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A SHELL OF ITS FORMER SELF: CAN *OSTREA LURIDA* CARPENTER 1864 LARVAL SHELLS REVEAL INFORMATION ABOUT A RECRUIT'S BIRTH LOCATION?

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ABSTRACT Despite the interest in restoring remnant populations of the Olympia oyster, *Ostrea lurida* Carpenter 1864,† little is known about connectivity among populations. Identifying the sources of settling larvae could broaden our understanding of the degree to which particular populations are reliant on their neighbors for their persistence. Calcified structures such as the otoliths of fish and statoliths of invertebrates are increasingly being exploited as useful "natural tags" that help track individual movements and, when applicable to larvae, could help to pinpoint important source populations. In controlled laboratory culturing experiments, we explored the prospects for using the chemistry of larval shells (prodissoconchs) as natural tags of larval source by examining whether larval shells record shifts in seawater element chemistry, whether larval shells undergo ontogenetic shifts in element uptake, and whether the chemistry of the shell formed during brooding is compromised by subsequent shell thickening during the planktonic phase. Results from a two-way ANOVA examining the effect of seawater element concentration and ontogeny showed that element/Ca in the shell increased in response to increasing seawater elemental concentrations for Ba, Ce, Pb, and Mn, whereas shell Cu/Ca did not change. *Ostrea lurida* shell chemistry also showed strong ontogenetic shifts in element/Ca for Mg, Sr, and Cu during the transition from larva to settler. Settler shell Mg/Ca strongly increased compared with planktonic shell, whereas Sr/Ca and Cu/Ca showed the opposite pattern. Further, the chemistry of the shell formed during brooding (at the birth location) did change as a function of environmental conditions experienced during the planktonic phase for the elements Ba and Ce, but that change was limited to regions of the brooded shell just adjacent to the planktonic shell. When the brooded portions of larval shells were sampled closer to the umbo, the brooded shells' chemistry remains intact. The combined results suggest that larval *Ostrea lurida* shells act as recorders of environmental change and show promise as tools to track larval movements.

KEY WORDS: Olympia oyster, Ostrea lurida, shell formation, larval dispersal, larval shell chemistry

INTRODUCTION

Many marine species (e.g., lobster, fish, oysters) produce free-swimming larvae that have an obligate developmental period in the water column before they can recruit into adult habitat. During this planktonic phase, larvae can be carried away from their birthplace by oceanic currents. How far do larvae disperse; where do they settle? Predicting larval dispersal destinations has proven challenging for marine biologists, because larvae are microscopic and possess complex swimming behaviors that could substantially alter their transport routes (Young 1995, Stobutski & Bellwood 1997, Shanks & Brink 2005). Resolving the mystery of where larvae go would make tremendous contributions to our understanding of population connectivity (Crowder et al. 2000), population dynamics and community structure (Caley et al. 1996), and could provide substantial useful information to restoration efforts for any species.

Calcified structures such as the otoliths of fish and statoliths of invertebrates are increasingly being exploited as useful "natural tags" that help track individual movements and, when applicable to larvae, such natural tags can help to resolve the mystery of where larvae go. Otoliths/statoliths are calcified ear stones that start forming early in development and increase in size through daily incremental deposition of calcium carbonate. As each new increment forms, elements with an ionic radius similar to Ca (e.g., Sr, Ba, Pb, see Campana 1999 for review) can substitute into the calcium carbonate matrix as a function of temperature, salinity, and element concentration in seawater (e.g., Radtke & Shafer 1992, Secor et al. 1995, Bath et al. 2000). Thus, as an individual travels across gradients in environmental factors, it may be "recording" its dispersal pathway in discreet time slices in the chemistry of its otolith. Otoliths and statoliths of juvenile fishes and squid have been used very successfully to provide a record of migratory pathways (Ikeda et al. 2003, Cairns et al. 2004), to sort migratory fishes into unique stocks (Campana et al. 2000, Arkhipkin et al. 2004, Volpedo &Cirelli 2006) and to identify spawning or nursery habitats (Milton et al. 1997, Gillanders & Kingsford 2000, Thorrold et al. 2001). The prospect to use the larval "cores" or segments of otoliths and statoliths as "recorders" of larval movements has proven technically challenging because of limited amounts of material to analyze, but nonetheless, a handful of studies have successfully extracted information about larval dispersal trajectories using these larval parts (Radtke et al. 1990, Swearer et al. 1999) or have demonstrated their potential usefulness (Zacherl et al. 2003a, Zacherl 2005) as natural tags that can uncover the birth locations of incoming settlers. Larval shells of bivalves (Becker et al. 2005; Becker et al. 2007) show similar promise as tags of source populations.

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†The taxonomy of the Olympia oyster has been in dispute since Harry (1985) proposed synonymy of *Ostrea lurida* Carpenter 1864 and *Ostrea conchaphila* Carpenter 1857. Polson et al. (2009) provide molecular evidence that the Olympia oyster refers to the nominal species, *Ostrea lurida* Carpenter 1864. In view of their genetic data, and for consistency, the original taxon, *Ostrea lurida*, is used throughout this volume to refer to the Olympia oyster, which is distributed from approximately Baja California (Mexico) to southeast Alaska.

The approach for using calcified tags to identify source populations is straightforward in theory, particularly for larvae whose calcified structures develop in egg masses while being brooded before the larvae enter their planktonic phase. To use calcified tags to identify the birth locations of postsettlement individuals at a particular site, one must first identify all potential source populations that could supply larvae to the site of interest. Then, spatial variation in the chemistry of the tags of pre-release larvae among those potential sources must be documented and mapped. Tags that are not temporally stable may require the compilation of a library of element tags over the time-period of interest to encompass a reasonable amount of temporal variation for each potential source population (Gillanders 2002). Finally, the element tag in the calcified structure of the postsettlement individual may be analyzed and matched to tags of potential source populations (e.g., Thorrold et al. 2001, Becker et al. 2007).

