

Environmental effects on elemental signatures in eastern oyster *Crassostrea virginica* shells: using geochemical tagging to assess population connectivity

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ABSTRACT: We evaluated the utility of geochemical tagging methods to discern larval connectivity among an invertebrate metapopulation within a large (~5000 km²) temperate estuary. Specifically, we examined how estuarine-scale gradients in temperatures (21° to 26.5°C), salinities (12.5 to 20 ppt), and trace metal concentrations (ambient, +16 ppb Mn and 0.16 ppb Pb, or +32 ppb Mn and 0.32 ppb Pb) affect *Crassostrea virginica* larval-shell signatures of Mn, Sr, Ba, and Pb in controlled mesocosms. We also utilized field-collected, newly settled oysters across Pamlico Sound, NC, USA, to explore signature variability among natural temperature and salinity gradients and examine the spatial resolution at which geochemical signatures can be used to discriminate between collection regions. Mesocosm experiments revealed environmentally and statistically significant interactive effects between temperature and salinity on elemental ratios in larval oyster shells, favoring higher Sr concentrations in cooler, fresher water, but no effects of these factors on Ba signatures. Mesocosm trials also showed increased Mn signatures in larval shell following from spiking mesocosms with Mn solutions; however, this relationship did not hold for Pb following analogous elemental spikes. Our field collections of recent settlers showed similar patterns of high Sr at relatively low salinities and temperatures, without clear environmental gradients for Ba. Overall, we found that across regional (35 km) spatial scales, environmental variables, such as salinity and temperature, can generate distinct multi-elemental signatures between putative natal sites. However, if sites are close together or located in similar environments, discrimination among sites appears greatly reduced. We suggest that geochemical tagging provides a promising approach for characterizing larval connectivity among subpopulations within whole-estuarine systems.

KEY WORDS: Bivalve larvae • Connectivity • *Crassostrea virginica* • Geochemical tagging • Larval dispersal • Laser ablation ICP-MS • Oysters • Salinity • Temperature

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INTRODUCTION

Researchers have long been interested in the complex larval dispersal patterns that govern early life history and distribution patterns of marine organisms (Young 1990). Current management strategies have

bolstered the impetus to discern population distribution patterns that are driven by larval dispersal, as successful marine reserve design is contingent upon the levels of larval input (e.g. immigration and self-recruitment; Jones et al. 1999, Puckett & Eggleston 2012) and export (e.g. spillover; Gerber et al. 2003,

Gaines et al. 2010). Recently, there has been substantial progress in deciphering ranges of dispersal and the degree of self-recruitment within marine populations and ecosystems (Cowen & Sponaugle 2009, Puckett et al. 2014), which has allowed researchers to question traditional concepts of connectivity, i.e. the degree to which marine populations are demographically open or closed. One of the key challenges in determining the role of larval connectivity in population dynamics and applying this knowledge to management is the ability to test predictions of larval connectivity, especially under variable environmental conditions. For example, larval dispersal is highly dependent on physical factors (e.g. current patterns and tidal forcing), which fluctuate annually, seasonally, and even daily, causing dispersal distances to be highly variable among years or even among neighboring habitats within the same temporal scales (O'Connor et al. 2007, Puckett et al. 2014, Qian et al. 2014).

Estuaries comprise an important domain for connectivity studies, as they are characterized by high environmental spatiotemporal variation (e.g. multiple freshwater input sources) and encompass varying geomorphological components (e.g. creeks, salt water inlets, and marshland). Estuaries also function as important nursery, juvenile, and even adult habitat for many marine organisms (Beck et al. 2001), resulting in the development of distinct subpopulations with varying amounts of larval exchange and connectivity. Finfish connectivity has been examined over many spatial scales, including intra- and inter-estuarine dynamics on both the east and west coasts of the USA (e.g. Able 2005, Fodrie & Levin 2008). However, invertebrate dispersal across estuarine scales has not been as intensively examined, with only a few studies exploring connectivity across estuarine environmental gradients (Becker et al. 2007, Cathey et al. 2012, Puckett et al. 2014 and references therein).

Bivalves, such as the eastern oyster *Crassostrea virginica*, provide an important model organism for the study of estuarine-scale larval connectivity because of their early life history characteristics and ecological role as a reef-building, foundation species. *C. virginica* also persists throughout a range of temperatures and salinities commonly found in estuarine systems (Davis 1958). Following successful fertilization, oyster larvae progress through an approximately 2 to 3 wk planktonic veliger phase (Medcof 1939) in which they begin to develop an aragonite-rich prodissoconch shell that is retained after an individual settles on suitable benthic habitat (most

typically, gregariously on other adult oyster shells; Stenzel 1964). Recently, biophysical models that simulated the dispersal of oyster larvae among a network of 10 reef units in Pamlico Sound, North Carolina (NC), USA, reported that dispersal distance varied around 5-40 km, which limited both inter-reserve connectivity and local retention (Puckett et al. 2014). However, there are no empirical data on oyster larval connectivity and demographic rates within this reserve system. The present study, which evaluates the efficacy of geochemical signatures in shells of larval stage oysters and recent settlers in the waters of Pamlico Sound and beyond, is a key step in testing model predictions of larval connectivity for this and similar bivalve metapopulations.

Because of *C. virginica*'s calcium carbonate shells, geochemical tagging methods can be used to empirically assess larval dispersal and connectivity (Carson 2010, Fodrie et al. 2011). Geochemical tags are based on unique physical and chemical environments experienced by organisms during their larval and post-larval life-history stages. As the organism grows, elements present in natal environments are accreted and stored in calcium carbonate structures (e.g. otoliths in fishes, shells in bivalves), usually through the substitution of anions²⁺ for Ca²⁺ or the entrapment of other contaminants (Bath et al. 2000). Environmental (e.g. temperature and salinity) variations have been shown to further affect the incorporation of trace elements into calcium carbonate structures (e.g. Becker et al. 2005, Martin & Thorrold 2005, Strasser et al. 2008b). These signatures can then be analyzed through the use of specialized mass spectrometry techniques to discriminate natal origin in both fishes and bivalves (Carson et al. 2013). Due to its exposure to various environmental conditions as larvae are retained within an estuary, the eastern oyster can also be used to further understand the mechanisms of geochemical tagging, via the relationship between salinity, temperature and elemental concentrations, as well as the application of elemental signatures to assess connectivity. Applications of this approach may be used to assess connectivity, thereby improving management and restoration efforts for the species.

The ultimate goal of this study was to develop geochemical tagging as an empirical tool to assess oyster larval connectivity. A requisite for achieving this goal was to first ground-truth tagging methods for oysters via spatially implicit laboratory experiments with larval oysters, coupled with spatially explicit field collections of recent settlers. In laboratory mesocosms, we conducted a fully crossed, 3-way, experiment to

investigate the effects of temperature, salinity, and seawater concentrations of Mn and Pb on larval (prodissoconch) shell signatures (i.e. elemental ratio, X:Ca). Next, we collected recently settled oysters (hereafter 'spat') from sites within the Bogue-Back-Core-Pamlico Sound estuarine system in North Carolina (NC), USA, and examined signatures present in larval shells and outermost portions of settler shells. These geochemical signatures were used to examine natural elemental variability in shells, with respect to salinity and temperature, and to explore discriminatory ability and resolution between sample sites or regions.

