that distinct elemental signals exist in hard clam juvenile shell and are detectable at small spatial scales (~12 km)(Fig. 3A & 3B and Tables 2 & 3). Our results further suggest that elemental signals are likely to exist in larval shell (PDI) that is produced overt the first 24-48 h of a clam's life and may be used to indicate natal origin. Mn, Ba, and Mg were the only elements present in juvenile shell that differed significantly among water masses on both sampling dates. DFA revealed that Mn was the primary component of our elemental signal explaining the bulk of among site variance during both fall (91.2%) and spring (71.9%). The observed contribution on Mn is not unexpected as this element has been used to help discriminate the natal origin and/or site of collection for crab zoea, juvenile bivalves, pelagic bivalve veligers, and encapsulated gastropod veligers (DiBacco and Levin, Becker et al. 2005, Dunphy et al. 2011, Becker et al. 2007, Zacherl 2005).

In estuarine systems, Mn is delivered via fluvial inputs and the reduction and solubilization of MnO_2 within anoxic sediments (Evans et al. 1977). Once dissolved Mn has been delivered or released into the water column it has been reported to behave both conservatively and non-conservatively along the estuarine salinity gradient (Evans et al. 1977, Morris et al. 1982, Dorval et al. 2005*a*). Unfortunately, Mn was below the limits of detection in sampled estuarine waters preventing a direct comparison with salinity or concentrations within juvenile shell. This limitation is further compounded by elevated Mn

concentrations in muddy sediments within our study area compared to the overlying water column (Evans et al. 1977). This complicates the assumption that hard clam larval shell will reflect the Mn signal observed in juvenile shell due to a decreased proximity to the sediment water interface. However, in the current study, juvenile clams were collected from sandy habitats where metal entrainment is minimal compared to fine sediments (Douglas et al. 2007). Riggs et al. (1991) investigated sediment Mn concentrations associated with various substrate types within an estuarine system adjacent to our study area (Neuse River Estuary). Data obtained from this investigation demonstrates that Mn is reduced substantially in substrate that is predominately sand (Fig. 7). Work by Zacherl et al. (2009) demonstrates a positive linear relationship that acts independently of ontogeny between Mn concentration in culture water and incorporation into larval and juvenile shell of the Olympic oyster *Ostrea lurida*. If the incorporation of Mn into hard clam carbonate also operates independently of ontogeny, the distinct Mn signal observed in juvenile shell may also be present in the larval form.

Ba has also been successfully employed to help discriminate the natal origin and/or site of collection for multiple invertebrate models (DiBacco and Levin 2000, Becker et al. 2005, Dunphy et al. 2011, Becker et al. 2007, Zacherl 2005). The delivery of Ba into estuarine systems is driven predominately by fluvial inputs and is reported to mix conservatively along the estuarine salinity gradient (Coffey et al. 1997, Dorval et al. 2005*a*). Our results demonstrate that within our study area dissolved Ba is proportional to salinity (Fig. 6D). Studies

have demonstrated a positive linear relationship between the Ba content of culture water and incorporation into invertebrate biominerals (Gilliken et al. 2006, Zacherl et al. 2003*b*, Zacherl et al. 2009). However, the Ba content of juvenile shell collected during October differed among locations despite an absence of significant differences in Ba concentration among estuarine waters (Fig. 5E). The analysis of the entire biomineral in solution could potentially explain this contradiction as it provided a cumulative signal generated over the entire juvenile phase that went undetected in individual water samples (Campana 1999, Becker et al. 2005).

There is also evidence to suggest that Ba may be associated with phytoplankton biomass, particularly diatoms. The demonstration of dissolved Ba being highly concentrated on diatom frustules via adsorption onto iron oxyhydroxides (Sternberg et al. 2005) prompted Thébault et al. (2009) conclude that dissolved Ba associated with diatoms desorb in the bivalve gut due to low pH (< 8) and absorb through the gut epithelium into internal fluids to be incorporated into shell material. Analysis of sampled estuarine waters occurred after a series of filtrations to remove particulate fractions while maintaining colloidal and dissolved fractions. Thus any Ba associated with primary production via adsorptive processes could be entrained during filtration thereby reducing estimates of dissolved Ba available in ambient estuarine waters. Future studies would benefit from the simultaneous analysis of filtered and unfiltered water to provide a more holistic interpretation of elemental species available for incorporation into molluscan biominerals.

Juvenile shell also exhibited significant differences in Mg concentration among water masses on both sampling dates. In estuarine waters, Mg is reported to behave conservatively along the salinity gradient (Stecher et al. 1996, Dorval et al. 2005*a*). This trend was supported in our study area as evidenced by consistently reduced concentrations in the Newport River compared to all other water masses of investigation (Figs. 5B & 6A). Concentrations of Mg in hard clam juvenile shell collected during October parallel this observation hinting that a relationship may exist between ambient Mg availability and incorporation into juvenile shell (Fig. 5B and Table 1). Work by Lorens and Bender (1980) support this relationship by demonstrating a directly proportional relationship between Mg content of bivalve aragonite and the Mg content of the water in which it formed.

The elements Ti, Co, Cu, Zn, Al, and Pb within juvenile clam shell differed among water masses during June sampling only (Table 1). DiBacco and Levin (2000) used Cu, Zn, Al to discriminate crab zoea collected from San Diego Bay. Similarly, Becker et al. (2005, 2007) used Pb to discriminate both mussel recruits and outplanted veligers from San Diego Bay. These authors postulate industrial and urban runoff as the primary factors influencing the availability of these elements. Laboratory experiments conducted by Zacherl et al. (2009) demonstrate a positive relationship between Pb concentrations in culture water and incorporation into juvenile and larval oyster shell and an absence of any such relationship with Cu. The well-mixed nature of our study area coupled with differential land use patterns including non-point pollution from the adjacent municipalities of Morehead City and Beaufort and extensive agricultural

development suggests that anthropogenic inputs may be influencing the availability and incorporation of heavy metals into juvenile clam shell (Swearer et al. 2003).

Finally, Sr represents an element that did not differ within juvenile clam shell among sites during October despite consistently reduced concentrations within the Newport River compared to all other water masses (Fig. 5D and Table 1). These findings are in agreement with other investigations concerning Sr incorporation into molluscan biominerals (Lloyd et al. 2008, Zacherl et al. 2009, Zumholz 2007). Sr incorporation within abiogenic carbonate is highly influenced by precipitation rate (Lorens 1981, Morse and Bender 1990). Additionally, work by Lorrain et al. (2005) demonstrates that kinetic effects strongly influence Sr incorporation into *Pecten maximus* shell. Similar kinetic effects could potentially explain the absence of any relationship between the Sr content juvenile hard clam carbonate and the Sr content within the estuarine waters in which it formed.

Temporal Variation of Elemental Fingerprints

11 minor and trace elements were present in juvenile clam shell on both sampling dates. Of these elements Ti, Cu, Zn, Mg, Mn, Co, Ni, Mg and Pb differed significantly between sampling dates. These differences are not unexpected given the dynamic nature of estuarine systems (Swearer et al. 2003). Work by Fodrie et al. (2011) demonstrated monthly and seasonal variation in the elemental composition of Mg, Cu, Sr, Mn, Cd, Pb, and U of juvenile shell from two species of *Mytilus*. Similarly, Dumphy et al. (2011) demonstrated seasonal variation regarding the Ni, Cu, and Ba content of juvenile green-lipped mussels

Perna canaliculus. A combined DFA using clams from both sampling dates suggests that temporal differences in elemental composition confounded our signal as evidenced by a reduction in overall classification success (81.9%) (Fig. 3C and Table 4). Importantly, Mn still explained the bulk of our among group variance (89.9%) and only differed significantly ($p < 0.05$) in the North River between sampling dates. Additionally, Mn within juvenile shell was consistently different among water masses on both sampling dates suggesting that the primary component of our elemental signal is reproducible through time (Figs. 4A & 4B). However, given that DFA from individual sampling events yielded perfect classification success (100%) of individual sampling sites the collection of larval reference samples during seasonally elevated periods of dispersal could maximize the applicability of elemental fingerprinting to identify the natal origin of unknown recruits (Figs. 3A & 3B and Tables 2 & 3). Ideally, a reference map of larval trace element signatures for individual water masses would be generated and monitored for intra and inter-annual variability. Pending the stability of elemental signals encountered it may be possible to collect a recruited hard clam, analyze the elemental composition of the PDI, then compare it to the reference map of known location to potentially identify the natal origin and dispersal trajectory of an individual clam.

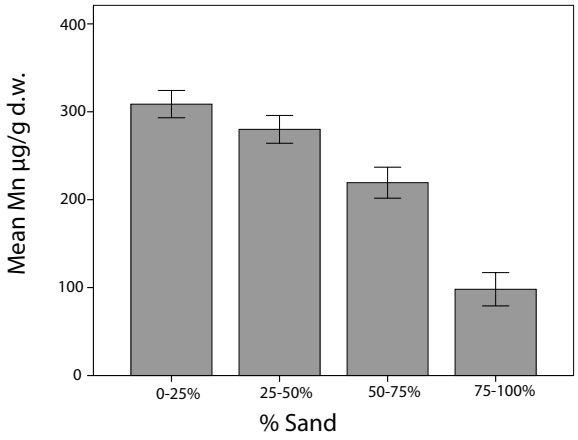
The incorporation of minor and trace elements into bivalve shell is strongly influenced by vital and kinetic effects (Stecher et al. 1996, Carré et al. 2006, Takasue et al. 2008, Schöne 2008, Lorrain et al. 2005). In North Carolina recruited hard clams can be assigned to spring or fall spawning events if they fall

between16-26 mm or below 15 mm length thresholds respectively (Peterson 2002). All sampled recruits were <10 mm in length allowing us to assume that they are representative of seasonal spawning events. However, the growth of juvenile hard clams is highly variable and may be influenced by interactions within or among exogenous and endogenous factors (as reviewed by Carriker 2001). Within our study area growth of hard clams is highly influenced by both habitat type and genetic composition (Peterson and Beal 1989). Given these factors, we can only assume that juvenile clams collected during October settled at some point during the reproductive season of 2010, while clams from June sampling settled at some point during the spring of 2011. To reduce the bias of exogenous factors on juvenile clam growth, samples were collected from similar habitat types (subtidal sand) in each water mass. However, it is difficult to account for genetic variation among sites given the planktonic duration of hard clam larvae (~11-20 d) and the well mixed nature of our study area (Carriker 2001, Leuttich et al. 1999). Given that juvenile clams collected during the spring were significantly larger ($t_{47} = -3.62$, $p = 0.001$) than individuals collected during the fall it is possible that differences in shell growth influenced rates of elemental incorporation between sampling dates.