The life history of the Olympia oyster, *Ostrea lurida* Carpenter 1864, is ideally suited to examining patterns of larval transport using calcified tags. First, this species is distributed among discrete populations in bays and estuaries (Baker 1995) that are likely to exhibit unique seawater characteristics caused by differences in watershed usage and differences in temperature and salinity profiles. These unique environmental profiles can be reflected in the chemistry of calcified structures forming at each location. Second, because females brood the larvae for 10–12 days before they are released into the plankton (Coe 1931), larvae form their calcified larval shell (prodissoconch) at their birth location and retain this structure after settlement, with the animal's birth location potentially recorded into the shell. *Ostrea lurida* larvae are believed to spend from 2 (Couch & Hassler 1989) to 8 wks (Breese 1953) in the plankton, providing adequate time for exchange among locations within estuaries, and perhaps even among distantly placed estuaries, though several studies have shown evidence for self-recruitment in species whose planktonic larval duration provides the potential to disperse widely (see Swearer et al. 2002 for review). Thus, it is reasonable to expect exchange of propagules among locations, and not unreasonable to suspect retention of larvae within a single source location. Further, the larvae settle gregariously on hard substrate, including on conspecific shells, making settlers easy to locate. Last, there are compelling reasons to study larval exchange among populations for this species. *Ostrea lurida* is the only oyster species native to the west coast of the United States, with a range extending from Sitka, AK to Cabo San Lucas, Baja California Sur, Mexico (Dall 1914). In the 19th century, it was a commercially important harvested species and was cultured at some locations in Oregon and Washington until natural populations were depleted in the 1930s because of a combination of overharvesting, dredging, pollution, and filling and draining of wetlands (Hopkins 1931, Bonnot 1935, Baker 1995, Conte 1996). For the following decades, commercial oyster fisherman lost interest in the Olympia oyster in favor of the much larger introduced Pacific oyster, *Crassostrea gigas*. However, in recent years, ecologists and commercial oyster farmers have expressed renewed interest in this species motivated by the Olympia oyster's potential to be a tantalizing "specialty fishery product" (Wong 2004) and the desire to understand what factors will facilitate the oyster's comeback. Despite the interest in restoration projects by agencies such as NOAA and California Department of Fish, little is known about this species' genetic structure at a large geographic scale (but see Polson et al. 2009) and nothing is known about per-generation exchange of larvae among populations. Thus, identifying the sources of larvae using calcified tags, coupled with detailed genetic studies, could broaden our understanding of how particular populations are reliant on their neighbors for their persistence, and would aid in identifying critical populations for restoration efforts.

Two complicating factors could interfere with the use of *O. lurida* prodissoconch as a tag of birth location. First, Strasser et al. (2007) completed isotope analyses of the shells of *Mya arenaria*, a free-spawning bivalve, which indicated that larval shell is progressively thickened during the planktonic phase, complicating the ability to sample uncontaminated tags of "source." In *O. lurida*, the prodissoconch is formed before release into the planktonic phase, but as subsequently deposited shell material is formed (and potentially under the influence of very different environmental conditions), some portion of the prodissoconch is probably thickened. This leaves open the possibility that the "tag" of birth location has been compromised. Second, when establishing the "atlas" of chemical tags among source locations, it is impossible to determine which oysters are gravid without sacrificing many of them to find the few containing brooded larvae awaiting release. This is not ideal for a species whose populations are in need of restoration. One possibility for eliminating the wastage involved with cracking open multiple oysters would be if recent settler or adult oyster shells could be sampled as a proxy tag of that of the brooded larva. That would require no ontogenetic shifts in element uptake into shells, however it has been known for decades that ontogenetic variation in bivalve shells might be problematic (Rosenberg 1980), and there is building evidence from field *in situ* studies of fish otoliths that ontogenetic shifts in element uptake do occur (e.g., Chittaro et al. 2006), ruling out the possibility for using later stage calcified structures as proxies for larval structures (also see Ruttenberg et al. 2005).

Thus, to explore the prospects for using larval and settler shells of the Olympia oyster, *Ostrea lurida*, as tags of birth location, we used controlled laboratory experiments to answer several questions. First, we explored whether seawater elemental concentration influences the chemistry of larval and settler shells for the elements barium (Ba), lead (Pb), manganese (Mn), copper (Cu), and cerium (Ce). If larval shells do record shifts in seawater chemistry, the portions of the shells formed prior to release into the plankton might act as tags of birth location useful for identifying productive source populations of recent settlers and for examining within and among-estuary exchange of larvae. Second, we explored whether shells undergo an ontogenetic shift in element uptake between planktonic and settler stages for the elements Mg, Sr, Ba, Pb, Mn, Cu, and Ce. If ontogenetic shifts do occur, it would be difficult to use later stage shell as a proxy for larval shell when building an "atlas" of tags. Last, to address whether shell thickening compromises larval shell formed during brooding, we explored whether the chemistry of the shell formed during brooding (at the birth location) changes as a function of environmental conditions experienced during the planktonic phase.

METHODS

Larval Culturing: Effects of Seawater and Ontogeny

We tested whether the shells of planktonic oyster larvae and recent settlers can record changes in seawater chemistry, and

whether shells undergo ontogenetic shifts in element uptake using controlled laboratory culturing experiments. Multiple *O. lurida* individuals were collected from Tomales Bay, CA in June 2003, rinsed with 0.2-μm filtered seawater to remove sand fragments and algal growth, and then cracked open to identify, which oysters were brooding "black sic" veliger larvae that were competent for release into the plankton. We measured the lengths of a subsample of the larvae ($n = 20$); maximal length averaged 174 μm (range 163.2–180.0). These black sic larvae from several broods were mixed and then split into 6 acid-rinsed glass culture jars, each containing 3 L of seawater. Groups of two culture jars were randomly assigned to each of three spiking levels (ambient, ~3 × and ~6 ×) of the elements Ba, Pb, Mn, Cu, and Ce.