MATERIALS AND METHODS

Temperature, salinity, and trace metal manipulations

To investigate environmental effects on larval (prodissoconch) shell signatures, we manipulated temperature, salinity, and elemental concentration of the water surrounding developing oyster larvae. Individual tanks were set up with the following treatments: low (21°C) or high (26.5°C) temperature; low (12.5 ppt) or high (20 ppt) salinity; and ambient (no addition), mid spike (+16 ppb Mn and 0.16 ppb Pb addition), or elevated spike (+32 ppb Mn and 0.32 ppb Pb) in concentrations of aqueous Mn and Pb. These elements were chosen because of their previous use and importance in elemental tagging studies (e.g. Zacherl et al. 2003, Strasser et al. 2008b). Temperature and salinity treatments were selected based on representative high and low observations in Pamlico Sound at the time of the experiment (late-summer). Trace metal spikes were calculated to increase the ambient levels of Mn and Pb in seawater, as measured by Statham & Burton (1986) for Mn and Wu & Boyle (1997) for Pb, by 400 and 800 % for mid and elevated spike levels, respectively.

Three-day old *Crassostrea virginica* larvae were obtained from the University of Maryland's Horn Point Laboratory in Cambridge, Maryland, USA. These larvae were mass spawned from a total of 21 males and 28 females and reared in a hatchery system until shipment to the Institute of Marine Sciences (IMS) in Morehead City, NC. Upon arrival, larvae were divided equally into two 1.21 aerated holding tanks filled with a 12.5 ppt seawater mix (ultrapure H₂O added to filtered seawater from Bogue Sound, NC). Over the next 4 d, larvae were acclimatized, with one tank receiving a salinity increase of -2 ppt

d⁻¹, resulting in a final salinity of 20 ppt, while the other tank remained at 12.5 ppt.

After the acclimatization process was complete, larvae from both holding tanks, now 7 d old, were divided equally into 72 'larval homes', with -1.6×10^4 larvae per home (21.2 larvae cm⁻³). Larval homes were constructed from hollow PVC tubing capped on each end with nitex cloth, with a 30 µm mesh opening, to allow for the flow of water and food into the home, but to prevent larvae from escaping. Homes were then placed into 24 aerated aquarium tanks (35 l), with 3 homes per tank. All tubing, PVC, air stones, and nitex were soaked in a HNO₃ solution and then rinsed thoroughly with ultrapure H₂O prior to its use in the experiment.

Temperatures were maintained at either a high or low level by 150 W Aquatop aquarium heaters, and salinity levels were established by mixing filtered seawater with ultrapure H₂O until desired salinity was reached. Mn and Pb concentrations were spiked by the addition of 545 µl of Mn + 5.45 µl of Pb or 1090 µl of Mn + 10.90 µl of Pb from 1000 ppt Fisher Scientific reference standard solutions, for mid and elevated spike treatments, respectively. Individual treatments (temperature, salinity, and Mn/Pb spiking) were crossed, to produce a full factorial design with 12 total treatment combinations. Water changes were conducted every other day by removing one-third (-12 l) of water from the tank and replacing it with a freshly made mix. To account for trace element dilution when un-spiked water was added during water changes, tanks with mid or elevated spike treatments were re-spiked with one-third of the original spike (182 µl Mn + 1.82 µl Pb or 363 µl Mn + 3.63 µl Pb). Immediately following water changes, larvae were fed by depositing dilute Instant Algae Shellfish Diet 1800 (Reed Mariculture) into larval homes via syringe. The experiment ran for 7 d, until the larvae were 14 d old.

Dissolved oxygen, temperature and salinity were monitored daily with a HACH HQ40d dual input, multi-parameter portable water quality meter. Dissolved oxygen, pH, salinity and temperature measures remained consistent among the treatments throughout our laboratory experiments. Mean ± SE dissolved oxygen and pH were 8.68 ± 0.025 mg l⁻¹ and 7.72 ± 0.032 , respectively. Mean salinity for high and low salinity treatments were 20.7 ± 0.091 and 12.8 ± 0.120 ppt, respectively. Mean temperature for high temperature treatments was $25.7 \pm 0.157^\circ\text{C}$ and $21.3 \pm 0.104^\circ\text{C}$ for low temperature treatments.

Although Pb and Mn were manipulated throughout the duration of the experiment, water chemistry

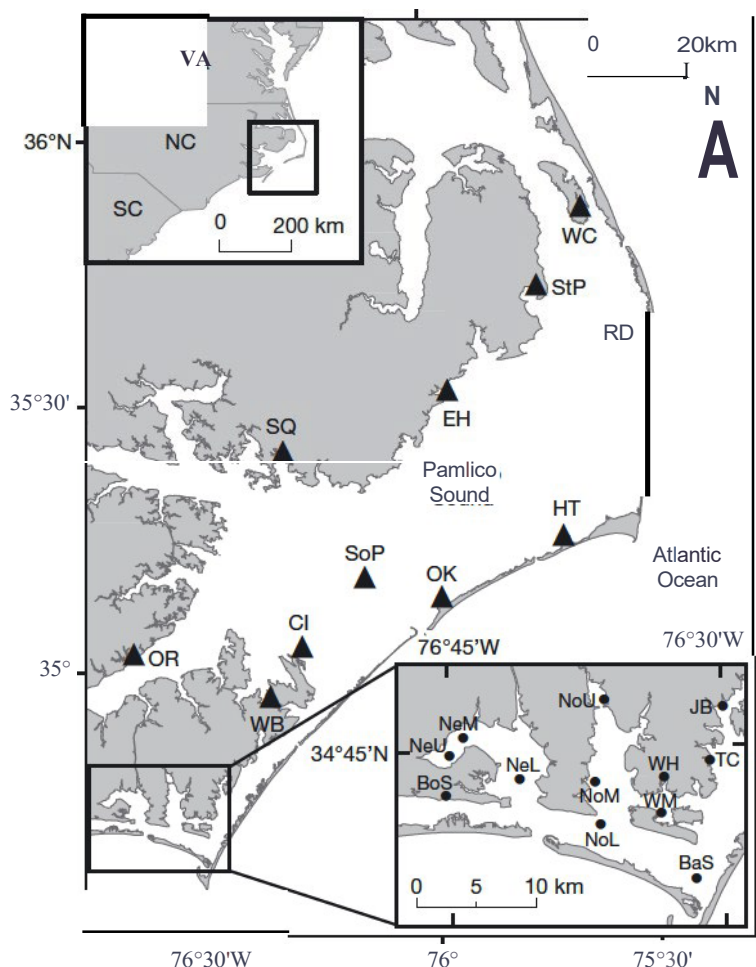


Fig. 1. Spat settlement collection sites within Pamlico Sound (●) and Bogue-Back-Core Sound (e), NC, USA, study system. For full site details see Table 1

was not analyzed. Previous mesocosm work has shown that salinity often affects the relative amounts of specific trace metals in seawater (e.g. Mn and Sr), whereas temperature is a less consistent factor (Martin & Thorrold 2005). Salinity fluctuations are often a result of freshwater inputs, which dilute seawater trace metal concentrations and can therefore be corrected for with our replicable spiking procedure (Martin & Wuenschel 2006). While measurements of specific elements are possible (e.g. Pb), determining the bioavailability of these elements within specific environments can be more challenging (Eggleston & Thomas 2004). Furthermore, the addition of larval oyster food into our mesocosms may complicate traditional elemental detection methods (Martin & Wuenschel 2006). However, larval diet was distributed uniformly to all tanks, and thus, while the chemistry of the actual treatments was not verified, we do have reason to assume consistency among treat-

ments. The larval diet used, Shellfish Diet 1800, was cultured in artificial seawater (with a deionized water base), which precludes any suspicions that it may contain above average levels of trace elements.