The possibility that juvenile clams collected during October sampling could have settled at any point during 2010 also suggests that ontogenetic effects may be influencing elemental incorporation. Zacherl et al. (2009) investigated the influence of ontogeny on the elemental incorporation of multiple elements into oyster shell and demonstrated that Mg, Sr, and Cu exhibit ontogenetic effects.

Specifically, Mg was elevated in settled individuals while Sr and Cu were increased in the larval form. Additionally, Cu has been shown to exhibit a similar ontogenetic effect regarding assimilation into hard clam somatic tissue (Larson 1979). These studies highlight that the extrapolation of elemental signals within juvenile carbonate to pelagic larvae must be taken with great care.

Figure 7



Mean concentration of Mn within sediments sampled from the Neuse River Estuary containing various fractions of sand. Units are micrograms/gram dry weight. Error bars ±1 standard error. Data from Riggs et al. (1991)

CHAPTER III: SPATIOTEMPORAL STABILITY OF TRACE AND MINOR ELEMENTAL SIGNALS WITHIN THE EARLY LARVAL SHELL OF THE HARD CLAM MERCENARIA MERCENARIA

The objective of this chapter was to investigate the temporal stability of trace element compositions resident within generations of D-stage larval hard clams, and the extent to which these may be used as geospatial tracers of natal estuaries. I tested the hypothesis that the incorporation of trace elements into hard clam larval shell will be insufficient to spatially or temporally discriminate among shellfish hatcheries separated by local and regional spatial scales (~1-50 km). To assess spatial distinction, D-stage larvae were spawned within a five-day interval from three shellfish hatcheries separated by ~1-50 km. The temporal variability (stability) of trace element compositions incorporated into larvae associated with a particular hatchery was assessed by collecting D-Stage larvae from four tri-weekly spawning events, each from a single hatchery. The validation of geospatially distinct elemental signals within larval bivalve shell has typically been accomplished by *in situ* culturing, or outplanting (Becker et al. 2007). Here, we capitalize on the existing infrastructure of commercial shellfish hatcheries. Hatcheries provide a unique and cost-effective opportunity to investigate spatiotemporal chemical variability in rapidly formed invertebrate larval biominerals. At the hatchery, newly spawned larvae (i.e., hard clam) incorporate trace elements during growth (biomineralization) within stable environments hosted by natural waters taken directly from adjacent estuaries. This sampling approach, here termed *inplanting*, is thus beneficial because it

avoids logistical difficulties (including large scale mortality) associated with outplanting larvae into estuaries within temporary enclosures.

Shell major, minor and trace element chemistry was analyzed by solution-mode inductively coupled plasma mass spectrometry (ICP-MS). Our goal was to determine the temporal variability (stability) of larval shell trace element compositions from natal hatcheries, and the extent to which larval shell trace element compositions can be used to discriminate natal origin (hatchery locality). If trace element compositions of larval shell (PDI) formed in individual hatcheries do not vary significantly through time during spawning events and allow for spatial discrimination between hatcheries, then PDI elemental signatures offer the potential to track patterns of hard clam larval dispersal and population connectivity, and to identify subpopulations that may differentially contribute to overall population dynamics by disproportionately supplying larvae. PDI tracer capabilities, if verified, could thus have significant implications for the management, conservation, and restoration of hard clam populations.

Methods

Sample Collection and Preparation

During February of 2012, three commercial shellfish hatcheries (Cherrystone Aquafarms, Cheriton, VA; Cherrystone Aquafarms, Willis Wharf, VA; and J.C. Walker Brothers Inc., Willis Wharf, VA) (Fig. 8) provided three replicates of D-stage hard clam larvae (>10^6 larvae per replicate) that had been spawned within a 5 d interval. Additionally, J.C. Walker Brothers provided 3 replicates of D-stage larvae from each of 3 subsequent, tri-weekly spawning events that spanned February 12th-April 10th. Culturing conditions at Cherrystone Aquafarms were 23.3ºC and a salinity of 30 and larvae from J.C. Walker Brothers were reared at 25ºC and a salinity of 28. Samples were frozen in culture water and maintained at 0ºC until processed for elemental analysis. To minimize potential cleaning artifacts, samples were not treated with cleaning agents to remove organics (Krause-Nehring et al. 2011).

The isolation of larval shell was conducted in a class 100 Laminar flow hood. Pooled larvae from each replicate were placed on 20 µm mesh, rinsed with (18.2 Ω) ultrapure water, and transferred into 1.5 ml acid-washed polypropylene vials (7% HNO_3 (v/v) Optima Grade; Fisherbrand). Larvae were then suspended in 1 ml (18.2 Ω) ultrapure water, vortexed for 30 s, sonicated for 5 min, and finally centrifuged for 3 min at 6,000 rpm. This method results in a stratification of material such that lighter larval somatic tissue and periostracum overlies heavier larval shell. Organic tissue and (18.2 Ω) ultrapure water were

removed and larvae were re-suspended in 1 ml (18.2 Ω) ultrapure water and treated an additional 9 times using the aforementioned methodology. Larvae were then dried under laminar flow and inspected for residual somatic tissue, which when encountered was removed using an additional 5 treatments. Once dry, pooled larvae from each replicate were weighed to the nearest mg. Shells were dry stored in acid-washed polypropylene vials (7% HNO_3 (v/v) Optima Grade; Fisherbrand) until analysis.

Elemental Analysis of Clam Shells using ICP-MS

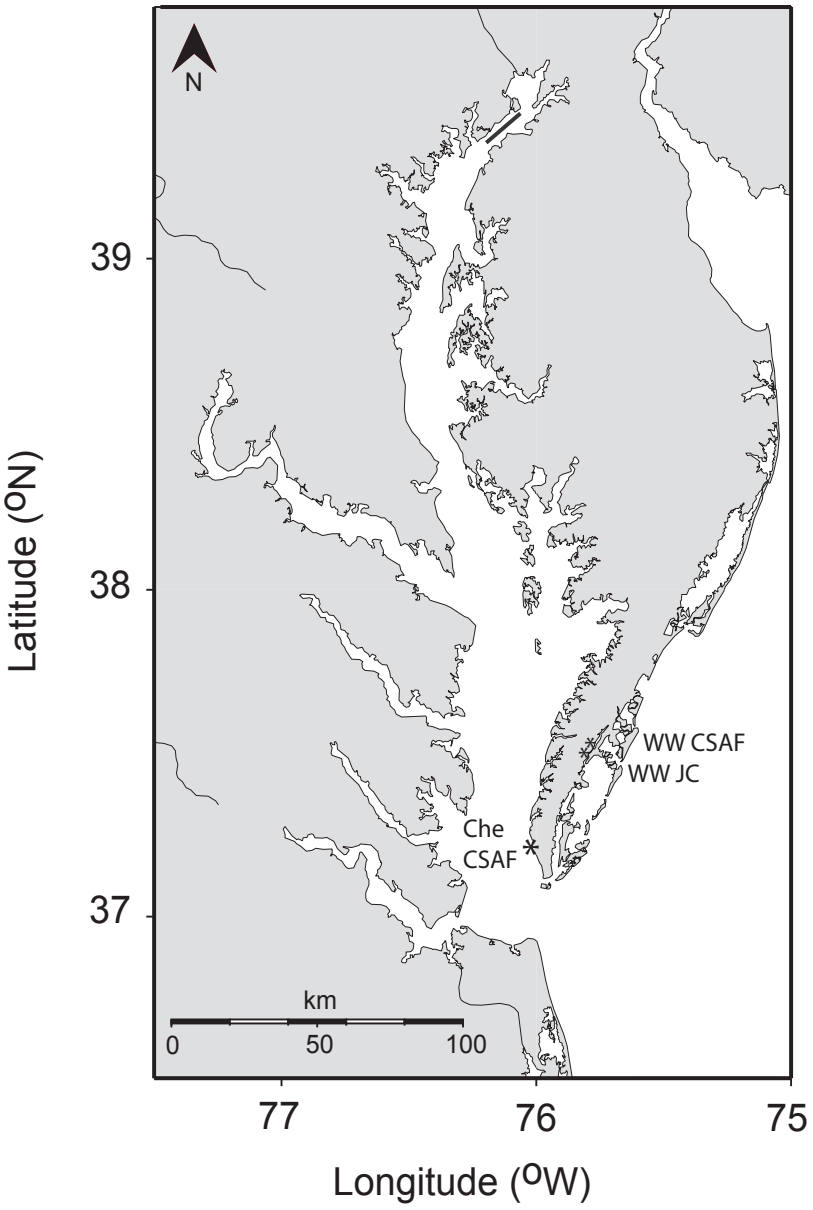
Juvenile bivalve shell (n = 18) concentrates, weighing 0.7 to 17.9 mg, were reacted for 12 hours with 1.25 mL of 0.9 M HNO_3 (Fisher trace metal grade) in acid-washed polypropylene microvials, then centrifuged (5 min @ 11,000 rpm) to settle undissolved residues. The supernatant (1.1 mL) was transferred to acid-cleaned microvials from which aliquots were diluted to levels appropriate for measurement of trace and minor elements (~2350x) and major elements (~82,000x). The acid strength and volume used for digestions was capable of dissolving all carbonate in the sample (based on starting weight) by factors of 3 to 80 (median = 8.9). However, samples typically had prominent tan-colored insoluble residues after centrifugation. To evaluate the non-carbonate insoluble residue content of larval bivalve shells, we determined insoluble residue contents of three representative sample splits (0.7 to 1.1 mg). Resulting insoluble residues varied between 26.0 and 43.8 wt% (avg: 33.7 ± 9.1 wt%), indicating that organic shell matrix comprised a substantial proportion of shell concentrate samples.