We chose to spike with elements that showed promise for discriminating among locations in previous larval tracking studies (e.g., Ba, Pb, Mn, Ce, Zacherl 2005) or in pollution monitoring studies (e.g., Cu, Richardson et al. 2001), whether the particular element might be expected to vary in estuaries across the range of the Olympia oyster (e.g., Ba, Ce, Pb, Cu) caused by geology, atmospheric deposition and/or anthropogenic influences, and whether they were detectable in our pilot studies of shell elemental chemistry. The ranges of concentrations for elements in our seawater typically bracketed the ranges of concentrations from the published literature. For Ba, our concentrations ranged from 6.9 ppb to 31 ppb, approximating the actual range in the world's oceans and estuaries (~4.8–30 ppb, Chan et al. 1977, Elsdon & Gillanders 2006). Our Pb concentrations ranged from 0.007–0.03 ppb compared with values in the world's oceans from 0.001–0.22 ppb (Schaule & Patterson 1981, Landing et al. 1995). Manganese ranges from ~0.01–0.05 ppb in the Pacific Ocean (Landing & Bruland 1980), but has been measured at much higher concentrations (up to 60 ppb) in recent studies of estuarine waters (Callaway et al. 1988, Elsdon & Gillanders 2006). Our values ranged from 1.3–4.7 ppb. Our Cu concentrations ranged from 0.3–2.7 ppb compared with 0.03–1.0 ppb in open-coast and estuarine waters (Scoullos et al. 2006, Bruland 1980). Last, there is a relative paucity of literature on Ce concentrations in seawater. Compared with published values from seawater near Japan (Alibo & Nozaki 1999) our Ce concentrations were an order of magnitude higher (0.01–0.04 ppb), however we suspect that higher levels of Ce found in our seawater samples from coastal CA relates to the anthropogenic influence of mining of Ce-rich minerals like bastnasite and monazite for use in the petroleum industry (Olmez et al. 1991). In sum, with the exception of Ce and the highest Cu spike, the spiking levels of all other elements fell within the range of measured variation in seawater.

Larvae were cultured at 21°C in a temperature-controlled walk-in incubator, with 100% water changes every other day. For all of the culturing water used in the experiment, we archived 0.2- μm filtered ambient seawater (collected from the flow-through seawater system at Bodega Marine Laboratory) into acid leached carboys. Carboys were stored in the walk-in incubator, and were spiked to the appropriate levels prior to introduction into the culture jars. We haphazardly sampled seawater from carboys just before introducing it into cultures for a total of four times per treatment. Seawater samples showed no significant change over time in the concentration of any element. After each water change, the oyster larvae were fed a mix of two algae, *Isochrysis galbana* (45,000 cells/mL) and *Dunaliella* sp. (5,000 cells/mL) for a combined concentration of 50,000 cells/mL in each culture jar. Cultures were maintained until larvae developed a conspicuous eyespot that indicated that they were competent to settle. At that point, we added glass slides to the cultures, continued to perform daily water changes, and terminated the experiment several days after oysters settled onto the glass slides. The glass slides were then frozen for storage.

Larval Culturing: Shell Thickening

In a separate culturing experiment, we examined whether the chemistry of the shell formed during brooding (at the birth location) changes as a function of environmental conditions experienced during the planktonic phase. Specifically, we determined whether all brooded shell is compromised, or just the newer portion of the brooded shell immediately adjacent to shell formed during the planktonic phase. Black sic larvae from several broods were again mixed and then split into 8 acid-rinsed glass culture jars. Groups of four culture jars were randomly assigned to each of two spiking levels (ambient, 6 ×) of the elements Ba, Pb, Mn, Cu, and Ce. Larvae were cultured until competent to settle as described earlier. We again sampled culture water as described earlier. Competent larvae were filtered out of the culture jars before settlement; they were then concentrated using a mesh filter, and frozen into 1.5 mL eppendorf tubes for storage.

Sample Prep for ICP-MS Analysis

For analysis, larval shells from both experiments were cleaned for 10 min at 65°C using 10 mL of a peroxide cleaning solution of an equal volume mixture of 30% H_2O_2 buffered in 0.1 N NaOH. After cleaning, shells were rinsed twice using ultrapure H_2O (resistivity >18.1 MΩ·cm), were acid-rinsed once by adding 0.5 mL 0.001N HNO_3 (Optima grade), and then were rinsed five times with ultrapure H_2O. All of the isolation steps were performed in a clean laboratory equipped with class 100 laminar flow hoods. All glassware used in the culturing experiment and cleaning procedures was cleaned with Citranox soap, rinsed 5 times with distilled H_2O (resistivity >2 MΩ·cm), soaked overnight in 1 N trace-metal grade hydrochloric acid and then rinsed 5 times with ultrapure H_2O (resistivity >18.1 MΩ·cm). Cleaned shells were mounted onto acid washed acrylic plastic slides using double-sided tape (Scotch) to secure them to the slide, and their chemistry analyzed using laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS).

ICP-MS Analysis

We assayed for a suite of 7 elements: calcium (Ca), magnesium (Mg), strontium (Sr), barium (Ba), lead (Pb), manganese (Mn), cerium (Ce), and copper (Cu); all elements reliably yielded detectable concentrations. From the first culturing experiment, transects from five haphazardly chosen planktonic shells and five haphazardly chosen settler shells from each replicate culture were introduced *via* a VG-UV microprobe Nd:YAG laser, frequency-quadrupled to 266 nm with a nominal beam width of 20 μm, into a Finnigan Element 2-sector field inductively-coupled plasma mass spectrometer (ICP-MS) using a microflow nebulizer at 20 μL min^{-1}. For the second experiment, we applied the exact same protocol, except this time we