At the conclusion of these mesocosm incubations, larvae from each home were filtered using nitex cloth (30 μ m) and then resuspended in 15 ml of water from their respective tank. A 0.5-1 ml subsample of each larval resuspension was removed and the number of whole larvae was counted. The remaining larval solution was then frozen at -23°C until sample preparation for geochemical analysis.

Spat settlement sampling and site prediction

We collected recently settled spat from sites (see next paragraph) across the Bogue-Back-Core-Pamlico Sound (BBCPS) estuarine system of NC (Fig. 1) to assess whether unique elemental signatures existed among estuarine regions that could be used to accurately predict collection sites of individual spat.

Spat settlement collectors were constructed by affixing 2 or 3 wire strings, each containing 12 adult oyster shells, to private and public docks or stand-alone wooden pilings, throughout the BBCPS study system. Settlement collectors were deployed on June 7 and 21 and again on August 1 and 16, 2012 and retrieved ~2 wk after each deployment as part of an ongoing settlement sampling program (D. B. Eggleston & B. J. Puckett unpubl. data). Recovered settlement collectors were frozen until individual spat could be counted and removed from adult oyster shells with a tungsten probe.

Sample preparation and laser ablation inductively coupled plasma mass spectrometry (LA ICP-MS)

Frozen larvae from the laboratory experiments were thawed and ~1000 larvae were obtained representing each replicate home. The larvae were then rinsed with ultrapure H_2O , and shells were inspected for any remaining tissue. The process of freezing, thawing, and rinsing larvae appeared to remove most soft tissue, and therefore acid and peroxide

(which could degrade shells) were not needed nor employed. If larvae were highly translucent (i.e. no tissue present), they were mounted as a concentrated mass on a labeled glass microscope slide covered in double-sided tape. This process continued until larvae from each home were mounted on a slide in haphazard order (i.e. each home was represented by 1 mound of shells; total $N = 72$). The slides then stored in a laminar flow hood until analysis.

Spat from the field settlement collections were thawed and placed individually in 2 ml centrifuge tubes filled with 100 ml of 15 % H_2O_2 solution buffered in 0.05 N ultrapure NaOH. Samples were sonicated for 10 min to remove organic material. The H_2O_2 solution was then removed and replaced with a 100 ml solution of 1 % ultrapure HNO_3 (OPTIMA grade; Fisher Scientific). Samples were then sonicated for 5 additional min to dissolve any remaining tissue and surface contaminants. Spat were then rinsed 3 times with ultrapure H_2O and dried overnight in a laminar flow hood. After drying, spat were mounted in haphazard order onto a glass microscope slide with double-sided tape and stored until analysis.

Both larval and spat samples were analyzed using a Thermo-Fisher Element 2 inductively coupled plasma mass spectrometer with a Teledyne ATLex 300si-x 193nm Excimer laser ablation unit (LA ICP-MS). To correct for mass bias and instrument drift, NIST-certified standards (Reference Material 612, 614, and 616) were run at the beginning and end of every 4th slide sequence (~140 burns). Concentrations of the following elements were quantified from laboratory larval samples: ^{48}Ca , ^{55}Mn , ^{88}Sr , ^{138}Ba , and ^{208}Pb ; and from field-collected spat: ^{26}Mg , ^{48}Ca , ^{55}Mn , ^{63}Cu , ^{88}Sr , ^{118}Sn , ^{138}Ba , and ^{208}Pb . These elements were all analyzed in low-resolution mode and were chosen because of their previous use in uptake and tagging studies of fish otoliths and bivalve shells (Martin & Thorrold 2005, Strasser et al. 2008a,b, Fodrie et al. 2011).

Larval slide-mounts from the laboratory experiment were ablated 3 times in bulk, using side-by-side line transects of 150 μm with 40 μm spot size and 80% laser intensity. Line transects covered ~2-3 shell lengths, following Becker et al. (2005), and were used instead of burning several individual larvae to reduce the likelihood of pseudoreplication. To determine elemental signatures of the spat collection sites, the outermost (most recently formed) section of the settler shell was also ablated twice with 150 μm end-to-end transects with 40 μm spot size and 80% intensity. The larval portion of settler shells was also analyzed to examine potential elemental variation in

larval source signatures. Larval shell of each spat sample was identified and sampled in duplicate with side-by-side line transects of 110 μm with 40 μm spot size and 80% intensity. Isotope intensities for replicate burns were averaged and then converted into elemental ratios (X:Ca) for each home or spat/larval shell, following Becker et al. (2007). For ease of comparison between laboratory and field experiments, and because X:Ca ratios can yield the same statistical results and significance as partition coefficients in bivalves (Strasser et al. 2008b), we opted to only utilize and report X:Ca ratios in our analyses.

Data analyses

Temperature, salinity, and trace metal manipulations

A 2-way ANOVA was used to test the effects of salinity and temperature on elemental ratios for the elements that were not spiked during the laboratory experiment (Sr and Ba). Due to the large amount of zero values in certain cases (e.g. undetectable amounts of Ba), Sr ratios and Ba ratios were transformed using a Box-Cox transformation to meet assumptions of normality and homogenous variances. After ensuring no interactive effects of Mn and Pb spikes with Sr or Ba signatures, or nesting effects for homes within individual tanks (using intraclass correlation), all tanks were included in this analysis with individual larval homes treated as replicates ($N = 6$) and temperature and salinity treated as fixed factors. For spiked elements (Mn and Pb), a 3-way ANOVA was used to test the effects of salinity, temperature, and spike level on elemental ratios. Mn ratios were transformed with a Box-Cox transformation, while Pb ratios were transformed logarithmically to meet assumptions of normality. After ensuring no nesting effects of individual tanks, homes were treated as replicates ($N = 6$); temperature, salinity and spike level were treated as fixed factors. For all 4 elements, Tukey's HSD tests were used post-hoc to explore differences within and among treatment groups.

Spat settlement sampling and site classification

Means and standard errors for field-collected larval and settlement shell Sr:Ca and Ba:Ca ratios were calculated and plotted by site to assess spatial variation in geochemical signatures among collection sites. Signatures from larval shells were used to

Table 1. Mean temperature (\pm SE) and salinity (\pm SE) measurements for spat collection sites over the collection periods in summer 2012

Site name	Site ID	Temperature (°C)	Salinity (ppt)
Back Sound	BaS	26.7 \pm 2.00	36.9 \pm 1.27
Bogue Sound	BoS	24.4 \pm 1.33	35.0 \pm 0.98
Cedar Island	CI	26.7 \pm 1.67	24.5 \pm 2.50
Engelhard	EH	26.9 \pm 0.44	20.1 \pm 0.47
Hatteras	HT	20.6 \pm 3.34	31.0 \pm 1.00
Jarret Bay	JB	27.8 \pm 0.62	35.5 \pm 0.51
Newport Lower	NeL	25.6 \pm 2.32	35.0 \pm 1.04
Newport Middle	NeM	25.6 \pm 0.87	26.0 \pm 1.65
Newport Upper	NeU	24.5 \pm 1.03	15.3 \pm 2.75
North Lower	NoL	25.6 \pm 1.76	36.0 \pm 1.00
North Middle	NoM	27.4 \pm 0.73	30.0 \pm 1.32
North Upper	NoU	28.9 \pm 0.95	33.7 \pm 1.21
Ocracoke	OK	24.5 \pm 0.41	25.0 \pm 1.52
Oriental	OR	30.1 \pm 0.36	20.7 \pm 0.95
Rodanthe	RD	17.2 \pm 1.52	26.0 \pm 1.24
South Pamlico	SoP	27.6 \pm 1.04	25.1 \pm 1.92
Stumpy Point	StP	24.8 \pm 1.85	24.8 \pm 2.52
Swan Quarter	SQ	27.4 \pm 1.21	18.6 \pm 0.78
Tusk Creek	TC	27.8 \pm 0.62	36.0 \pm 0.25
Wanchese	WC	26.1 \pm 0.56	15.7 \pm 1.45
West Bay	WB	22.2 \pm 1.11	25.0 \pm 1.00
West Mouth	WM	29.4 \pm 0.72	35.0 \pm 0.94
White Hurst Creek	WH	29.4 \pm 0.05	35.0 \pm 1.15