Elemental concentrations (B, Na, Mg, Al, Si, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, As, Se, Rb, Sr, Zr, Mo, Ag, Cd, Sn, Sb, Cs, Ba, Tl, Pb, Bi, Th, and U) were determined using an Agilent 7500ce ICP-MS at the University of Texas at Austin. (Dept. of Geological Sciences). The instrument was optimized for sensitivity across the AMU range, while minimizing oxide production (< 1.9%). The analytical method employed an octopol reaction system (ORS), operated in helium (collision-mode) and hydrogen (reaction-mode) atmospheres for removal of polyatomic interferences. Internal standards, mixed into unknowns via in-run pumping, were used to compensate for instrumental drift and internal standard sensitivity variations were well within QA tolerances (± 50%). Limits of detection, based upon the population of blank (2% HNO_3) analyses interspersed throughout the analytical sequence were typically better than 0.173 ppb (median = 0.009 ppb) for analytes measured in optimal modes (with or without the ORS). Analyte recoveries obtained for replicates of two independent quality control standards were typically within 2% of certified values. Relative precisions (n = 2-3) obtained for these quality control standards were typically within 0.4 to 1.6% of replicate averages. Matrix spikes, performed on two randomly selected samples, had analyte recoveries of 97-98%, indicating that matrices of diluted samples ionized comparable to calibration and quality control standards.

Ca concentrations calculated based on starting shell weights averaged 56 ± 18% of concentrations expected in stoichiometric $CaCO_3$. These are systematically low values consistent with samples having appreciable insoluble residue contents (in agreement with the range of our insoluble residue

determinations described above). Accordingly, larval shell cation concentrations were normalized according to Ca concentration and assuming a stoichiometric $CaCO_3$ composition. This is supported by consistent Ca concentrations within juvenile hard clam shell mineralogy (393.6 ± 10.5 mg/L (Cathey et al. 2012). As long as leachate cations derive predominantly from shell carbonate the metal to calcium ratios considered subsequently are unaffected by mineral phase composition.

Figure 8


N
39
38
37
Latitude (°N)
WW CSAF
WW JC
Che
CSAF
km
0
50
100
77
76
75
Longitude (°W)

Map of the Chesapeake Bay and Delmarva Peninsula. Asterisks denote hatcheries that supplied D-stage hard clam larvae. Cherrystone Aquafarms Cheriton (Che CSAF), Cherrystone Aquafarms Willis Wharf (WW CSAF), and J.C. Walker Brothers Willis Wharf (WW JC)

Data Analysis

When assumptions of normality and homoscedacisity were met, ANOVA was used to investigate differences in the mean concentration of metal:calcium (hereafter Me:Ca) within pooled larval shell among sampling locations. When these assumptions were not met, non-parametric Kruskall-Wallis tests were used. Similarly ANOVA and Kruskall-Wallis tests were used to investigate differences in the Me:Ca of larval shell among sampling dates. Discriminant function analysis (DFA) was employed to investigate the ability to classify larval clams from different hatcheries based on their multivariate Me:Ca ratios. DFA is a multivariate statistical test used to produce a predictive model composed of discriminant functions derived from linear combinations of the independent variables (Me:Ca of larval shell) that provide the best discrimination among our dependent variables (hatchery of origin and date of collection) (Manly 2005).

Results

Spatiotemporal variability of elemental signals

Seventeen of the 34 trace and minor elements investigated were present in the larval shell at detectable levels in all samples (Figs. 9 & 10). For D-stage larvae sampled from February 2012, ANOVA ($p < 0.01$) revealed significant differences in the means of Mg:Ca, Ti:Ca, Co:Ca, Ni:Ca, Zn:Ca, Se:Ca, and Rb:Ca among hatcheries and Kruskall-Wallis tests ($p < 0.01$) revealed significant differences in the means of Al:Ca, V:Ca, Cr:Ca, Mn:Ca, Cu:Ca, Sr:Ca, Ba:Ca, Pb:Ca, and U:Ca among hatcheries (Fig. 9). A (DFA) with Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, Pb Ca, Al:Ca, Mn:Ca, and Cr:Ca serving as independent variables assigned larval clams to their hatchery of origin with 100% success (Fig. 11a and Table 6). Cr:Ca was the primary driver of our elemental signal, responsible for 78.1% of the observed variance among natal locations.

Significant temporal differences were detected in the Me:Ca of pooled larval shell for samples collected tri-weekly from J.C. Walker Brothers between February 12th-April 10th. ANOVA ($p < 0.05$) revealed differences in Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, and Pb:Ca. Similarly, Kruskall-Wallis tests ($p < 0.05$) revealed significant temporal differences in Al:Ca, Mn:Ca, and Cr:Ca (Fig. 10). DFA using Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, Pb:Ca, Al:Ca, Mn:Ca, and Cr:Ca as independent variables correctly assigned pooled individuals to their date of

collection with 100% success (Fig. 11b Table 10). Al:Ca explained 81.4% of the observed variance among collection date.

Table 6

Classification Results[a]

			Predicted Group Membership			
		Site	Cheriton CSAF	Willis Warf JC	Willis Warf CSAF	Total
Original	Count	Cheriton CSAF	3	0	0	3
		Willis Warf JC	0	3	0	3
		Willis Warf CSAF	0	0	3	3
	%	Cheriton CSAF	100.0	.0	.0	100.0
		Willis Warf JC	.0	100.0	.0	100.0
		Willis Warf CSAF	.0	.0	100.0	100.0

a. 100.0% of original grouped cases correctly classified.

Classification success using larval shell microchemistry to determine natal hatchery from J.C.Walker Bros in Willis Wharf (WW JC), Cherrystone Aquafarms in Willis Wharf (WW CSAF), and Cherrystone Aquafarms in Cheriton (Che CSAF). Grouped as WW JC, WW CSAF, and WW CSAF. Rows denote actual grouping using discriminant function anlysis (DFA) model

Table 7

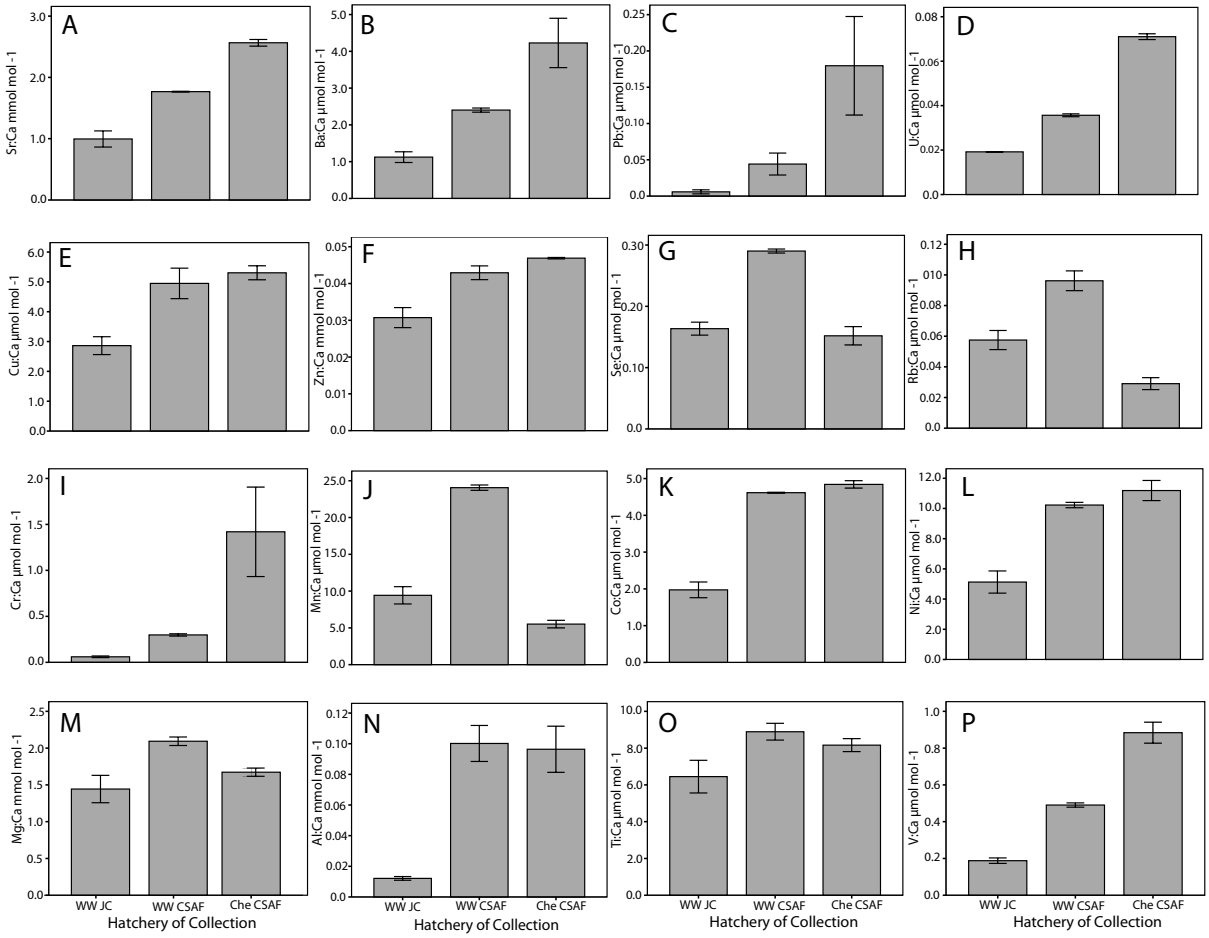
Classification Results[a]

			Predicted Group Membership						
		Date	WW JC S1	WW JC S2	WW JC S3	WW JC S4	WW CSAF T1	Ch CSAF T1	To
Original	Count	WW JC S1	3	0	0	0	0	0	
		WW JC S2	0	3	0	0	0	0	
		WW JC S3	0	0	3	0	0	0	
		WW JC S4	0	0	0	3	0	0	
		WW CSAF T1	0	0	0	0	3	0	
		Ch CSAF T1	0	0	0	0	0	3	
	%	WW JC S1	100.0	.0	.0	.0	.0	.0	1
		WW JC S2	.0	100.0	.0	.0	.0	.0	1
		WW JC S3	.0	.0	100.0	.0	.0	.0	1
		WW JC S4	.0	.0	.0	100.0	.0	.0	1
		WW CSAF T1	.0	.0	.0	.0	100.0	.0	1
		Ch CSAF T1	.0	.0	.0	.0	.0	100.0	1

a. 100.0% of original grouped cases correctly classified.