sampled brooded shell only, but in two locations—nearest the umbo (hereafter referred to as "Earlier") and nearest the transition to planktonic shell (hereafter referred to as "Later"). Instrument sensitivity was approximately 1×10^6 counts s^{-1} for 1 ppb indium (In) solution. Samples from each treatment were analyzed in a random order to minimize the effects of instrument drift. The plasma conditions were kept constant during analysis of standards, instrument blanks and laser-ablated samples by constantly aspirating a 1% HNO3 solution, which was the matrix for both the standards and the blanks (see Zacherl et al. 2003b). We used matrix matched solution-based standards of known element/calcium ratio and applied a mass bias correction to determine the element/calcium ratio of the sample (Rosenthal et al.1999). To check the accuracy and precision of our solution-based measurements, we used a consistency standard containing Ca, Mg, Sr, Ba and Mn (Spex Certified primary standard solutions). Mg/Ca (mmol/mol) = 1.9%, Sr/Ca (mmol/mol) = 1.8%, Ba/Ca (μmol/mol) = 1.8%, Ce/Ca (μmol/mol) = 3.6%, Pb/Ca (μmol/mol) = 7.7%, Mn/Ca (mol/mol) = 5.3%, Cu/Ca (μmol/mol) = 28.0%. We also used National Institute of Standards and Technology (NIST-612) glass standards as reference materials from which we could estimate the precision of the laser ablation method. The reproducibility (% relative standard deviation, % rsd) estimate for the laser method was determined using the results of laser ablated NIST612 standard reference material, Mg/Ca (mmol/mol) = 8.6%, Sr/Ca (mmol/mol) = 3.1%, Ba/Ca (μmol/mol) = 6.7%, Ce/Ca (μmol/mol) = 5.8%, Pb/Ca (μmol/mol) = 7.6%, Mn/Ca (μmol/mol) = 11.3%, Cu/Ca (μmol/mol) = 15.1% (n = 8 runs). We quantified elemental concentrations using our matrix matched standard solution instead of the NIST standard, because the NIST glass lacks the aragonite-dominated matrix of larval shell and the calcite-dominated matrix of settler shell. The good quantitative agreement between aragonite-dominated matrix matched materials analyzed in solution *versus* laser-based modes suggests that our use of solution-based standards is robust (Thorrold et al. 1997). However, the lack of certified matrix-matched solid reference materials is problematic because it means that our data should be compared with other studies with caution (Campana 1999). Following Swearer et al. (2003), we calculated detection limits for each element by calculating the standard deviation (SD) of the intensities of the elements in 1% nitric acid (HNO_3) instrument blanks, and then multiplying the SD by 3. That value was then added to the blank mean for each element and used as the minimum detectable signal. The intensities of blank-subtracted samples averaged > 125 × the detection limit for ^{48}Ca, > 1280 × for Mg, >19 × Sr, >20 × Ba, >1 × for Pb, >69 × for Mn, > 95 × for Ce and >7 × for Cu. All element concentrations were standardized to Ca to control for variation in the rate of sample removal during ablation.

Seawater Analysis

Seawater samples were analyzed *via* ICP-MS using serial dilutions of the samples. For barium, we applied a method of isotope dilution using a standard enriched in ^{135}Ba (Oak Ridge National Laboratory $Ba(CO_3)_2$ salt), and collected the ^{135}Ba/^{138}Ba ratio. For the other elements analyzed, we used a method of standard additions (Spex Certified primary standard solutions) with internal standards; Sc was the internal standard for Ca and Mn, whereas Y was the standard for Sr, and In was the standard for Pb, Cu, and Ce.

Statistics—Effects of Seawater and Ontogeny

The effects of seawater element concentration and ontogenetic shifts on element uptake into planktonic and settler shells was analyzed *via* a two-way ANOVA for each element. All data were first checked to ensure they conformed to the assumptions for ANOVA. Because all data met the assumptions, the analysis was performed on untransformed data. Where significant effects of seawater element concentration were found, we tested whether uptake was linear for each treatment group (planktonic, settler) using regression analysis. In the case of Mg/Ca and Sr/Ca, where seawater concentrations were not manipulated, we could not test for linear uptake, but rather used posthoc Student t-test comparisons to test for differences among treatment groups. Statistical analyses were conducted using JMP (SAS) statistical software (versions 5.1 and 7.0.1).

Statistics —Shell Thickening

We tested whether the chemistry of the shell formed during brooding (at the birth location) changes as a function of environmental conditions experienced during the planktonic phase and whether the change occurs throughout the brooded shell by performing a two-way ANOVA that tested the effects of seawater treatment, shell region sampled, and the interaction between seawater treatment and shell region. All data were first checked to ensure they conformed to the assumptions of ANOVA; Ce/Ca was log-transformed to correct for heteroscedasticity. When significant main effects and/or interaction effects were found, we used posthoc Student's t comparisons to test for differences among treatment groups. Statistical analyses were conducted using JMP (SAS) statistical software (version 7.0.1).

RESULTS

Effects of Seawater and Ontogeny

For elements that were spiked in culture seawater (Ba, Pb, Mn, Cu, and Ce), element/Ca ratios in the planktonic and settler shells were significantly influenced by changes in seawater elemental concentrations except in the case of Cu (Table 1 and Fig. 1). Planktonic and settler shell Ba/Ca and planktonic shell Pb/Ca showed linear increases with increasing element concentration in seawater; none of the other elements showed statistically significant linear relationships ($r^2 < 0.61$, $P > 0.05$, Fig. 1). For planktonic shell Ba/Ca, the linear relationship ($r^2 = 0.99$, $P < 0.0001$) was $Ba/Ca_{shell} = 0.36 + (1.2 \times 10^7)Ba_{SW}$. For settler shell Ba/Ca, the linear relationship ($r^2 = 0.95$, $P = 0.001$) was $Ba/Ca_{shell} = 0.22 + (1.3 \times 10^7)Ba_{SW}$. For planktonic shell Pb/Ca, the linear relationship ($r^2 = 0.82$, $P = 0.01$) was $Pb/Ca_{shell} = 0.05 + 36.25Pb_{SW}$. Seawater Mg and Sr concentrations were not spiked in the experimental design, and their element/Ca ratios in shell did not change significantly among seawater spike treatment groups ($P > 0.05$, Table 1).

Ostrea lurida shell chemistry also showed significant ontogenetic shifts in elemental concentrations during the transition from larva to settler for Mg/Ca, Sr/Ca, and Cu/Ca; results for Mn/Ca were marginally insignificant and Ba/Ca, Pb/Ca, and

TABLE 1.