examine possible temperature and salinity gradients present among natal sites (Table 1). Additionally, contour plots were used to explore how settler shell elemental concentrations of Mn, Sr, Ba, and Pb varied with temperature and salinity. Contour plots were created using the *graphics* package in R (version 3.0.3). Multiple regression models were then used to quantitatively assess the relationship between salinity, temperature and shell signatures in a natural environment. Because some collection sites did not produce any spat over a given collection period, spat were grouped only by site to increase the sample size and statistical power of our results. A logarithmic transformation of elemental ratio was used as the response variable.

Linear discriminate function analysis (DFA) was performed on Box-Cox transformed ratios to examine spatial variability in settler shell geochemistry and to determine the viability of using geochemical fingerprints to assess connectivity in oyster populations. All 23 sites were used in preliminary DFAs; however, the classification success was low, directing us toward independent examination of Pamlico Sound (PS) sites from the Bogue-Back-Core Sound (BBCS) sites. Because of spatial autocorrelation in temperature and salinity, PS sites were then grouped by geo-

graphic quadrant within PS: Northwest (NW; WC, EH, StP), Northeast (NE; RD, HT), Southeast (SE; OK, CI, WB), and Southwest (SW; OR, SoP, SQ). Each quadrant contained a diagonal of ~35 km to the centroid of PS. BBCS sites were similarly broken up into 5 groups based on geomorphology and site location: Bay (JB, WM), Creek (WH, TC), Newport (NeU, NeM, NeL), North (NoU, NoM, NoL), and Sound (BoS, BaS) (Fig. 1). Jack-knifed classification matrices, without sample replacement, were compared to expected classification matrices, based on random chance, to assess classification success. Sites were additionally grouped based on similar temperature and salinity profiles; however, classification success did not improve significantly over geomorphological quadrants so analysis did not continue with these groupings. Because natal origins are unknown and modeled dispersal pathways for the area (e.g. Haase et al. 2012) have not been empirically validated, no DFA was performed on larval signatures.

RESULTS

Temperature, salinity, and trace metal manipulations

Of the initial 1.60×10^4 larvae per home, a mean of 8390 ± 920 larvae were recovered, with an average of 128 ± 22.5 motile larvae per home. While estimated larval survival was low (based on presence of moving larvae), 0.80 ± 0.14 %, survival did not vary significantly by treatment ($p = 0.524$) and was consistent with published values of *Crassostrea virginica* larval survival (Davis 1958).

We found a significant interactive effect of temperature and salinity on Sr concentrations in larval shells ($F = 4.23$, $df = 3$, $p = 0.041$; Fig. 2a). Highest larval Sr concentrations, 5.51 ± 0.752 mmol mol⁻¹, were present in the low salinity (12 ppt), low temperature (21°C) treatment, representing an average increase of 35.1% over the mean concentrations of the other treatments. A similar pattern was observed in Ba concentration with a 572% increase in the low salinity, low temperature treatment as opposed to mean Ba concentrations of the other treatments combined. However, this trend was not statistically significant due to high variance within the treatment, coefficient of variation $c_v = 0.991$ ($F = 1.02$, $df = 3$, $p = 0.383$; Fig. 2b).

Larval shell Mn concentrations increased significantly with spike level. Mean concentration increased from 0.111 ± 0.015 to 0.568 ± 0.079 mmol mol⁻¹

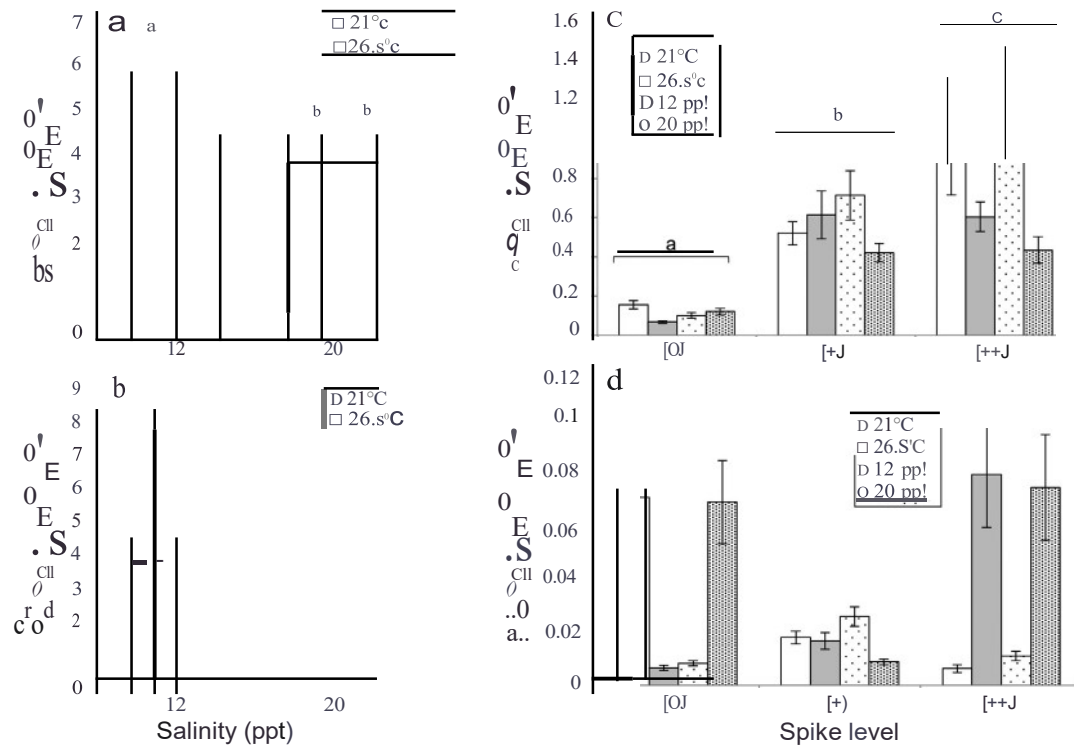


Fig. 2. Average elemental ratios (X:Ca; \pm SE), determined by laser ablation inductively coupled plasma mass spectrometry (LA ICP-MS), for larvae exposed to high and low temperature ($^{\circ}$ C) and salinity (ppt) and ambient (OJ, mid [+], and elevated [++]) concentrations of: (a) Sr, (b) Ba, (c) Mn, and (d) Pb. Sr and Ba were not spiked

between ambient [OJ and mid [+I spike levels, and to 0.802 ± 0.236 mmol mol $^{-1}$ at elevated[++] spike levels, with a 621% mean increase in concentration from ambient to elevated treatments ($F = 59.6$, $df = 11$, $p < 0.001$; Fig. 2c). Temperature and salinity did not influence overall Mn concentration ($F = 1.46$, $df = 3$, $p = 0.228$).

