Measuring Calcification in Biological Experiments – Mollusks

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Outline

- Calcification in Mollusks – Overview and Background
- Indirect Measures of Calcification
  - Larval Shell growth (Light microscopy, Image Analysis, SEM)
  - Alkalinity Anomaly Method (net calcification as a function of changes in TA)
- Measuring Ca Directly
  - Inductively Coupled Plasma/Optical Emissions Spectrophotometry (ICP/OES)
- Present some of our research on larval oyster growth and calcification in mesohaline conditions: (18 ppt, TA ~1225 μmol/kg-SW)
Calcification in Mollusks - Bivalves
Bivalve anatomy

- Periostracum
- Hinge
- Mouth
- Anterior adductor muscle
- Foot
- Ctenidia (gills)
- Shell
- Posterior adductor muscle
- Exhalant siphon
- Inhalant siphon
- Mantle
Bivalve in cross-section

Jacob et al. 2008
Components of calcification in mollusks

- Mineralization environment is isolated from outside world
- Extracellular, biologically controlled, process in mollusks, bryozoans, some foraminifera, etc.
- Includes creation of an organic matrix:
  - Site of nucleation and mineralization
  - Stabilizing environment for amorphous calcium carbonate (transient precursor to aragonite)

Figure 1. Life cycle of the eastern oyster, Crassostrea virginica.
Onset of calcification ~20 h post-fertilization

Wallace 2001

Figure 1. Life cycle of the eastern oyster, Crassostrea virginica.
Figure 1. Life cycle of the eastern oyster, Crassostrea virginica.
Early Embryo – Trochophore (first 20 hrs)

- Ectodermal cells in shell gland produce initial periostracum (outermost organic shell layer)
- Shell gland turns inside out and becomes mantle epithelium
- Onset of Calcification
- Mantle epithelium produces:
  - Periostracum/ organic matrix
  - Calcification of shell (lengthening & thickening)

Weiss et al. 2002
Biologically controlled, extracellular mineralization

Mantle epithelial cells
Extrapallial space
Site of mineralization (outside cell)

Weiner and Dove 2003
Biologically controlled, extracellular mineralization

Addadi et al. 2006

Amorphous CaCO₃??

Addadi et al. 2006
Model of shell forming organic matrix

Organic Matrix cartoon

- Chitin structure
- Silk fibroin gel
- Glycoproteins (nucleation sites)
Organic Matrix cartoon

- Chitin structure
- Silk fibroin gel
- Glycoproteins (nucleation sites)
- Transient colloidal Amorphous CaCO3
Nucleation and aragonite crystallization occur at expense of ACC → Biomineral

Biomineralized aragonite with ACC and glycoprotein occlusions
Amorphous CaCO$_3$ as transient precursor

- ACC is unstable – tends toward spontaneous crystallization
- Glycoproteins and Mg stabilize ACC (inhibit crystallization)
- Glycoproteins also serve as nucleation sites for initiation of crystal growth
- Gel filled organic matrix controls orientation and extent of crystallization

Schematic model of nacre formation

Addadi et al. 2006

(After mineralization)

(After mineralization)
Larval Shell Architecture

D-stage or Prodissoconch I

Umbo-stage or Prodissoconch II

Carriker & Palmer 1979
Amorphous CaCO$_3$ precursor to aragonite

- Larval *Mercenaria mercenaria* and *Crassostrea gigas*
- Applied the multiple techniques to shell cross-sections to determine crystalline and amorphous CaCO$_3$ forms:
  - Polarized light microscopy
  - Infrared spectroscopy
  - Raman imaging spectroscopy
  - Scanning Electron microscopy
- Evidence of ACC to aragonite transformation

Weiss et al. (2002)
Ultrastructure of *Mercenaria* larval shell

3 days old

9 days old

Weiss et al. (2002)
Mercenaria larval shell etched in DI water

Weiss et al. (2002)
Crassostrea gigas – 9 days old

Weiss et al. (2002)
C. virginica - 28 days old

- Larval shell cross-sections
- Clear prismatic and granular layers, suggests presence of crystalline aragonite and ACC
Comparative Oyster Larval Experiments in Mesohaline Environments (2)

*Crasostrea ariakensis*  *Crasostrea virginica*

Amanda Reynolds, Cristina Sobrino, Fritz Riedel – SERC
Mark Luckenbach and Stephanie Bonniwell – VIMS Eastern Shore Lab
Hypotheses

- Increased CO$_2$ will make carbonate less bioavailable and calcification energetically more costly
- Oyster larvae grown under high CO$_2$ conditions will grow and calcify more slowly
  - Larval shells will be smaller
  - Larval shells will contain less CaCO$_3$
- Effects will be similar for *Crassostrea virginica* and *C. ariakensis*
Experimental Conditions
(Summer in the Chesapeake Bay)

- Salinity = 18 ppt
- TA titration (1225 μmol/kg-SW) to set pH targets
- Temperature = 25°C
- Light/Dark cycle = 14hr/10hr
- Diet = Isochrysis galbana (controlled amount daily)
- Water changed every 48hrs
- pCO₂ adjusted continuously/ pH tracked hourly
- DIC measured every 2-3 days (pCO₂ tracking)
- Treatment targets: (280), 380, 560, 800 matm CO₂
Experimental Treatments