Two-way ANOVA testing for effect of seawater element concentration and ontogeny on *Ostrea lurida* shells. DF = degrees of freedom, SS = sum of squares, SW = seawater. Bold results indicate significance.

Element	Source	DF	SS	F	Prob > F
Mg	SW	2	41.04	1.72	0.2570
	Ontogeny	1	1,203.85	100.83	**<0.0001**
	SW*Ontogeny	2	20.60	0.86	0.4684
	Error	6	71.64		
	Total	11	1,337.14		
Sr	SW	2	0.01	0.07	0.9310
	Ontogeny	1	1.46	40.18	**0.0007**
	SW*Ontogeny	2	0.07	1.00	0.4218
	Error	6	0.22		
	Total	11	1.75		
Ba	SW	2	10.47	94.21	**<0.0001**
	Ontogeny	1	0.00	0.08	0.7857
	SW*Ontogeny	2	0.03	0.30	0.7524
	Error	6	0.33		
	Total	11	10.85		
Pb	SW	2	1.14	5.38	**0.0459**
	Ontogeny	1	0.07	0.70	0.4360
	SW*Ontogeny	2	0.07	0.33	0.7277
	Error	6	0.64		
	Total	11	1.92		
Mn	SW	2	2,376.96	5.95	**0.0377**
	Ontogeny	1	1,173.22	5.87	0.0517
	SW*Ontogeny	2	53.98	0.14	0.8763
	Error	6	1,199.43		
	Total	11	4,803.59		
Cu	SW	2	16.41	1.63	0.2724
	Ontogeny	1	118.52	23.51	**0.0029**
	SW*Ontogeny	2	11.38	1.13	0.3836
	Error	6	30.25		
	Total	11	176.56		
Ce	SW	2	9.85	7.44	**0.0237**
	Ontogeny	1	0.07	0.11	0.7511
	SW*Ontogeny	2	0.17	0.17	0.8475
	Error	6	3.97		
	Total	11	14.12		

Ce/Ca showed no ontogenetic shifts (Table 1). Settler shell Mg/Ca strongly increased by at least 5× when compared with planktonic shell (Fig. 2), whereas Sr/Ca (Fig. 2) and Cu/Ca (Fig. 1) ratios decreased.

Shell Thickening

The chemistry of the shell formed during brooding changed significantly as a function of seawater treatment group (ambient *vs*. spiked) during the planktonic phase for Ba/Ca, and Ce/Ca, but for Ba/Ca that change was limited to "Later" regions of the brooded shell just adjacent to the planktonic shell (SW × Shell region interaction, $P = 0.0401$, Table 2, Fig. 3). For Ce/Ca the pattern was not quite as clear; there was a significant effect of seawater treatment, with higher Ce/Ca in spiked treatments ($P = 0.0291$, Table 2, Fig. 3), but posthoc Student's t comparisons showed that the spiked treatments were not significantly different than one of the ambient treatments. For Mn/Ca, there was no effect of seawater treatment but there was a shell region treatment effect ($P = 0.0044$, Table 2, Fig. 3), with higher Mn/Ca near the umbo relative to brooded shell further away from the umbo. There was no change in brooded shell chemistry because of seawater treatment during the planktonic phase for Cu/Ca and Pb/Ca ($P > 0.05$, Table 2).

DISCUSSION

Shells as Calcified Tags

We provide evidence that larval *Ostrea lurida* shells act as recorders of environmental change and, in this regard, show promise as tags to track larval movements. Specifically, we demonstrated that increases in concentrations of some elements in seawater will result in increases in the element/Ca ratios in larval shell. The presumption that bivalve and other mollusc shells could record characteristics of the environment has been accepted for decades (see Richardson 2001 and references therein for review), based primarily on convincing correlative field and partially controlled laboratory studies that linked variation in shell chemistry to variation in environmental parameters such as element concentrations (e.g., Carriker et al. 1980, Pitts & Wallace 1994), temperature and productivity (e.g., Vander Putten et al. 2000), and based on the wealth of empirical data from other calcified structures such as foraminifera (e.g., Lea et al. 1999, Lea & Spero 1992) and otoliths (e.g., Bath et al. 2000, Elsdon & Gillanders 2003, Farrell & Campana 1996, Milton & Chenery 2001). However, few empirical studies have actually tested these assumptions about mollusc shell chemistry in a completely controlled laboratory setting using manipulative experiments (e.g., Lorens & Bender 1980) and fewer still on larval shell (Zacherl et al. 2003b). Our findings of increased Ba/Ca and Pb/Ca in larval shell with increasing concentrations in seawater concur with earlier findings for gastropod larval calcified structures (Zacherl et al. 2003b, Lloyd et al. 2008), and to our knowledge this is the first empirical evidence generated for Ce and Mn in mollusc larval shells. In the cases of Ce and Mn, the relationship between seawater element concentration and shell element uptake was not linear, but rather, appeared to asymptote at the highest seawater elemental concentrations.

Cu/Ca in shell did not change with increase in seawater Cu. This result is consistent with the notion that copper uptake is likely under strict physiological control, as seems to be the case with gastropods (e.g., Langston & Zhou, 1986), though Richardson et al. (2001) found elevated levels of Cu in mussel shells in contaminated sites relative to control sites, presumably because of elevated Cu concentrations in contaminated site seawater. Our results indicate that copper would not be a useful discriminator in natural calcified tags of the Olympia oyster.