Larval shell concentration of Pb was highly variable, with no change in overall concentration with spike level and an overall mean of 0.034 ± 0.014 mmol mol $^{-1}$ ($F = 1.02$, $df = 2$, $p = 0.361$; Fig. 2d). There was a significant interactive effect of temperature, salinity and spike level ($F = 3.369$; $df = 11$, $p = 0.0374$) seen in the ambient and elevated Pb treatments. Specific comparisons for all examined elements and treatments are provided in Table 2.

Settler signatures and site prediction

Both settler and larval shells from field-collected spat showed robust spatial variability in Sr signatures (Fig. 3), while elemental concentrations of Sr were typically higher in larval shell than in settler shell {e.g. 73.9% increase in intensity from settler to larval shell at SQ}. Strong Sr:Ca gradients were present,

Table 2. Analysis of variance (ANOVA) table summarizing the effects of temperature (T), salinity (S), and Mn/Pb spike factor (I) on *Crassostrea virginica* larvae in laboratory experiments. Elemental ratios: X:Ca; N = no. of larval

homes. 'p' < 0.05

Element (X:Ca)	Factor	N	df	F	p
Sr	T	36	1	2.18	0.142
	S	36	1	2.60	0.108
	TxS	18	3	4.23	0.041'
Ba	T	36	1	1.01	0.316
	S	26	1	1.00	0.317
	TxS	18	3	1.05	0.306
Mn	T	36	1	0.417	0.519
	S	36	1	0.710	0.401
	I I	24	2	56.9	<0.001'
	TxS	18	3	1.46	0.228
	Tx I I	12	4	2.19	0.115
	S XI I	12	4	2.62	0.075
	TX S XI I	6	11	2.15	0.119
	T	36	1	0.731	0.393
	S	36	1	0.096	0.757
Pb	I I	24	2	1.02	0.361
	TxS	18	3	0.676	0.412
	Tx I I	12	4	0.177	0.838
	S XI I	12	4	0.588	0.556
	TX S XI I	6	11	3.37	0.037'

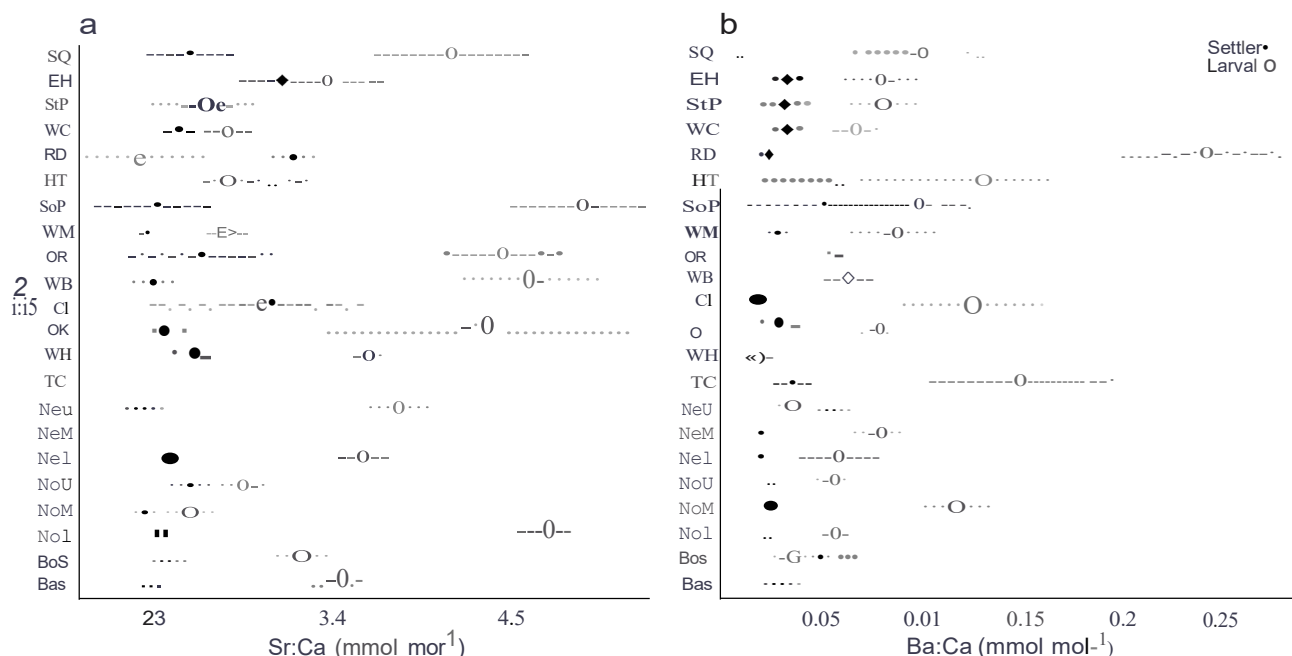


Fig. 3. Element ratios (X:Ca; \pm SE) of settler and larval components of collected spdt shell, by site, for (a) Sr and (b) Ba. Sites are arranged moving east to west within the northern and southern Pamlico Sound and grouped by geomorphological features in the Bogue-Bedc-Core Sound. Not enough intact spdt were collected from Jarret Bay (JB) for this analysis. See Table 1 for full site names

with increasing Sr settler shell concentrations when moving northward (e.g. from SQ to EH, a 30.2% increase) and eastward toward inlet openings (e.g. RD and HT, 31.1% increase from SQ). High larval Sr:Ca concentrations were present in the southern PS (e.g. SoP, OR, WB), mean $4.72 \pm 0.654 \text{ mmol mol}^{-1}$ when compared to concentrations in the northern PS (e.g. EH, StP, WC), mean $3.28 \pm 0.292 \text{ mmol mol}^{-1}$.

Generally, settler shells displayed less explicit spatial variation with respect to Ba:Ca ratios, although there was a trend of higher intensities at sites closer to freshwater inputs (OR, NeU), with a combined mean of $0.044 \pm 0.014 \text{ mmol mol}^{-1}$ at these sites, when compared to the overall mean of $0.037 \pm 0.016 \text{ mmol mol}^{-1}$. Larval shell Ba:Ca was fairly homogeneous along the north-south axis of the PS. However, eastern sites near inlets (RD, HT and even TC) exhibited higher Ba concentrations (e.g. a 127% increase when moving from SQ to RD).

Settler shell elemental concentrations varied greatly along natural temperature and salinity gradients (Fig. 4). For Mn:Ca, greater concentrations ($>3.5 \text{ mmol mol}^{-1}$) were found in settler shell collected from mid-salinity (26 ppt), mid-temperature (26°C) sites, with concentrations declining at lower temperatures and higher salinities ($<2 \text{ mmol mol}^{-1}$; Fig. 4a). A multiple-regression model verified this, as Mn concentrations were negatively correlated with salinity ($p < 0.001$)

and positively correlated with temperature ($p < 0.001$), with an R^2 value of 0.101. Sr concentrations were greatest ($>3.8 \text{ mmol mol}^{-1}$) in low temperature ($<22^\circ\text{C}$) and low salinity ($<21 \text{ ppt}$) waters, with concentrations decreasing with increasing salinity and temperature (Fig. 4b). Multiple-regression analysis validated this, showing strong, negative correlations between Sr signatures and temperature ($p < 0.001$) and salinity ($p = 0.007$), with an R^2 value of 0.091. Conversely, observed Ba concentrations were greatest ($>0.06 \text{ mmol mol}^{-1}$) at either end of the temperature range ($<18^\circ\text{C}$ or $>28^\circ\text{C}$) and at high salinity ($<30 \text{ ppt}$; Fig. 4c). Pb concentrations were the greatest in higher salinity water ($>24 \text{ ppt}$). However, concentrations varied across a wide range of temperatures ($>16^\circ\text{C}$ and $<29^\circ\text{C}$), with highest levels in mid temperature water (Fig. 4d). We found no significant correlations between Ba:Ca or Pb:Ca ratios and salinity and temperature.