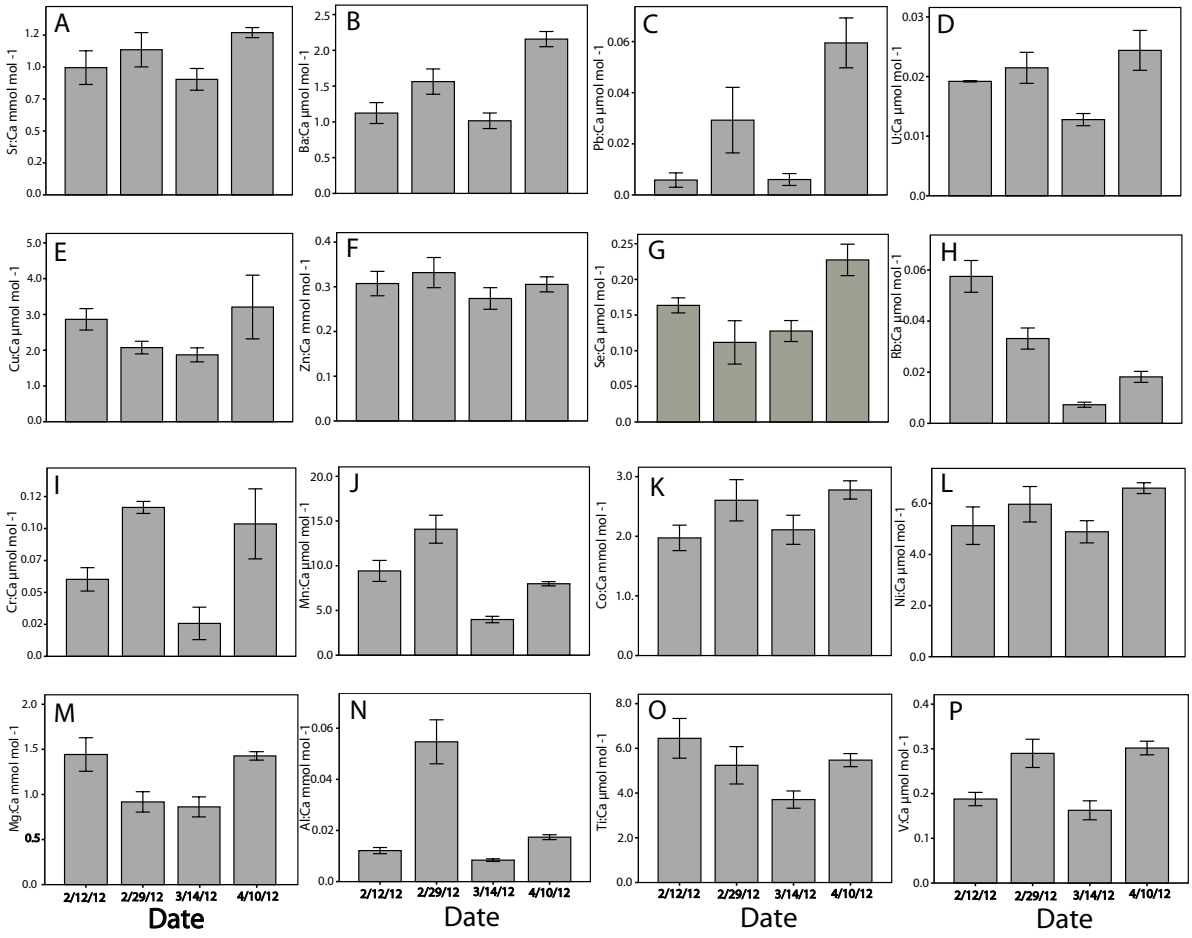
Classification success using larval shell microchemistry to determine spawning date for for hard clam larvae collected from J.C.Walker Bros in Willis Wharf from each of 4 triweekly spawning events spanning February 12th-April 10th (ww JC S1-S4) and clams collected from Cherrystone Aquafarms in Willis Wharf (WW CSAF), and Cherrystone Aquafarms in Cheriton (Che CSAF). Grouped as WW JC, WW CSAF, and WW CSAF. Grouped by date spawned and site of collection. Rows denote actual grouping using discriminant function anlysis (DFA) model

Figure 9



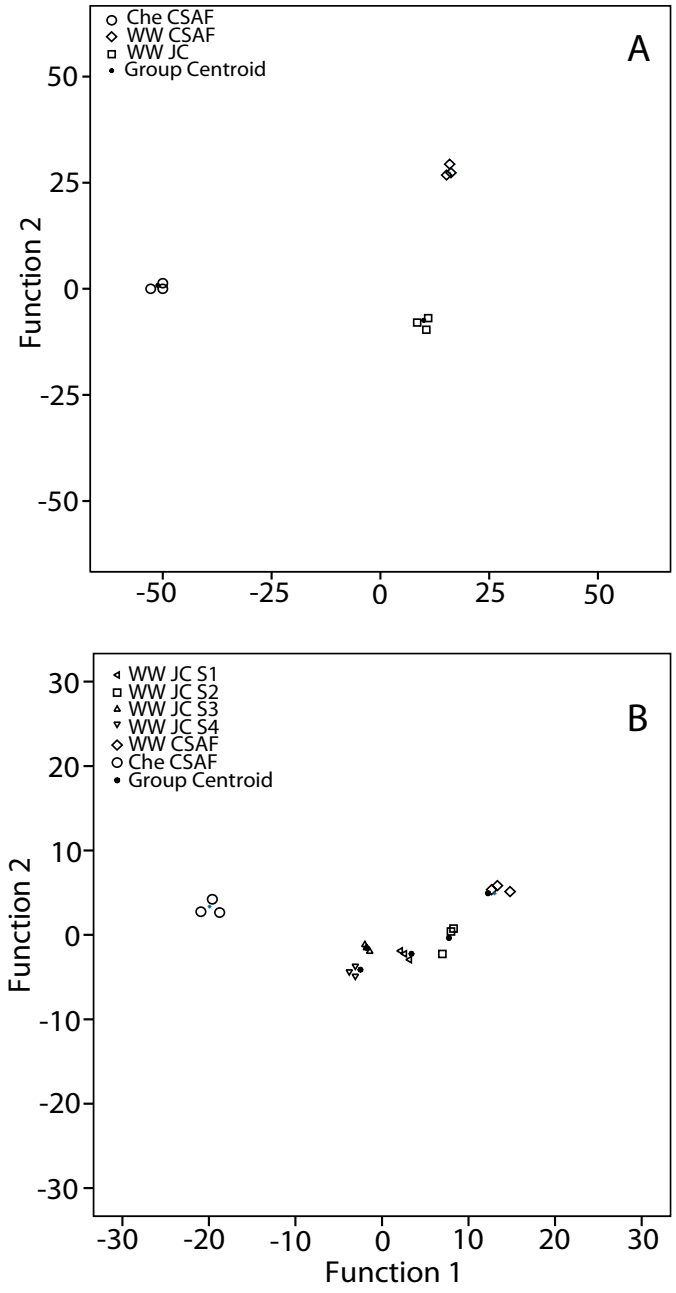
Mean (± 1 SE) of Me:Ca within pooled D-Stage hard clam larvae collected from J.C.Walker Bros in Willis Warf (WW JC) , Cherrystone Aquafarms in Willis Warf (WW CSAF), and Cherrystone Aquafarms in Cheriton (Che CSAF). (A) Sr:Ca, (B) Ba:Ca, (C) Pb:Ca, (D) U:Ca, (E) Cu:Ca, (F) Zn:Ca, (G) Se:Ca, (H) Rb:Ca, (I) Cr:Ca, (J) Mn:Ca, (K) Co:Ca, (L) Ni:Ca, (M) Mg:Ca, (N) Al:Ca, (O) Ti:Ca, and (P) V:Ca

Figure 10



Mean (± 1 SE) of Me:Ca within pooled D-Stage hard clam larvae collected from J.C.Walker Bros in Willis Wharf from 4 triweekly spawning events spanning Feb 12th-April 10 th. (A) Sr:Ca, (B) Ba:Ca, (C) Pb:Ca, (D) U:Ca, (E) Cu:Ca, (F) Zn:Ca, (G) Se:Ca, (H) Rb:Ca, (I) Cr:Ca, (J) Mn:Ca, (K) Co:Ca, (L) Ni:Ca, (M) Mg:Ca, (N) Al:Ca, (O) Ti:Ca, and (P) V:Ca

Figure 11



A) Scatterplot of discriminant function analysis (DFA) scores of element (Mg, V, Se, Rb, Ba, Pb, Al, Mn, and Cr) to Ca ratios in hard clam larvae collected from J.C.Walker Bros in Willis Wharf (WW JC), Cherrystone Aquafarms in Willis Warf (WW CSAF), and Cherrystone Aquafarms in Cheriton (Che CSAF). Grouped as WW JC, WW CSAF and WW CSAF. (B)

Scatterplot of discriminant function analysis (DFA) scores of element (Mg, V, Se, Rb, Ba, Pb, Al, Mn, and Cr) to Ca ratios in hard clam larvae collected from J.C.Walker Bros in Willis Wharf (WW JC with S1-S4 denoting each of 4 triweekly spawning events), Cherrystone Aquafarms in Willis Wharf (WW CSAF), and Cherrystone Aquafarms in Cheriton (Che CSAF). Grouped as WW JC S1-4, WW CSAF and WW CSAF

Discussion

Spatial distinction of elemental fingerprints

Our results demonstrate for the first time the existence of spatially distinct elemental signals within hard clam larval shell (Fig. 9). Concentrations of Mg:Ca, Sr:Ca, Zn:Ca, Pb:Ca, U:Ca, Ba:Ca, and Mn:Ca within pooled larval shells were consistent with values reported for pooled protoconchs from encapsulated gastropod veligers (Zacherl 2005). Substantial spatial variability of Me:Ca within larval shell among the three hatcheries allowed a DFA using Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, Pb Ca, Al:Ca, Mn:Ca, and Cr:Ca to classify larvae to their natal hatchery with 100% accuracy (Fig. 11a Table 9). A subsequent DFA using only most effective discriminators from our original model (Cr:Ca > Co:Ca > Ba:Ca) also correctly assigned pooled larvae to their hatchery of origin with 100% success. The ability to discriminate among natal location with a reduced suite of elements will serve to reduce operating costs as well as increase the speed of future analyses (Dunphy et al. 2011).

The results of this investigation are supported by a growing body of literature suggesting that elemental fingerprints within invertebrate larval biominerals can be used to assign individuals to their site of collection at regional spatial scales (~30-50 km) (DiBacco and Levin 2000, Zacherl 2005, Becker et al.