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<tr>
<th>280 Low &lt;1800</th>
<th>380 Ambient 2008</th>
<th>560 Mid 2050</th>
<th>800 High 2100</th>
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- Each aquarium inoculated with 15,000 three day old oyster larvae
CO₂ Control and Delivery

- 1% CO₂
- Solenoid/valve
- Soda lime
- Air Pump
- pH controllers
- Incubator with aquaria
Hourly pH readings/ continuous CO₂ control
Hourly pH Control Conditions
(Targets: 800 = 7.70; 280 = 8.10)

Time (Hrs)

pH (NBS)
Mean Hourly pH (+/- 1 SD) over 574 h
(Targets: 800μatm = 7.70; 280μatm = 8.10)
C. virginica veligers

D-Stage Larva

Umbo Stage Larva
Mean shell height (± 1 SEM) by age (C. virginica; n = 30 larvae/treatment)
Mean shell height (± 1 SEM) by age (C. ariakensis; n = 30 larvae/ treatment)
Image Analysis (Scion Image)
Area measurements
Cumulative size frequency of larval shells @ 30 d (μm²/shell; n= 205/replicate)

Miller et al. 2009
Inductively Coupled Plasma/Optical Emission Spectrophotometry used for detection of trace metals - Ca
ICP/OES procedure

- Tissue removed by exposure to weak bleach solution (2.5%) and agitation for 10-15 mins
- Shells rinsed with DI water to remove bleach and salts
- Known no. shells dissolved in trace metals grade HCl and diluted to known volume
- ICP/OES used to determine mean Ca content per shell
- Mean CaCO$_3$ calculated.
Shell area and per capita CaCO$_3$ mass

(A) *Crassostrea virginica*

(B) *Crassostrea ariakensis*

(C)

(D)

Miller et al. 2009
Conclusions

- Eastern oysters showed differences in growth and calcification at varied $\text{CO}_2$
- Suminoe oysters showed no significant $\text{CO}_2$ effects
- When $\Omega_{\text{arag}} < 1.0$, both species have net growth and calcification, indicating some degree of biological resiliency to acidification
Alkalinity Anomaly Method  
(Smith and Key 1975)

- Estimating net rates of calcification ($G$) by measuring changes in TA

\[ G = -\frac{\Delta TA}{2} \]

- Precipitation of 1 mole of CaCO$_3$ consumes 2 moles of HCO$_3^-$, decreasing TA by 2 equivalents

- In mollusks, respiration and calcification cause changes in pH and pCO$_2$

- Method is sensitive and can be applied in incubations that last only a few hours (e.g., Gazeau et al. 2007)
Scanning Electron Microscopy

*C. virginica* larva, 250X
280 μatm treatment
Transition between Prodissoconch I and Prodissoconch II
What does dissolution look like?

- *C. pyramidata*, pteropod
- $\Omega_{\text{arag}} < 1.0$ for 48 hrs

Orr et al. 2005

- $\Omega_{\text{arag}} = 0.6$
- *M. mercenaria*
- Juvenile infauna

Green et al. 2009
SEM Lab at Smithsonian NMNH
No obvious evidence of deformities or severe dissolution (*C. virginica*)

Preindustrial, 280 µatm  
Year 2100, 800 µatm
Measuring Growth Rings

- 120 SEM photographs:
  - 60 photos each for both 280µatm and 800µatm treatments
- Measured the number of rings and the increments between rings
Measuring Growth Rings
Number of growth rings

- No difference in number of growth rings between treatments

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<th>280 µatm</th>
<th>800 µatm</th>
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<tr>
<td>Mean</td>
<td>22.879</td>
<td>23.933</td>
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<td>Standard Deviation</td>
<td>2.318</td>
<td>2.544</td>
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- Mean approximates the length of experiment, 28 days
  - Suggests daily growth rings
- Adult and larval daily growth lines in many bivalves
  - Growth rings correlate with tides, seasons, environmental conditions, diurnal changes
Mean Growth Increment

- **p** = 0.0001
- **t** = 4.753
- **n** = 60

C. virginica CO₂ Treatment (µatm)
Cumulative growth increment distributions

![Graph showing cumulative percentage distribution with size (μm) on the x-axis and cumulative percentage on the y-axis. The graph compares two distributions labeled 'Cumulative 280' and 'Cumulative 800'.]
Importance of Incremental growth

- Small differences in increments can lead to large differences in area
- Increment differences may account for the observed 16% decrease in area
Potentially important sites of energy expenditure
Implications & Questions

- Species react differently/ Different evolutionary histories and differences in environmental variability?
- Slower larval growth → longer time in the water column → greater vulnerability to predation and disease → greater pre-settlement mortality
- Does larval experience affect metamorphosis success and post-settlement growth and survival?
- Acidification may alter species interactions:
  - competition, predation, parasitism
  - community assembly
  - invasibility of benthic habitats by non-native species