Ontogenetic Shifts

Ostrea lurida shell chemistry showed strong ontogenetic shifts in elemental concentrations during the transition from larva to settler for Mg, Sr, and Cu and a marginally insignificant shift for Mn, whereas Ba, Pb, and Ce uptake did not appear to be under ontogenetic control. Fowler et al. (1995) generated the first solid evidence for ontogenetic shifts in Sr and Ba uptake into fish otoliths by raising fish under completely controlled laboratory conditions. Most other evidence has been correlative or has been generated under partially controlled conditions only

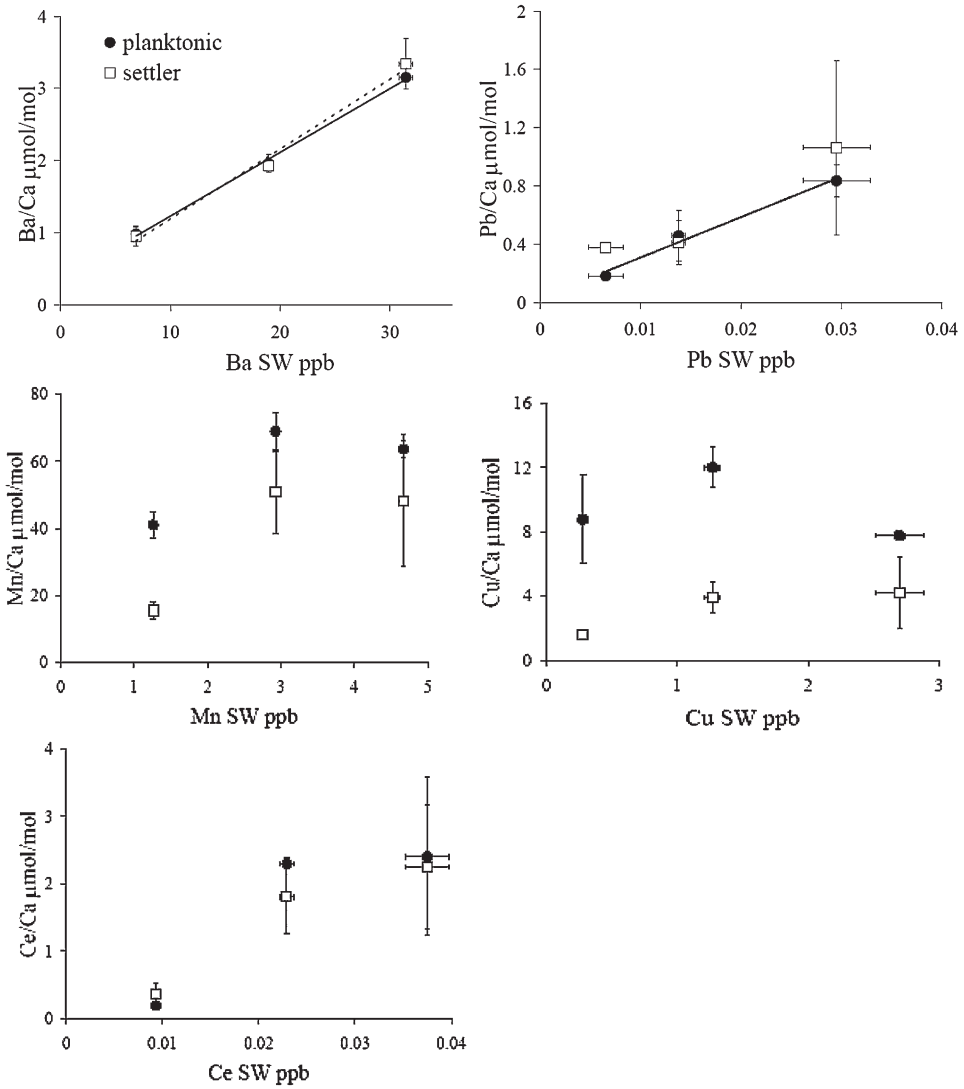

Figure 1. Mean element/Ca ratios (±1 SE) in planktonic larval (filled circles) and settler (open squares) shells of laboratory-reared *Ostrea lurida versus* element concentration (ppb) in seawater. Solid lines (planktonic) and dashed line (settler) indicate statistically significant linear relationships between seawater element and shell element concentrations. For planktonic shell Ba/Ca, $r^2 = 0.99$ ($P < 0.0001$); for settler shell Ba/Ca, $r^2 = 0.95$ ($P = 0.001$); for planktonic shell Pb/Ca, $r^2 = 0.82$ ($P = 0.01$).

(e.g., Chittaro et al. 2006, Otake et al. 1997, Carriker et al. 1996, Brophy et al. 2004). Our data generally concur with the findings of the earlier mentioned studies and others (e.g., Ruttenberg et al. 2005), with elevated elemental concentrations in the calcified structures of the youngest life stages (but see Elsdon & Gillanders 2005), though we did not find ontogenetic shifts for the same particular subset of elements. For example, unlike Chittaro et al. (2006), who found ontogenetic shifts in Ba, Pb, and Ce uptake, we did not find such shifts for the same suite of elements. Of course, the same mechanism may not be generating the ontogenetic shifts in differing taxonomic groups.

Scientists have speculated about several mechanisms that might generate ontogenetic shifts in metal uptake including, among others, differences in growth rates and changes in the crystal form of calcium carbonate. In our particular study, one simple mechanism may be invoked to explain the shift in chemistry; it may simply be a change in the crystal form of calcium carbonate. Otoliths, statoliths and larval shells can be composed of multiple crystal forms of calcium carbonate, including calcite, aragonite and vaterite, which are known to incorporate at least some elements (e.g., Mg, Sr, U) into the calcium carbonate matrix to varying degrees (Campana 1999, Ortega 2003, Deer et al. 1992). Switches between crystal forms, such as from aragonite during the pelagic period to calcite after settlement in *Ostrea edulis* larvae (Medaković et al. 1997), for example, would be expected to result in declines in Sr and increases in Mg. In *Ostrea lurida*, settler shell Mg strongly increased in concentration compared with planktonic shell, whereas Sr and Cu showed the opposite pattern. This shift in element concentration matches the prediction that the crystal form shifts from the aragonite to calcite in the shell.