Differences in settler shell geochemistry were not sufficient to discriminate among locations when including all sites in DFA (classification success of 18.3%). However, when considering only PS sites, jack-knifed classification success rose to 36.5% over a null expected classification success of 22.5%. When sites were divided into quadrants based on location within PS, we achieved an average classification success of 61.0%, a significant increase over

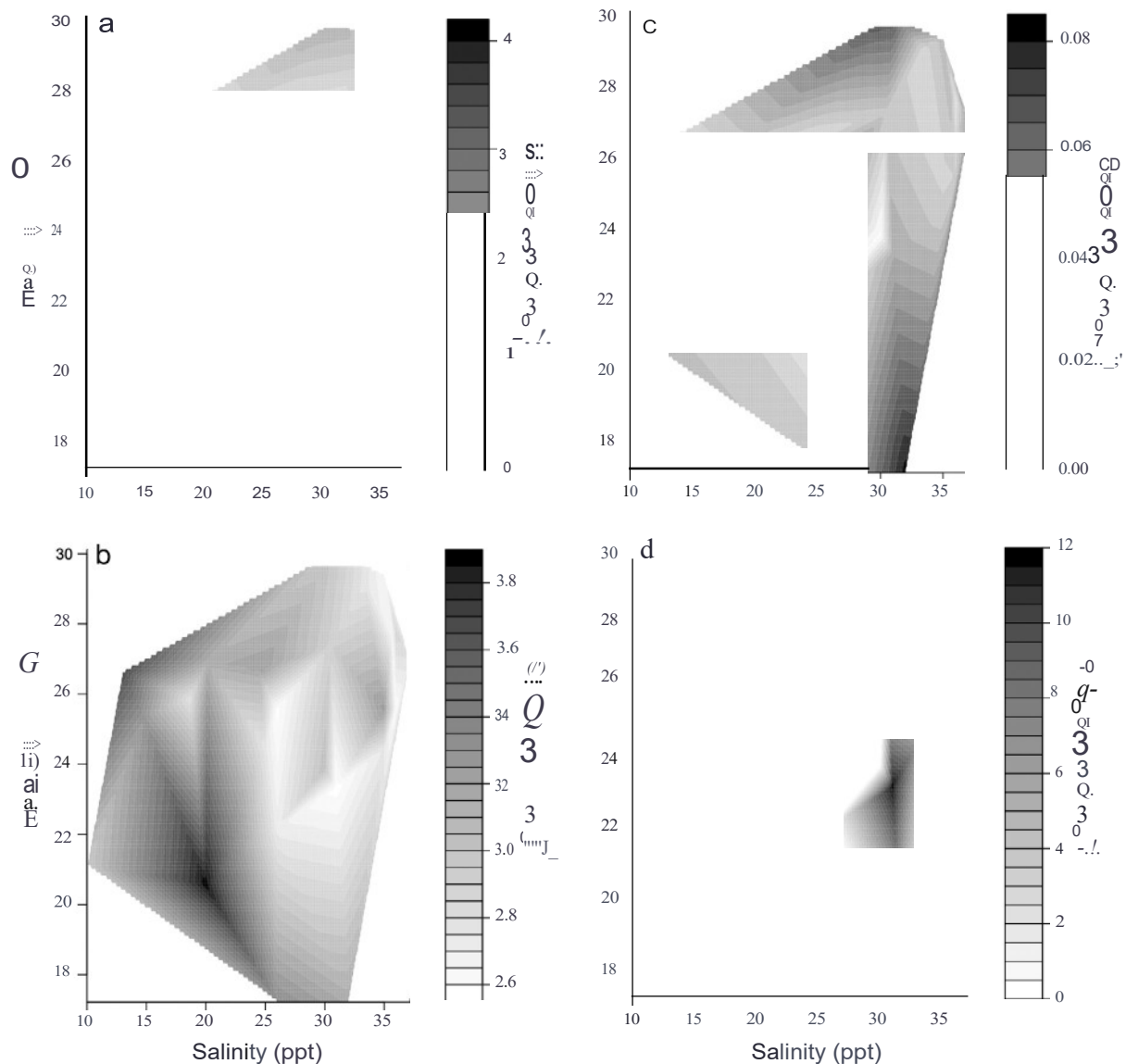


Fig. 4. Contour plots showing the distribution of elemental ratios for (a) Mn, (b) Sr, (c) Ba, and (d) Pb over observed temperature and salinity gradients for all collected spat shells throughout the Bogue-Back-Core-Pamlico Sound system

the null expected of 34.1% (Fig. 5a,b). Classification success for spat collection location varied greatly between sites and quadrants, ranging from 0 to 68 % correct assignments. The strongest discriminating elemental ratios for quadrant divisions were Sr:Ca, followed by Mn:Ca and Mg:Ca. For BBCS sites, discriminatory ability did not increase substantially when examining them without PS sites (classification success of 20.25 %). When dividing BBCS sites into geomorphological regions (e.g. Bay, Creek), there was a marginal increase in average classification success to 34.9% (Fig. 5c). Discrimination was driven, in order of predictive ability, by

Mn:Ca, Mg:Ca, and Sr:Ca ratios, based on forward stepwise variable analysis. BBCS sites were grouped into a single southern sites 'SS' grouping and including PS quadrants, jackknifed classification success rose to 76.5 % over a null expected of 23.8 % (Fig. 5d); however, classification success was still highly variable among sites, ranging from 96 % (SS) to 0% (NW). The strongest discriminating elemental ratios for these groupings were, again, Sr:Ca, followed by Mn:Ca and Mg:Ca, based on forward stepwise variable analysis (Fig. 6). For all grouping combinations, Pb:Ca was the least discriminatory element.