2005). Additionally, work by Cathey et al. (2012) demonstrates that newly recruited hard clam shell contains distinct chemical signals that can be used to assign individuals to their site of collection with very high levels of accuracy at even smaller spatial scales (~12 km). Importantly, our current results provide the first evidence that elemental fingerprints within invertebrate larval biominerals can be used to identify natal origin at local scales (~1 km). The strength of signal observed at such a small spatial scale could be the result of analyzing pooled larvae in solution, as this method serves to increase the concentration of elemental species present compared to microbeam assays such as laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) (Campana 1999). The refinement of microbeam techniques such as LA-ICP-MS to analyze the chemical composition of individual hard clam larval shell will be requisite for the application of elemental fingerprinting as a methodology to model the larval dispersal of hard clams (see Chapter IV). Finally, if the small-scale chemical signals observed in pooled samples are identified within individual larva, this will have significant logistical implications for the chemical characterization of all possible natal origins (Campana et al. 2000).

The incorporation of trace and minor elements into invertebrate biominerals is influenced by a complex interplay between geochemical and biological processes (Schöne 2008). The incorporation of some elements appears to be predictably influenced by the physical and chemical composition of the water in which they form (Gilliken et al. 2006, Zacherl et al. 2009). In the current investigation small differences were present in the physical and chemical

characteristics of the water in which hard clam larvae were cultured. Specifically, conditions at both Cherrystone Aquafarms hatcheries were 23.3ºC and 30 with larvae from J.C. Walker Brothers cultured at 25ºC and 28. Empirical evidence supports an inverse temperature effect regarding the incorporation of Ba, Pb, and Sr into gastropod and cephalopod statoliths (Zacherl et al. 2003, Lloyed et al. 2008, Zumholz et al. 2007). Significant differences in shell Mg:Ca, Se:Ca, Zn:Ca, U:Ca, V:Ca, Ni:Ca, Mn:Ca, Pb:Ca, Sr:Ca, Ba:Ca and Cu:Ca among all three hatcheries despite identical culturing conditions at the two Cherrystone hatcheries suggests the small temperature differences in culture water were likely negligible (Fig. 9). Additional experiments demonstrate that salinity does not appreciably influence the incorporation of Mn, Mg, Ba, and Sr into fish otoliths and cephalopod statoliths (Martin and Thorrold 2005, Zumholz et al. 2007). A reduced contribution of salinity is supported by significant differences in Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, Pb Ca, Al:Ca, Mn:Ca, and Cr:Ca among collection date from clams spawned under identical salinity regimes by J.C. Walker Bros..

Estuaries are dynamic systems that experience differential concentrations of trace elements due to varying geomorphology, atmospheric deposition, pollution, and inputs from local watersheds (Swearer et al. 2003, Thorrold et al. 2007). Laboratory experiments have demonstrated a positive linear relationship between the concentration of Mg:Ca, Pb:Ca, Ce:Ca, and Ba:Ca ratios within molluscan biominerals and the concentration of these elements within culture water (Lorens & Bender 1980, Lloyd et al. 2008). Unfortunately, due to logistical difficulties chemical analysis of culture water was not conducted in the current

investigation, thus preventing a direct comparison with elemental concentrations of hard clam larval shell. Importantly, elemental fingerprints within biominerals may be used as natural tags without a full comprehension of all factors influencing elemental incorporation (Gillanders and Kingsford 1996, Campana 1999). Future work will be critical to elucidate factors influencing the availability and incorporation of trace and minor elements into hard clam larval shell.

Temporal stability of elemental fingerprints

The present study is the first to investigate the temporal stability of elemental signals within pelagically formed bivalve larval shells (PDI). The protracted spawning period of our hard clam model coupled with the rapid formation of this larval biomineral (~24-48 h) within highly dynamic estuarine systems underscores the importance of validating any small-scale temporal variability of chemical signals (Carriker 2001, Peterson and Fegley 1986, Swearer et al. 2003). Evidence to date suggests that elemental signals within recruited bivalve shells can be relatively stable on weekly, monthly, and seasonal time scales (Becker et al. 2005, Dunphy et al. 2011, Cathey et al. 2012). In the current investigation, Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, Pb Ca, Al:Ca, Mn:Ca, and Cr:Ca differed among spawning dates (Fig. 10). DFA using Mg:Ca, V:Ca, Se:Ca, Rb:Ca, Ba:Ca, Pb Ca, Al:Ca, Mn:Ca, and Cr:Ca as independent variables correctly assigned pooled individuals to their date of collection with 100% success (Fig. 11B Table 10). Despite these results suggesting a high level of temporal variability in the chemical composition of hard clam larval shells, we were able to discriminate between J.C. Walker Bros. and both Cherrystone

Aquafarms facilities regardless of collection date (Fig. 11B Table 10). Furthermore, DFA scores plotted for independent spawning dates cluster more closely to one another than either of the Cherrystone hatcheries (Fig. 11A). Furthermore, work by Fodrie et al. (2011) demonstrated the ability to consistently spatially resolve elemental signals within recruited bivalve shell despite weekly variability in Mn:Ca, Cd:Ca, Ba:Ca, Pb:Ca, U:Ca, Cu:Ca and Sr:Ca. If the overall spatial resolution among hatcheries is maintained through time, any inter-location temporal variability could potentially be used to more precisely identify the date of birth for an individual recruit.

CHAPTER IV: LASER ABLATION INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY (LA-ICP-MS) AS A METHOD TO ANALYZE ONLY THE RETAINED LARVAL SHELL OF MERCENARIA MERCENARIA

The current chapter seeks to test the hypothesis that the microprobe assay LA-ICP-MS cannot accurately quantify the chemical composition of the retained PDI of the hard clam. Work to date suggests that distinct elemental signals exist within hard clam juvenile and larval shell at spatiotemporal scales relevant to fisheries managers (Cathey et al. 2012). Importantly, empirical validation of microprobe assays to target only the retained larval biomineral (PDI) is critical for the application of elemental fingerprinting (Strasser et al. 2007, Zacherl et al. 2009). Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) has emerged as the preferred methodology to quantify trace and minor elements within growth zoned biominerals (Campana 1999), but may be limited in its application to accurately analyze the larval shell (PDI) of recruited bivalves due to the three dimensional nature of shell growth. Specifically, shell material thickens through development such that juvenile shell underlies the retained larval shell (Fritz 2001). Given the ablation of material during LA-ICP-MS analysis, the potential exists to transcend the larval shell and introduce elemental contamination from underlying juvenile carbonate. If LA-ICP-MS can be refined to analyze only the retained larval shell (PDI) of the hard clam without introducing contamination from underlying juvenile carbonate, the application of elemental fingerprinting has the potential to become a tractable methodology to model hard clam metapopulation dynamics.

Methods

Experimental Design

During June, 2012, hard clam broodstock were obtained from and spawned within the Carteret Community College Aquaculture Facility, Morehead City, North Carolina. Gravid adults were induced to spawn by thermal stimulation. Larvae were cultured in filtered estuarine waters (~30 salinity) until the PDI had set (48 h post fertilization), at which time larval shell ranged from 78-95 µm in length. Larvae (~90,000) were then transferred into each of twelve NaCLO leached 19 L high-density polyethylene (HDPE) containers and randomly assigned to one of three treatments. The experimental design was identical to that used by Strasser et al. (2007) and consisted of three treatments each containing four replicates that varied in their exposure to the stable isotope ^{138}Ba in the form of $BaCl_2$. The first treatment received no ^{138}Ba enrichment and will be indicative of naturally occurring ^{138}Ba:^{137}Ba ratios within shell material ("Unspiked"). The second treatment was enriched with a ^{138}Ba at a concentration of 25 µg g^{-1} once the PDI had set 48 h ("Early Spike"). By eight days, the majority of larvae had begun to metamorphose and settle, at this time my third treatment also received a ^{138}Ba enrichment of 25 µg g^{-1} ("Late Spike"). Culture water was exchanged every two days at which time clams were fed *Isochrysis* spp. The ^{138}Ba enrichments of 25 µg g^{-1} for the early and late spike treatments were sustained for the duration of the experiment and occurred following water changes. After 25 days clams were sacrificed in frozen culture water until prepared for elemental analysis.

Sample Preparation for LA-ICP-MS

The isolation of recruited shell (n = 26, 380-520 µm shell length) was conducted in a laminar flow hood. To minimize potential cleaning artifacts, samples were not treated with cleaning agents to remove organics (Krause-Nehring et al. 2011). Clams

from each replicate were placed on 60 µm mesh and rinsed vigorously with (18.2 Ω) ultrapure water. Flesh was manually removed with acid washed (7% HNO_3 (v/v) Optima Grade; Fisherbrand) plastic probes. Juvenile shell was then transferred into 1.5 ml acid-washed polypropylene vials (7% HNO_3 (v/v) Optima Grade; Fisherbrand), suspended in 1 ml (18.2 Ω) ultrapure water, vortexed for 30 s, and sonicated for 5 min. Organic tissue and (18.2 Ω) ultrapure water were removed and clams were re-suspended in 1 ml (18.2 Ω) ultrapure water and treated an additional 9 times. Clam shells were then dried under laminar flow and inspected for residual somatic tissue, which when encountered was removed using plastic probes. Samples were mounted on glass slides using Scotch® double sided tape in an identical fashion to that used by Strasser et al. (2007). One valve from each clam was placed concave side up to allow access to the umbo and PDI, while the second valve was placed concave side down to allow access to the PDII and flattest section of the juvenile shell.

Elemental Analysis of Clam Shells using ICP-MS

Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) analyses of juvenile bivalves were performed using a New Wave UP193fx (193 nm, 4-6 ns pulse width) excimer laser coupled to an Agilent 7500ce ICP-MS at the University of Texas at Austin. The laser system has a large format two-volume ablation cell enabling

samples and standards to be simultaneously loaded (Fig. 12 A & B), with very fast (<1 s) washout times irrespective of location within the cell.