More importantly, this finding indicates that, at least for those elements showing ontogenetic shifts in metal uptake, the chemistry of juvenile or adult shell cannot be used as a proxy for a larval tag of birth location. To establish the map of potential source populations, one must sample brooded larvae and cannot

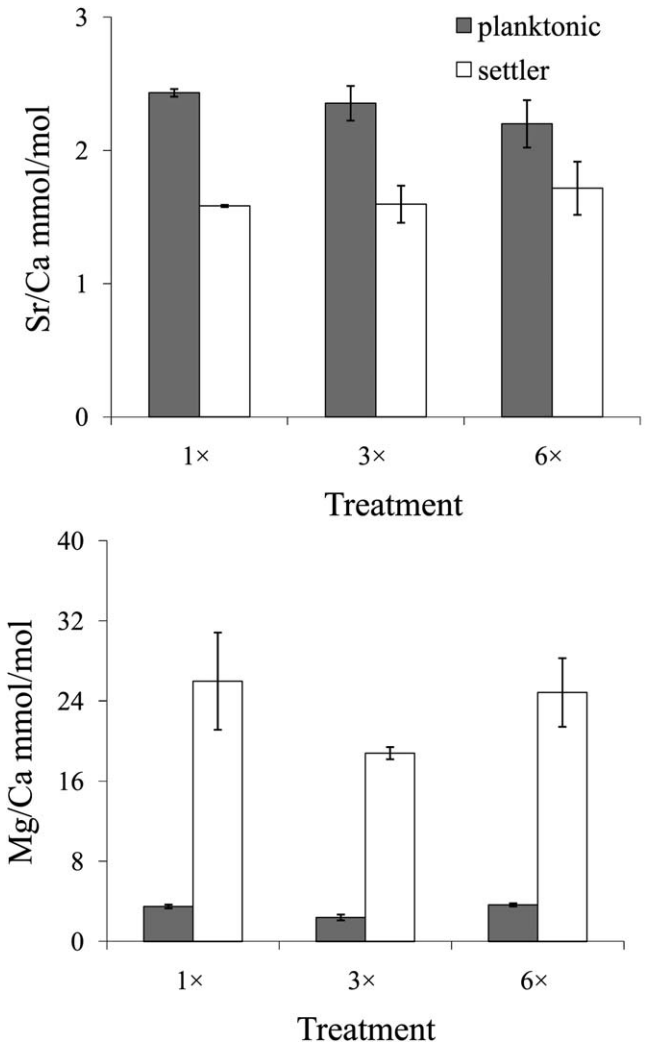

Figure 2. Mean element/Ca ratios (±1 SE) in planktonic larval (shaded bars) and settler (open bars) shells of laboratory-reared *Ostrea lurida* per treatment group for (A) strontium and (B) magnesium. Treatments refer to relative concentrations of elements (Ba, Pb, Mn, Cu, Ce) in laboratory-spiked seawater; Mg and Sr concentrations in seawater were constant among treatments. Letters above bars indicate significant differences based upon posthoc Student *t*-test comparisons.

TABLE 2.

Two-way ANOVA testing for effect of seawater element concentration and sampling region on uptake of elements during planktonic phase into brooded (already formed) shells *of Ostrea lurida* larvae. DF = degrees of freedom, SS = sum of squares, SW = seawater. Bold results indicate significance.

Element	Source	DF	SS	F	Prob > F
Ba	SW	1	1.34	9.98	**0.0082**
	Shell region	1	1.83	13.66	**0.0031**
	SW*Shell region	1	0.71	5.30	**0.0401**
	Error	12	1.61		
	Total	15	5.49		
Pb	SW	1	0.02	2.36	0.1502
	Shell region	1	0.01	1.97	0.1857
	SW*Shell region	1	0.00	0.02	0.8877
	Error	12	0.09		
	Total	15	0.12		
Mn	SW	1	138.30	1.06	0.3244
	Shell region	1	1,605.33	12.26	**0.0044**
	SW*Shell region	1	458.40	3.50	0.0859
	Error	12	1,571.55		
	Total	15	3,773.58		
Cu	SW	1	2.01	0.05	0.8328
	Shell region	1	164.30	3.80	0.0749
	SW*Shell region	1	0.70	0.02	0.9011
	Error	12	518.46		
	Total	15	685.47		
Ce	SW	1	0.68	6.14	**0.0291**
	Shell region	1	0.06	0.57	0.4657
	SW*Shell region	1	0.04	0.35	0.5668
	Error	12	1.33		
	Total	15	2.11		

rely on source tags generated by settler or adult shells. This poses logistical challenges for a species undergoing restoration efforts because it requires sacrifice of reproductive females to sample brooded larvae. Thus, we stress the importance of pinpointing exact reproductive windows for this species, particularly the time period when females have brooded larvae awaiting release, to minimize wastage of reproductive individuals.

Evidence for Shell Thickening

Strasser et al. (2007) indicated that larval shell of the bivalve *Mya arenaria* is progressively thickened during the planktonic phase, complicating the ability to sample uncontaminated tags of "source." In *O. lurida*, larval shell is formed prior to release into the planktonic phase, but as subsequently deposited shell material is formed, some portion of that initial shell (the prodissoconch) may be thickened. This is evident in our qualitative observations on the ablation dynamics and appearance of the shells of brooded larvae *versus* those that were allowed to develop further through their planktonic phase, with brooded larval shells being more "brittle" and more transparent. Our culturing data also suggested some thickening was occurring; the chemistry of the shell formed during brooding significantly changed as a function of treatment group (ambient *vs.* spiked) during the planktonic phase for Ba/Ca and Ce/Ca. However, in the case of Ba/Ca, that change was limited to "Later" regions of the brooded shell just adjacent to the planktonic shell (Table 2, Fig. 3). When the "Earlier" portions of brooded larval shells were sampled closer to the umbo, there was no significant difference in element/Ca ratios in ambient *versus* spiked treatments.