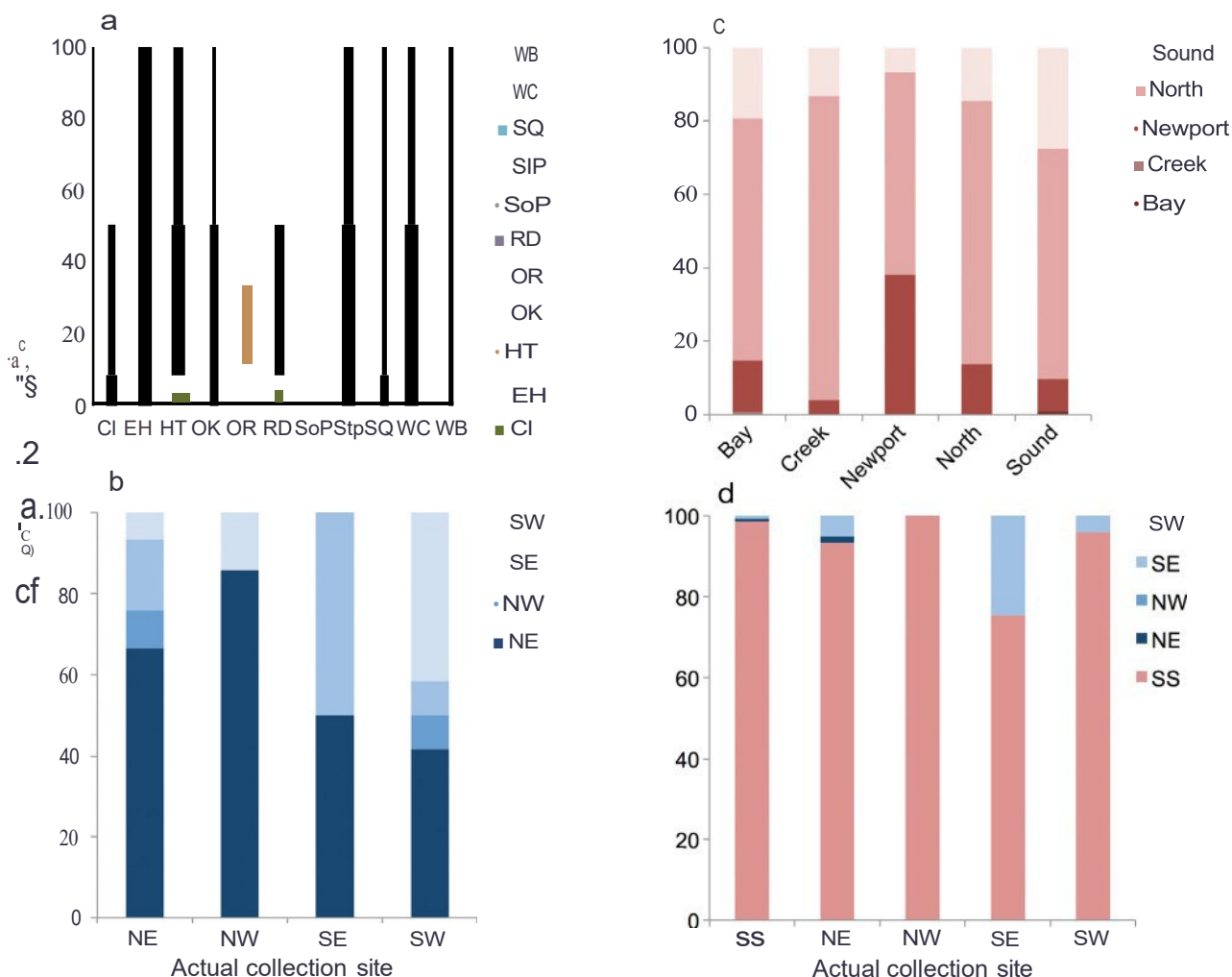


Fig. 5. Classification success determined by linear discriminant function analysis for (a) individual sites within Pamlico Sound, (b) regions within Pamlico Sound, (c) geographic regions among southern BBCS sites (SS), (d) and all groupings of sites where spat were collected. Colors represent the predicted collection sites, and cumulative percentage correctly identified is displayed on the y-axis. For full site names in (a) see Table 1; for sites included in each grouping see 'Materials and methods-Data analyses-Spat settlement sampling and site classification'

DISCUSSION

Geochemical tags, reflective of spatial gradients in environmental conditions, have been successfully used to identify natal origins, nursery use, and population-level connectivity patterns within a variety of teleost fishes (e.g. Patterson et al. 2005, Bradbury et al. 2011) and bivalves (Becker et al. 2007, Carson 2010, Cathey et al. 2012). The results of our study expand the use of elemental tags to the eastern oyster by providing the foundation from which to empirically assess population connectivity among estuarine sub-populations. Our study shows that environmental conditions necessary to impart distinct signatures within oyster shells are reliable over regional (35 km) spatial scales within a large estuarine complex. However, conditions of an individual

site (i.e. temperature and salinity) can vary greatly across time and space. Consequently, this approach may be better suited to predicting environmental conditions within a site at a given time, rather than discriminating between specific collection sites. Here, we consider the utility of geochemical signatures in discerning environmental condition over various scales within an estuarine system.

Environmental influence of trace metal signatures

It has been suggested that biological regulation of Sr ions has more influence on shell elemental concentration than salinity or kinetic effects of temperature (Gillikin et al. 2005, Strasser et al. 2008b). However, we observed significantly higher levels of Sr at

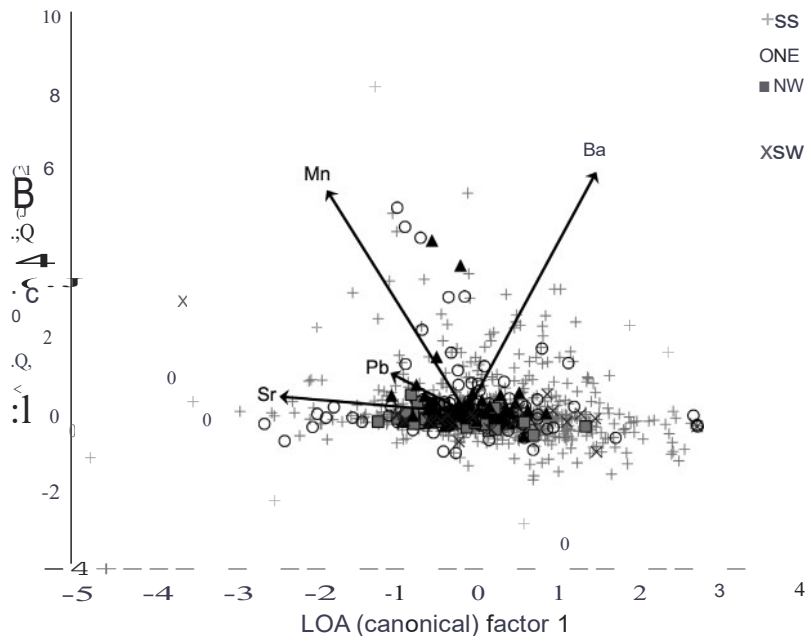


Fig. 6. Canonical score plots of the linear discriminant function analysis (LDA) for *Crassostrea virginica* settler shells grouped into Pamlico Sound quadrants and adjacent southern BCCS sites (SS)

low salinity and low temperatures in experimental larval oysters and field-collected settlers, supporting the utility of Sr as a marker of abiotic conditions experienced by an individual in tagging studies. If Sr incorporation into oyster shell is biologically regulated (as suggested by Strasser et al. 2008b), it follows that factors affecting metabolism (e.g. temperature) will likely impact Sr signatures. For example, cold water can lead to proportionally heavier calcium carbonate structures (Worthington et al. 1995) as well as altered precipitation rates and elemental incorporation (Martin & Thorrold 2005). For oysters in our study, lower temperatures may have slowed larval growth, resulting in increased proportional accumulation of Sr within the settler shells (sensu Martin & Wuensche 2006) and thereby allowing the possibility of dual biotic and abiotic regulation of Sr signatures.

Positive correlations between temperature and Ba, and no correlation between salinity and Ba, have been seen in Olympic oysters along the Pacific coast of the United States (Carson 2010). Our laboratory experiments exhibited no significant correlations between ambient Ba concentration and temperature and/or salinity. In the field, however, higher levels of Ba were detected at lower temperatures, a trend also found in clams (Strasser et al. 2008b) and neogastropod shells (Zacherl et al. 2003). There was an anomalous spike in Ba at higher temperatures (>26°C)

within the HT site; however, this site also experienced the greatest variance in Ba concentrations (Fig. 3b). While the specific mechanisms remain unclear, we believe Ba signature can be used dependably to effectively discriminate between temporal environments in geochemical tagging studies.