Figure 12

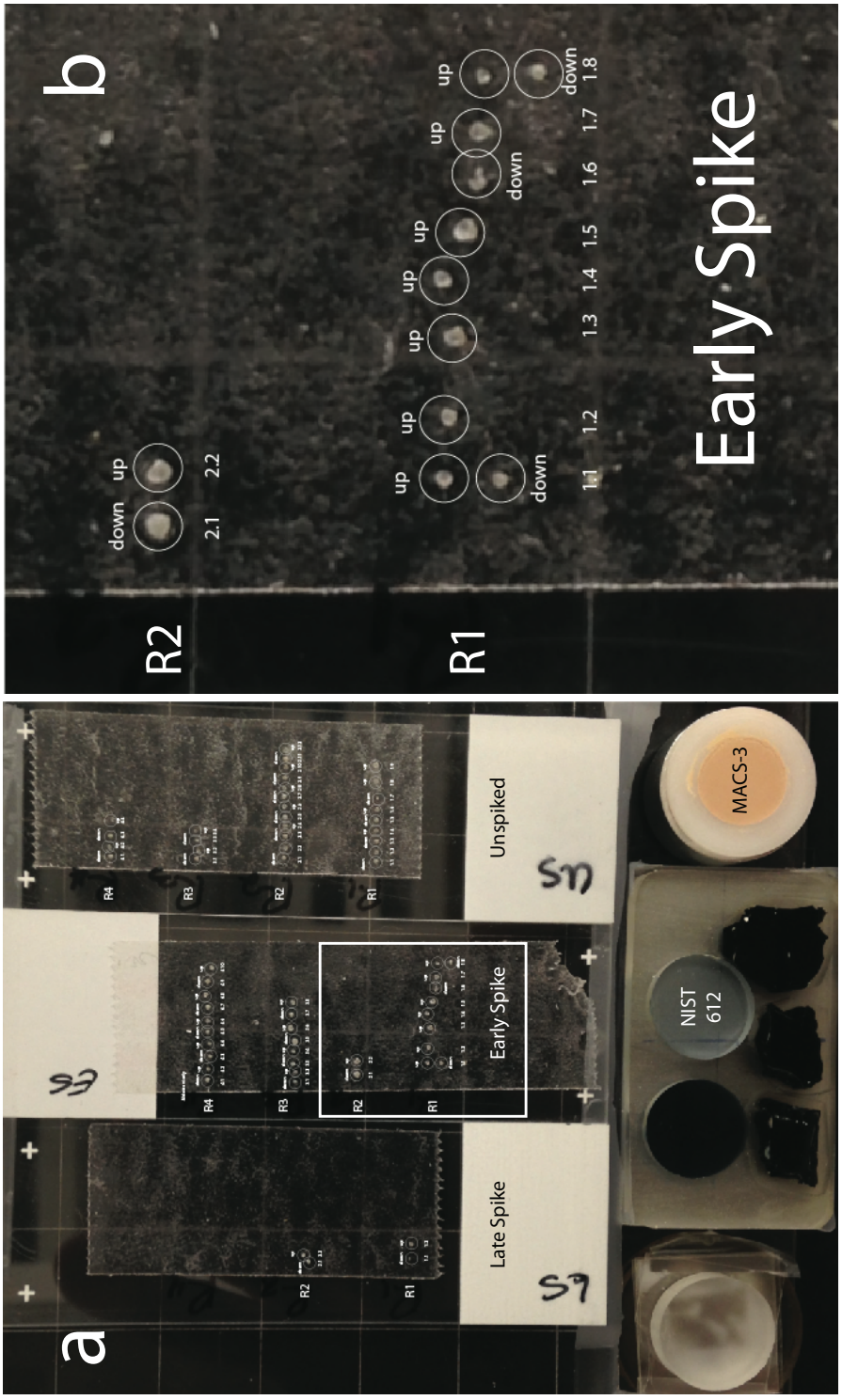


Photo showing (a) the loaded laser ablation drawer with labeled (spike) and unlabeled juvenile bivalves; (b) shows enlargement of the inset box shown in (a)

Laser ablation parameters were optimized based upon tests of unspiked valves. A large (100 μm) spot was selected to optimize sensitivity within the largest permissible extent (diameter) of prodissoconch II and dissoconch calcite, as expressed among representative individuals of the same age. Laser power, repetition rate, and He flow were modulated to obtain the most stable settings, providing for reasonably long dwell times prior to burn-through of the entire valve. The best settings were determined to be 30% laser power, 10 Hz repetition rate, and a He flow of 225 mL/min. A small flow of N_2 (5 mL/min) was added to the argon carrier stream (1.1 L/min) upstream of the ablation cell, which acts to increase the plasma temperature and enhance ionization. The 30% laser power setting corresponds to an average fluence of 3.75 J/cm^2. Pulse-to-pulse laser power variation was maintained within 3.84% of this value during all analyses. The curved shape of valves is likely associated with some variation in the amount of initially ablated material, prior to obtaining a flat-bottomed ablation crater.

The quadrupole method involves measurement of ^{43}Ca, ^{48}Ca, ^{137}Ba, and ^{138}Ba, using integration times of 10 ms for Ca, 40 ms for ^{137}Ba, and 30 ms for ^{138}Ba. The sampling period for all analytes (0.0992 s), corresponds to 90% measurement time and 10% settling and fly-back time; enabling hundreds of measurements to be made within the dwell interval of the laser (45 s). Robust measurement conditions can be obtained with such conditions (Longerich et al., 1996, p. 900). The optimized LA-ICP-MS settings are summarized in Table 8. The standards MACS-3 (synthetic aragonite) and NIST-612 were analyzed along with unknowns, although for this study, elemental quantification was not required. Ba/Ca intensity ratios, following background subtraction, were derived using Iolite software (Hellstrom et al., 2008).

Table 8

Laser Parameters (Spots)	
Gas blank	30 s
Dwell time	45 s
Laser power	30 %
Repetition rate	10 Hz
He cell flow	225 mL/min
N2 carrier gas flow	5 mL/min
Ar carrier gas flow	1.1 L/min
Spot diameter	100 µm
ThO/Th (oxide production)	< 0.2 %
ICP-MS Parameters	
RF power	1600 W
Cones	Ni
Sampling depth	8 mm
Extract 1	2.1 V
Extract 2	-140 V
Quadrupole	
43Ca int time	0.010 s
48Ca int time	0.010 s
137Ba int time	0.040 s
138Ba int time	0.030 s
Sampling time	0.0992 s

Summary of optimized LA-ICP-MS analytical conditions

Data Analysis

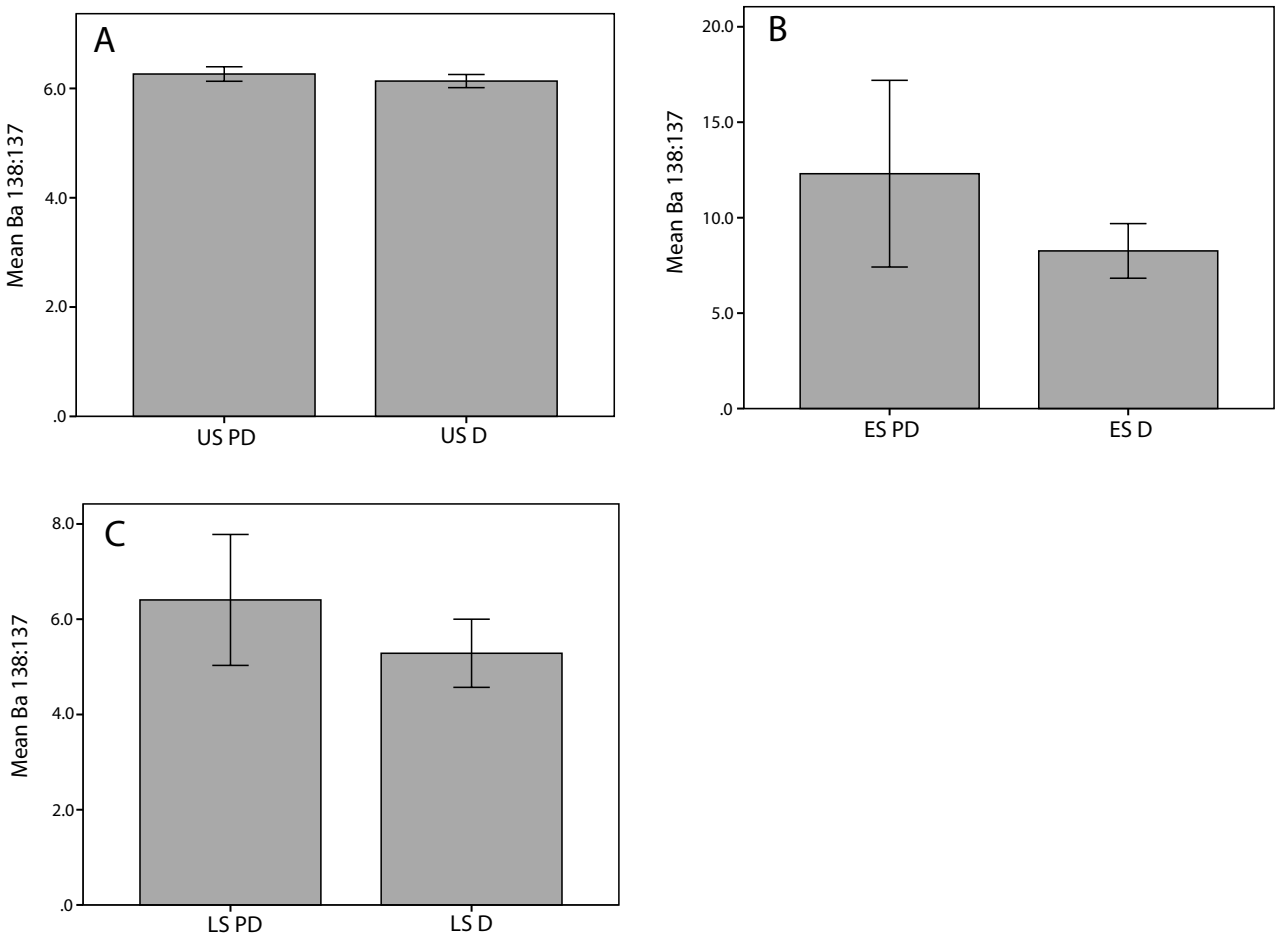
Initially, larval (PDII) and recruited juvenile shells from our unspiked treatment were analyzed to assess any naturally occurring ontogenetic influence of barium incorporation (as per Strasser et al. 2007, Zacherl et al. 2009). Larval shell was then

analyzed at the PDII and recruited juvenile shell of our early spike and late spike treatments to verify that the barium enrichment of culture water was being incorporated into underlying shell before LA-ICP-MS was used to analyze the PDI. When assumptions of normality and homoscedascity were met, *t*-tests were used to investigate potential differences in the mean concentration of $^{138}Ba:^{137}Ba$ within spiked and unspiked treatments for both larval and recruited juvenile shells. When these assumptions were not met, Mann-Whitney U-tests were employed. Finally, a Kruskall-Wallis test was used to investigate differences in the mean concentration of $^{138}Ba:^{137}Ba$ among the PDII and juvenile shells of all treatments.