These results suggest that larval shell of *O. lurida* nearest the umbo can be used as tags of natal source, but the shells must be sampled cautiously and precisely. Thus, it is imperative to fully characterize the extent of thickening and to accurately identify regions of the larval shell that remain uncompromised through subsequent development phases *via* further laboratory validation studies. For example, using seawater spiked with ^{138}Ba well above ambient concentrations (e.g., Strasser et al. 2007) would enable us to quantify the extent of thickening and to measure shell thickness in regions uncompromised *versus* compromised by subsequent thickening. These experiments should be performed across multiple temperature treatments relevant to field conditions experienced by this species to control for differences in shell deposition as a function of temperature. Point-sampling using laser ablation technology will enable us to identify regions

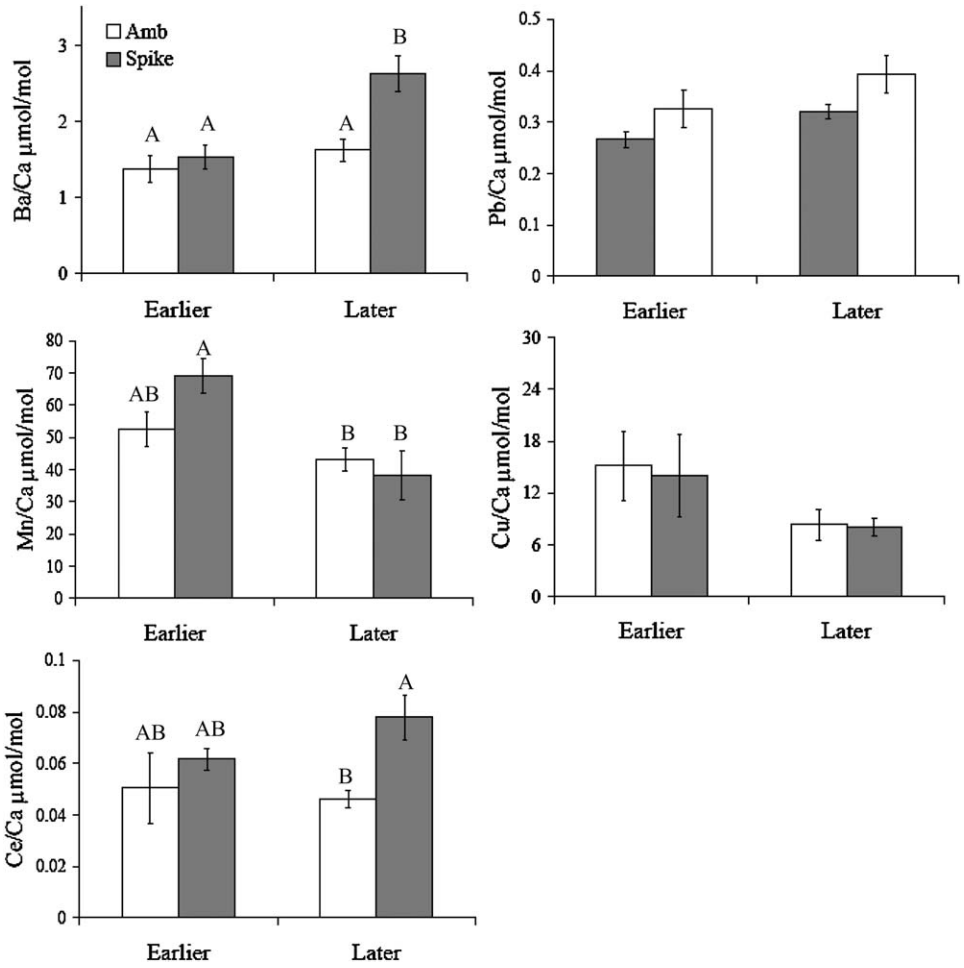

Figure 3. Mean element/Ca ratios (± 1 SE) in larval shells of laboratory-reared *Ostrea lurida* in seawater with ambient (open bars) and spiked (shaded bars) seawater measured at two locations (Earlier, Later) relative to the umbo. "Earlier" refers to samples collected adjacent to the umbo and thus deposited earlier in larval life, whereas "Later" refers to samples collected from larval shell further away from the umbo deposited later in larval life. Spiked seawater contained approximately four to six times the concentration of Ba, Pb, Mn, Cu, and Ce relative to ambient seawater. Letters above bars indicate significant differences based upon posthoc Student *t*-test comparisons.

of the shell that remain uncompromised and thin sections will allow measurements of thickness in all sections of the brooded shell. Careful application of this technique to track larvae should proceed only after such characterization studies have been complete.

CONCLUSION

The combined results demonstrate that larval *O. lurida* shells have potential as natural tags of natal origin that might help inform restoration efforts about important source populations of seed larvae for populations of interest. The true utility of using larval *Ostrea lurida* shells as calcified tags to track larval movement will depend on the extent of variation in the chemistry of those tags among locations of interest. Many studies using calcified tags have focused on species inhabiting estuarine habitats and have examined among-estuary exchange of individuals. Probably because of substantial among-estuary differences in salinity, temperature, and water chemistry, variation in calcified tags among estuaries has been significant (e.g., Thorrold et al. 1998, Swearer et al. 2003). Variation in estuarine water chemistry is the result of differences in watershed geology, variable inputs from local watersheds and locations of point sources of pollution and atmospheric deposition. The watersheds along the west coast of the United States show extreme variation in land-use and range from the Dominguez and Los Angeles watersheds in Los Angeles, CA, which are heavily urbanized and were extensively modified into massive storm drain systems, to San Mateo Creek Watershed near San Diego, CA, which has seen minor development because of the presence of Camp Pendleton and other restricted lands (Schiff & Stevenson 1996). Further, watersheds in Washington and Oregon regularly receive >100 inches of rain annually *versus* watersheds in southern California that rarely see >50 inches annually (National Weather Service, 2005; data from 1961–1990 average annual rainfall statistics). Because of this extreme variation in land use, geology and runoff contributions along the west coast of the United States, and because of the success of prior studies in finding differences in the chemistry of calcified structure in estuaries, this habitat may provide an ideal location for application of a calcified tag. Given the extreme gradients within estuaries, it is also possible

that within-estuary variation in physical-chemical conditions is strong enough to generate within-estuary site-specific tags. The application of this calcified tag, then, hinges on a full exploration of the extent of variation in tags among *O. lurida* populations of interest and their potential source populations.

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