Previous literature on the geochemistry of bivalve shells has been unable to define a specific relationship between Mn concentrations and temperature and salinity (Siegele et al. 2001, Strasser et al. 2008b). Similarly, Mn elemental ratios in our laboratory experiment did not show temperature or salinity effects; however, Mn elemental ratios did scale with increasing spike level. Mn can enter the marine environment via terrestrial runoff, particle re-suspension, and as a product of redox reactions occurring in low-oxygen environments (Limburg

et al. 2015). Therefore, we can expect that riverine inputs and localized phytoplankton blooms created hypoxic/anoxic zones that resulted in the strong discriminatory ability of Mn among our study regions. This also explains why higher concentrations of Mn were found within warmer, less oxygen-rich waters (e.g. OR).

While Strasser et al. (2008b) found results similar to ours with respect to Pb concentration in larval clams, i.e. no effects of temperature or salinity, they also assert that Pb signatures are more strongly influenced by seawater Pb concentration than temperature or salinity (as in Pitts & Wallace 1994). However, we did not find a relationship between seawater Pb concentration and shell signature in the laboratory, and settler shell patterns of Pb were similarly ambiguous. As a result, Pb was not an effective discriminator between collection sites, or quadrants, and the addition of Pb to our final DFA model did not significantly enhance prediction ability. Pb enters the marine environment via anthropogenic pollutants, but as there are no explicit point sources for Pb within the BBCPS system, it was improbable Pb would have as much discriminatory power as other trace metals. Furthermore, Pb in the water column is often adsorbed to sinking particles and scavenged very quickly by sediments; therefore, it is unlikely that much of it is bioavailable (Bruland & Lohan 2003).

Our initial larval cleaning methodology included rinsing larvae with a mild acid solution. However, significant degradation of shell material was observed, and remaining larvae were rinsed only with ultrapure H₂O. While shells were examined visually for signs of remaining tissue, it is possible that residual organic matter or surface contaminants influenced observed elemental patterns. As individual tank environments were monitored and held constant (with the exception of treatment factors), it is unlikely that specific tanks, larval homes, or larvae would have higher contamination risks than others. Nevertheless, differences in cleaning methodology may limit some comparisons of shell chemistry of mesocosm larvae with the larval shell of field-collected spat, which were cleaned with nitric acid. To avoid possible contamination and/or standardization issues, future larval mesocosm studies might consider developing and employing a methodology that utilizes mild acid-washing to clean larval shells.

Application of elemental tagging to assess oyster larval population connectivity

Among the established PS quadrants, elemental tags showed high discriminatory ability and accurately assigned juvenile oysters to their region of collection with a resolution of ~35 km. Comparatively, oysters failed to provide the same discriminatory ability as other bivalves studied in an overlapping area of NC, i.e. ~12 km resolution found by Cathey et al. (2012) for the hard clam *Mercenaria mercenaria*, but did deliver close to the 20-30 km resolution found for mussel species *Mytilus californianus* and *M. galloprovincialis* and the 25-75 km resolution found for the Olympia oyster *Ostrea lurida* in San Diego, CA, USA (Becker et al. 2007, Carson 2010). Several factors may be responsible for this dissimilarity in scales between hard clams and oysters, including differing ICP MS methods (dissolving shell in acid as in Cathey et al. 2012 is more integrative and incorporates longer time periods whereas laser ablation in the present study targets specific points in time), potential variations in uptake at the organismal level, and sample site selection and variability. Predicted dispersal distances for *Crassostrea virginica* larvae range from 0.1 km to up to 110 km (North et al. 2008, Puckett et al. 2014), so our results indicate that elemental tagging can be valuable for refining our understanding of estuarine-scale larval connectivity for these species in the PS as well as in similar estuarine environments (e.g. Chesapeake Bay).

To create our PS quadrants (NW, NE, SE, SW), the area was divided into distinct regions with varying exposure to salt/fresh water influxes and temperature gradients, which, based on our larval experiments, could directly affect individual elemental signatures. For example, SQ and OR sites within the 'SW' quadrant both receive low salinity inflows from the Tar and Neuse Rivers, which likely elevated levels of Sr in settler shells collected from those sites. Laboratory results indicated elemental signatures of Mn were more dependent on seawater concentration than temperature or salinity. As terrestrial runoff, particle re-suspension, and redox cycling are major inputs of Mn in estuarine environments (Morris et al. 1982), many river-adjacent sites may have uniformly high Mn inputs that degrade signature uniqueness and discriminatory ability. As Sr and Mn offered consistently high discriminatory ability, similar levels among sites within regional groupings provided greater uniqueness to the overall signature. However, at the scale of individual sites, site proximity and environmental similarity resulted in ambiguous elemental signatures.

Proximity to freshwater sources may also explain the low level of prediction accuracy within and among the BBSC and PS sites. When analyzed individually, the BBSC sites had very low prediction accuracy, driven by a large overlap in predicted site matching between the Newport and North Rivers. As the rivers are adjacent (~6 km apart) and experience comparable surrounding land usage, similar geochemical environments and signatures are to be expected. While including the PS sites (as quadrants) and the singular SS site into the geomorphological DFA (Fig. 5d) significantly improved classification accuracy, high between-site variability was likely an additional result of the connection between PS and the Newport and North Rivers.

In general, comparisons made between signatures in larval and settler shells should be interpreted with caution, as the composition of aragonitic larval shells and calcitic settler shells may favor the uptake of specific elements differently (Finch & Allison 2007, Strasser et al. 2008a,b). For instance, we found higher Ba and lower Sr concentrations in a majority of larval shells when compared to their corresponding settler shells (Fig. 3b). Larval shell patterns also indicate a potential departure from traditional models of connectivity within this system. Recent work in the Pamlico Sound suggests that inter-reef connectivity is very low (~2 %) and that local retention sustains the sub-populations (Puckett et al. 2014). However, the presence of north-south and east-west gradients in

larval shell Sr and Ba, respectively (Fig. 5), may indicate multiple larval sources among our study sites. Furthermore, high variability within sites (e.g. OK) may indicate multiple natal sources exist even within a single area or site where spat have settled.

Given the differences we recorded in larval and settler shell from individual spat, our findings support previous work demonstrating the importance of a larval shell atlas for exploring larval connectivity, such as that utilized by Becker et al. (2007). This is necessary to expand our understanding of larval connectivity and identify potential dispersal corridors within the BBCPS system. Larval outplant experiments would also allow for exposure to other environmental factors not examined in our laboratory experiments, such as such as ultraviolet radiation, localized primary production, and oxygen concentration, which may affect element uptake (e.g. Elsdon & Gillanders 2005). Finally, we recommend the coupling of geochemical tagging data (e.g. based on larval drifter studies) with expanded biological (e.g. surveys of adult oyster density and distribution), and physical (e.g. current and wind patterns) datasets to produce rigorous biophysical models, which can be used to predict dispersal and inform managers.

Acknowledgements. This work is supported by NSF project number OCE-042092122. We thank S. Alexandler of the University of Maryland Horn Point Laboratory for supplying us with IMVal oysters and helpful aquaculture advice and suggestions. We also thank A. Pool and L. Alexandler for assistance with experimental set-up and sample processing. M. Kenworthy, J. Byrum and H. Eggleston from North Carolina State University, and A. McCall from the Nature Conservancy provided assistance in deploying and collecting settlement collectors. M. Kenworthy also provided mapping expertise and N. Lindquist and R. Gudjardo provided temperature and salinity data for field sites. Finally, we thank P. Peterson and A. Murchetti for comments on early drafts of this manuscript.

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Editorial responsibility: Steven MorgBn,
Bodega Bay, California, USA

Submitted: April 30, 2015; Accepted: November 10, 2015
Proofs received from author(s): January 25, 2016