Results

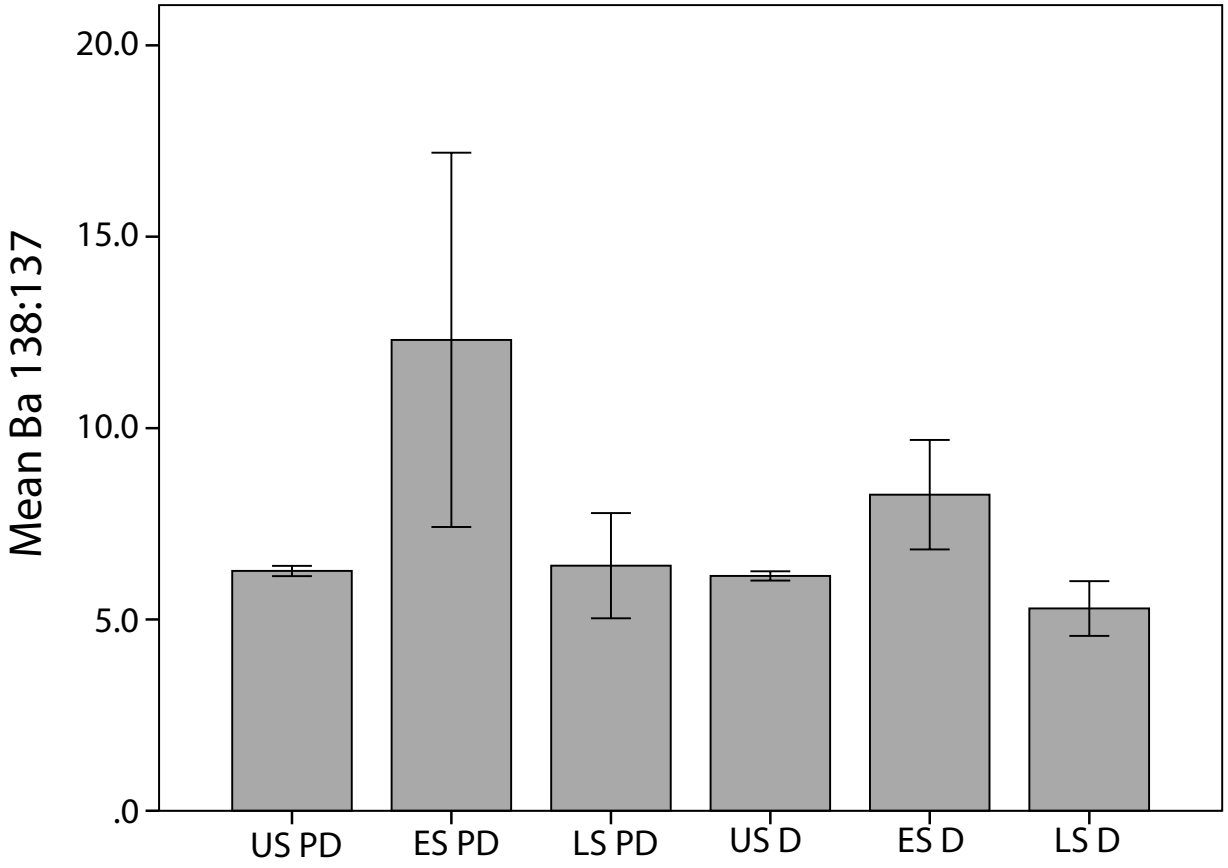
No significant differences in mean $^{138}Ba:^{137}Ba$ concentrations were observed between larval and juvenile shell of both unspiked treatment ($t_{25} = 0.724$, $p = 0.476$) and early spiked treatment ($t_{23} = 0.878$, $p = 0.389$) (Fig. 13A & B). Similarly, Mann-Whitney U tests revealed no significant difference in mean $^{138}Ba:^{137}Ba$ concentrations between larval and juvenile shell of our late spike treatment ($Z = -0.775$, $p = 0.439$) (Fig. 13C). Additionally, a Kruskal-Wallis test revealed no significant difference in the mean values of $^{138}Ba:^{137}Ba$ within larval and juvenile shells among all treatments ($p = 0.524$) (Fig. 14). Data obtained from the unspiked treatment ablated more consistently than either of the spiked treatments as evidenced by reduced variance (Fig. 14). The large range of $^{138}Ba:^{137}Ba$ values from the PDII (4.74 - 60.23) and juvenile shell (3.33 - 23.04) of our early spiked treatment suggests differential and inconsistent barium incorporation. Finally, scanning electron microscopy of ablated shell samples at the PDII revealed complete burn through in 38.5% of samples (Fig. 15 A & B).

Figure 13



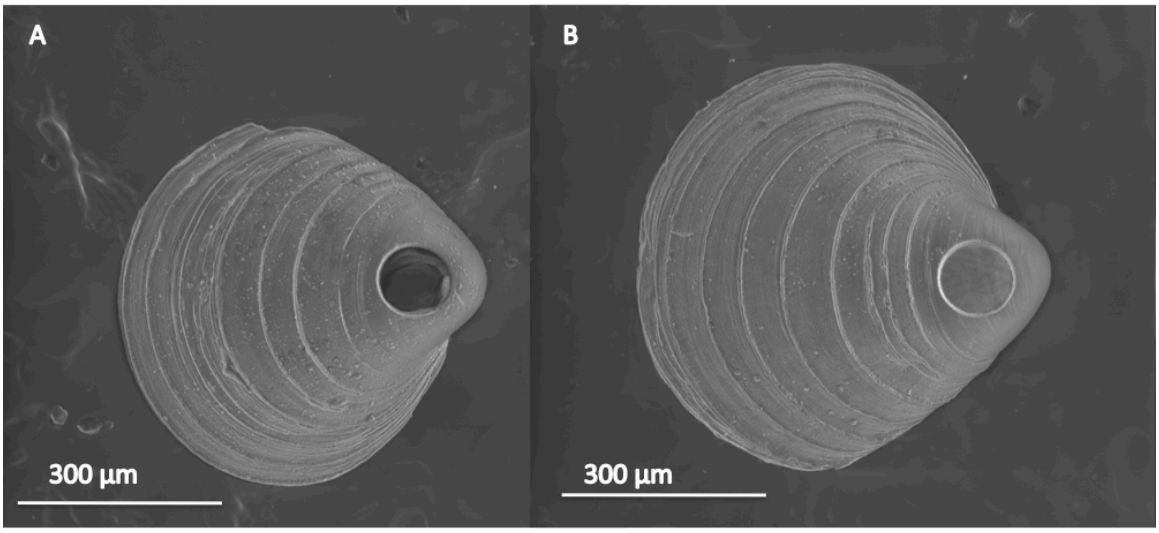
Mean (± 1 SE) of ^{138}Ba:^{137}Ba within larval shell PD and Juenile shell D (A) unspiked treatment (B) early spike treatment and (C) late spike treatment

Figure 14



Mean (± 1 SE) of ^{138}Ba:^{137}Ba within larval shell PD and Juenile shell D from all treatments (unspiked US PD, US D; early spike ES PD ES D; and late spike LS PD, LS D)

Figure 15



Scanning electron micrograph of juvenile hard clam shell showing laser ablation at the PDII. (A) Complete laser burn through (B) No laser burn through. Scale bar is 300µm

Discussion

Our results support the conclusions of Strasser et al. (2007) by demonstrating extensive laser burn through of larval (PDII) and underlying juvenile shell. The LA-ICP-MS analysis of samples at the PDII transcended both the larval and underlying juvenile shell in 38.5% of samples at a shell region that is thicker than that directly underlying the PDI. This suggests that additional analyses of the PDI using the same LA-ICP-MS instrument settings would likely produce similar results. Importantly, analysis of spiked PDII, as well as spiked juvenile shell material, is requisite to ensure that elevated Ba incorporation occurred relative to unspiked shell material (i.e, PDI). Without significant and consistent elevation of Ba within our spiked treatments it is impossible to evaluate the extent of laser burn through when sampling the PDI. The differential assimilation of barium within PDII and juvenile shell in our early spike treatment demonstrates that our

Ba enrichment was not consistently incorporated. The chaotic ablation (high frequency oscillation between high and low concentrations) of the labeled samples suggests that there might be high concentration domains within hard clam biogenic carbonate for Ba, that are not present in the unlabeled samples (Fig. 13 A & B &C). This is surprising given the repeated demonstration of a positive linear relationship between the concentration of Ba within culture water and its incorporation within the biominerals of teleost fish and multiple invertebetrate models (including gastropod and bivalve larvae) (Zacherl et al. 2003*b*, Gillikin et al. 2006, Bath et al. 2000, Zacherl et al. 2009). While the Ba enrichment in this experiment was identical to that used by Strasser et al. (2007) (25 μg g^{-1}), and was three orders of magnitude greater than concentrations found in the world's oceans and estuaries (4.8-30 ng g^{-1}) and was also larger than documented manipulations used to investigate its incorporation into larval molluscan biominerals (Elsdon and Gillanders 2006, Lloyd et al. 2008, Zacherl et al. 2009, Zacherl et al. 200*b*). The incorporation of Ba into molluscan biominerals occurs primarily within the mineral phase of the biogenic carbonate matrix. Ba is passively diffused through Ca channels where it is precipitated as $BaCO_3$ via substitution reactions with Ca (Carré et al. 2006). Importantly, hard clam larval shell (PDI and PDII) is comprised of amorphous calcium carbonate which transitions into aragonite as the shell thickens through development (Weiss et al. 2002). An analysis of insoluble residues from digested D-stage larval hard clam shells indicates that organic shell matrix comprises a substantial proportion of shell concentrate within larval hard clam carbonate, 26.0-43.8 wt% (see Chapter III). Thus, the differential and inconsistent Ba incorporation observed within our spiked treatments could be the result a high proportion of the biomineral being comprised of organic shell

matrix, which does not incorporate Ba as readily as the mineral fraction (Morrison and Brand 1986, Takesue et al. 2008). The inability to accurately detect our Ba enrichment within our spiked treatments in PDII and juvenile shell was not expected and prevents any attempt to quantify potential contamination from these underlying shell layers during laser ablation of the PDI.

The visual confirmation of complete laser burn through of PDII and underlying juvenile shell in 38.5% of samples unequivocally demonstrates the inability of LA-ICP-MS to sample only overlying larval shell (PDII) using the current instrument settings (Fig. 15). Very few studies have applied LA-ICP-MS to analyze the recruited shell of bivalves. Work by Fodrie et al. (2011) and Becker et al. (2007) investigated elemental signals within the PDI of recruited mussel spp. using LA-ICP-MS analysis and reported minimal burn through. Importantly, these authors had validated elevated Mg within recruited shell of their model that allowed for some estimate of potential laser burn through, but is not as accurate as empirical investigations of LA-ICP-MS efficacy (Becker et al. 2005, Strasser et al. 2007). Our results were similar to Strasser et al. (2007) who found substantial laser burn through of *Mya arenia* shells. Future refinement of LA-ICP-MS to analyze only the retained larval shell of the hard clam will be requisite before elemental fingerprinting can be reliably used to model larval dispersal in this species.

Chapter V: General Discussion

The results of this investigation have broadened existing knowledge on biomineralization within the early life stages of the hard clam by providing the first documentation of trace and minor elemental compositions from juvenile and larval shells. These results demonstrate the existence of spatially distinct elemental signals within juvenile and larval hard clam shell. Within juvenile shell, elemental fingerprints are seasonally reproducible and can be used to assign individuals to sites of collection at small spatial scales (~12 km), within a well-mixed estuarine-lagoonal system with high levels of success (100%), during elevated periods of larval dispersal (Fig. 3A & 3B and Tables 2 & 3). Similarly, I demonstrate for the first time the existence of spatially distinct elemental signals within the early larval shell of the hard clam (~1-50 km) (Fig. 11A Table 9). Despite temporal variability in elemental signals from larval shell samples, I was able to discriminate between J.C. Walker Bros. and both Cherrystone Aquafarms facilities regardless of collection date (Fig. 11b Table 10). These results suggest that elemental signals encountered within hard clam larval shell may be used as a tag of natal origin. A growing body of literature provides evidence that elemental fingerprints within the early juvenile and larval shell of bivalves are relatively stable on weekly, monthly, and seasonal temporal scales, and can be used to assign individuals to the habitat in which they formed at spatial scales on the order of (~40-80 km) (Becker et al. 2005, Becker et al. 2007, Dunphy et al. 2011, Fodrie et al. 2011). Importantly, these results further contribute to the

validity of using bivalves as model organisms for elemental fingerprinting studies by demonstrating the existence of small spatial (~1 km) and temporal (tri-weekly) differences in the elemental chemistry of hard clam larval shells. Finally, the results support the conclusions of Strasser et al. (2007) by demonstrating extensive laser burn through of the PDII, as well as underlying juvenile shell in 38.5% of the samples analyzed (Fig. 15). As such, the application of LA-ICP-MS using the current instrument settings is not a tractable methodology to accurately analyze the retained larval shell of the hard clam.

Limitations

While these results suggest that strong elemental signals exist within hard clam juvenile and larval shell, the mechanistic processes that influence the availability and incorporation of trace and minor elements from the surrounding environment into biogenic carbonate are less clear. For example, the influence of seawater regarding the elemental compositions within juvenile hard clam shell from Cape Lookout, NC (Chapter II) were disappointing and particularly troubling for Mn, which provided the bulk of our elemental signal and was below the limits of detection in estuarine waters on all sampling dates. Naturally occurring concentrations of Mn in seawater are very low (~5-26 ng/L) (Cabon et al. 1995). Furthermore, the limit of detection for the ICP-OES used in this study is on the order of 0.5 µg/L versus 0.0005 µg/L by ICP-MS. At UT-Austin, the ICP-MS detection limit for Mn is typically on the order of 10-70 ppt, however seawater needs to be diluted 100x (~5 salinity) prior to ICP-MS analysis and thus would likely be below detection. There are high matrix interfaces

techniques such as the application of magnesium hydroxide coprecipitation, which removes major cations such as K, Na, Ca, Mg that could potentially work, but is not currently available (Ardini et al. 2011). Finally, given that juvenile clams were collected from benthic habitats, it is highly probable that sediments were directly influencing the availability and incorporation of trace and minor elements into juvenile shell. Unfortunately, this investigation was operating on a very limited budget and focused on water chemistry (with a view toward subsequent larval work), thus did not analyze sediment Mn values. Attempting to reduce the contribution from sediments, samples were collected from sandy habitats; however, sediments cannot be ruled out as a factor influencing rates of elemental incorporation.

A lack of water chemistry data from the inplanting study (Chapter III) also obscures potential mechanisms driving the availability and incorporation of trace and minor elements from the environment into the larval shell of the hard clam. This study was completely reliant upon the cooperation of Tim and Kari Rapine of Cherrystone Aquafarms and Ann Gallivan of JC Walker Brothers. Given their generous donations of both time and D-stage larval hard clams for elemental analysis, the additional burden of collecting water samples was not feasible. Another limitation to this investigation was the analysis of pooled larvae in solution. The great strength of elemental fingerprinting is its potential to track the realized dispersal of an individual organism (i.e, fish, bivalve) (Campana et al. 1999, Thorrold et al. 2007, Levin 2006). While pooling individuals for analysis has been reported in the literature, it can only describe broad differences in the

elemental composition of biominerals among locations (Zacherl 2005). The decision to analyze pooled larvae in solution was necessitated by the inability to accurately analyze individual D-stage hard clam larvae using LA-ICP-MS. Using a single spot ablation, D-stage shell material was too thin and fragile to accommodate an ablation of sufficient duration to generate reliable statistics for quantification. Numerous experiments and instrument settings were applied including: spot sizes between 25 and 50 μm, laser energies between 1-10%, and repetition rates between 3-10 Hz. The best results were obtained by turning the laser power output down to the lowest possible setting 1% power (1.64 J/cm^2), which amounts to a fraction of a millijoule over a 35μm spot, and ablate at 5 Hz. A 35 μm spot seemed optimal, but only because fewer shells seemed to be annihilated. A 20 s dwell time was attempted, but only a single shell came close to providing a signal. The shells did not tolerate pre-ablation using a larger spot, thus both the middles and outer edges of the PDI were analyzed to see if a stable signal could be achieved, without success. Virtually no Co or Pb were recovered and other analytes (Mg, Al, Ca, Mn, Cu, Zn, Sr, Ba) only showed signals at the beginning of ablations, thus it was not possible to determine if elemental compositions were from the shell and/or surficial to the shell.

Broader impacts

Attempts to understand the fundamental ecological mechanisms structuring benthic marine invertebrate populations have highlighted recruitment events as critical periods in the life history of multiple bivalve species (Beukema and Dekker 2005). The continued overharvest of commercial bivalves has the

potential to facilitate recruitment limitation in such species as the Bay Scallop *Aequipecten irradians*, and the Hard Clam *Mercenaria mercenaria* (Peterson et al. 1996, Kraeuter et al. 2005). In North Carolina waters, populations of the hard clam *M. mercenaria* have been decimated due to extensive exploitation and are also believed to be recruitment limited (Peterson 2002). The importance of the pelagic life history stage in structuring the dispersion and sustainability of bivalve populations highlights the need to develop methods to more fully understand patterns of larval dispersal and population connectivity (Thorson 1950, Garland et al. 2002). This is particularly relevant given the importance of spatial dynamics in fisheries management and the establishment of marine protected areas. Despite the economic importance of the hard clam, amazingly little data are available concerning the influence of larval dispersal on this species population dynamics. Investigations to date have been limited to observational studies of larval periodicity as well as estimates of survivorship (as reviewed by Fegley 2001). Importantly, differences in genetic composition among hard clam populations are minimal and allozyme data suggests that the hard clam is homogeneous throughout its range (as reviewed by Hilbish 2001). Thus, population genetics are not applicable to investigate the exchange of individuals among hard clam populations and other methodologies must be explored. The current investigation supports elemental fingerprinting as a method to identify natal origin and potentially investigate the influence of dispersal on the population dynamics of the hard clam at spatial and temporal scales relevant to fisheries managers (~1-50 km). However, substantial method development of LA-ICP-MS

assays to exclusively target the PDI of larval, as well as recruited hard clams, will be requisite before this method is fully tractable to investigate larval dispersal. If this methodological hurdle can be overcome, elemental fingerprinting could facilitate the identification of subpopulations that may disproportionately supply larvae. These source populations would benefit the most from management, conservation, and restoration efforts.

Future directions

Future work should focus on the interplay between exogenous and endogenous factors that influence the availability and incorporation of trace and minor elements into hard clam larval shell. Of particular interest is the documented maternal transfer of elemental species from broodstock to progeny for teleost fish and gastropods (Lloyed et al. 2008, Thorrold et al.). Given that larval clams are dependent on yolk reserves and do not feed through the deposition of the PDI, it is possible that maternal effects could be influencing the availability and incorporation of trace and minor elements during biomineralization (Carriker 2001). Complementary studies should thoroughly investigate the role of water chemistry on the incorporation of elemental species during the secretion of molluscan larval biominerals, as ambient chemical concentrations have been documented to influence a suite of elements including: Ba, Pb, Ce, and Mg (Lorens and Bender 1980, Llyod et al. 2008, Zacherl et al. 2009). Additionally, work by Zacherl et al. (2009) demonstrates that the elemental composition of Olympic oyster *Ostrea lurida* larval shell is altered as the biomineral thickens through ontogeny. The documented ontogenetic

transition of hard clam larval shell from amorphous calcium carbonate to aragonite as the shell thickens further underscores the importance of validating any developmental differences between the elemental composition of the PDI in the pelagic verses the recruited form. Potential signals recorded in the water column may be modified during subsequent stages of shell deposition (thickening) in the recruited form (Weiss et al. 2002).

Reductions in commercial landings of hard clams have occurred in many areas and are thought to be driven by recruitment limitation stemming from reductions in adult standing stock biomass (Peterson 2002). The implementation of conservation, management, and restoration strategies will be critical to facilitate sustainable management of hard clam stocks. The stock status of the hard clam is currently unknown in many states and little data is available concerning the relationship between biology, ecology, and the population dynamics of this species. The current study represents the first attempt to examine shell trace and minor chemistry as a method to identify patterns of hard clam larval dispersal. Our results are very promising in that they demonstrate the existence of geospatially distinct chemical signals resident within hard clam juvenile and larval shell that are indicative of site of collection and natal origin, respectively. If LA-ICP-MS can be refined to analyze only the retained larval shell of a recruited hard clam, patterns of hard clam larval dispersal and population connectivity can be elucidated, thus the potential exists to identify subpopulations that may differentially contribute to overall population dynamics by disproportionately supplying larvae. Validation of this methodology will provide

an extremely powerful management tool that can be used to identify populations that should be conserved (source) vs. those that should be open to commercial harvest (sink) throughout this species entire range.

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