Measuring Calcification in Biological Experiments – Mollusks

OCB/ EPOCA Ocean Acidification Course

Woods Hole, MA

November 2 - 12, 2009

Whitman Miller

Smithsonian Environmental Research Center

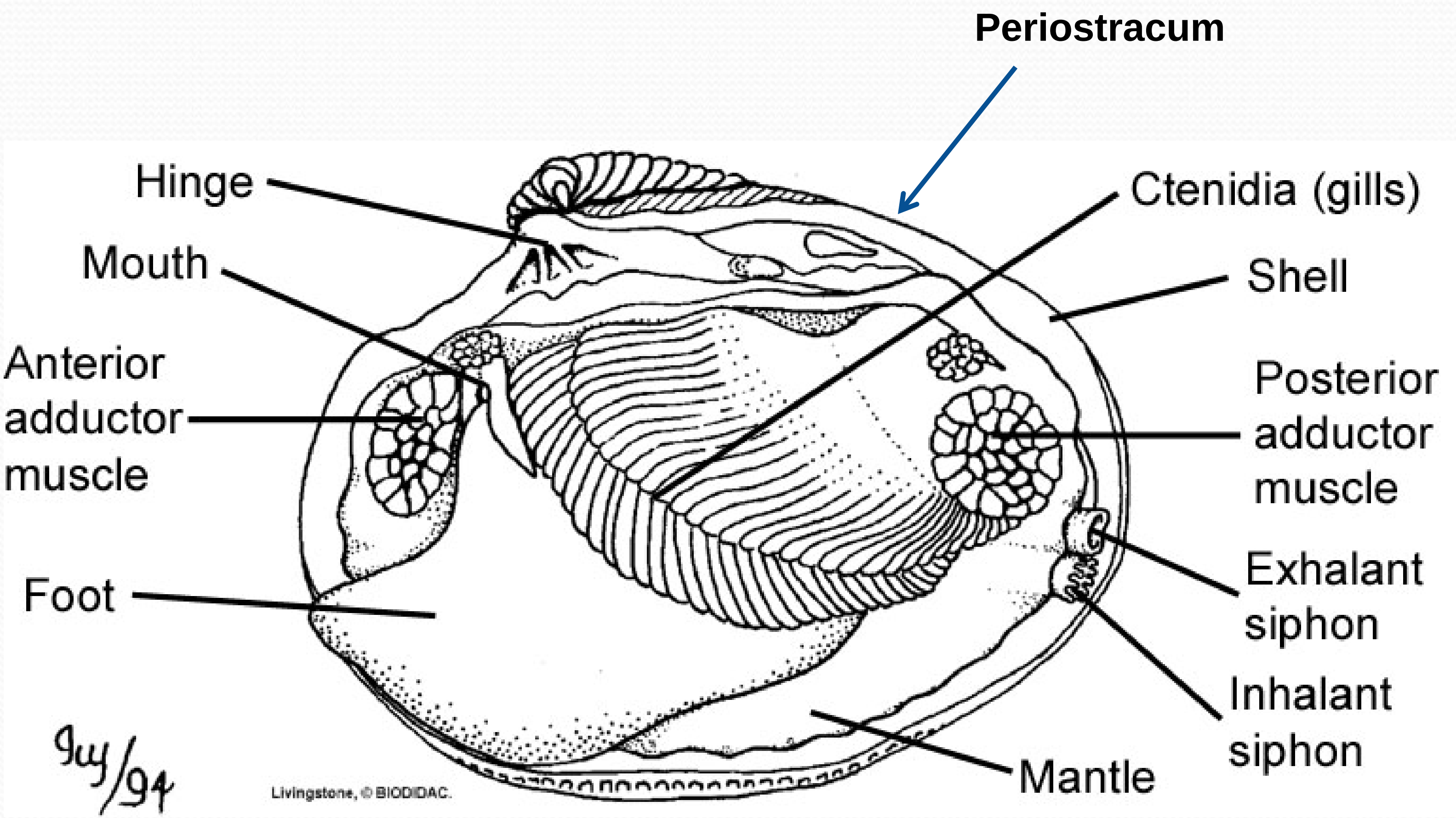
Edgewater, MD

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)

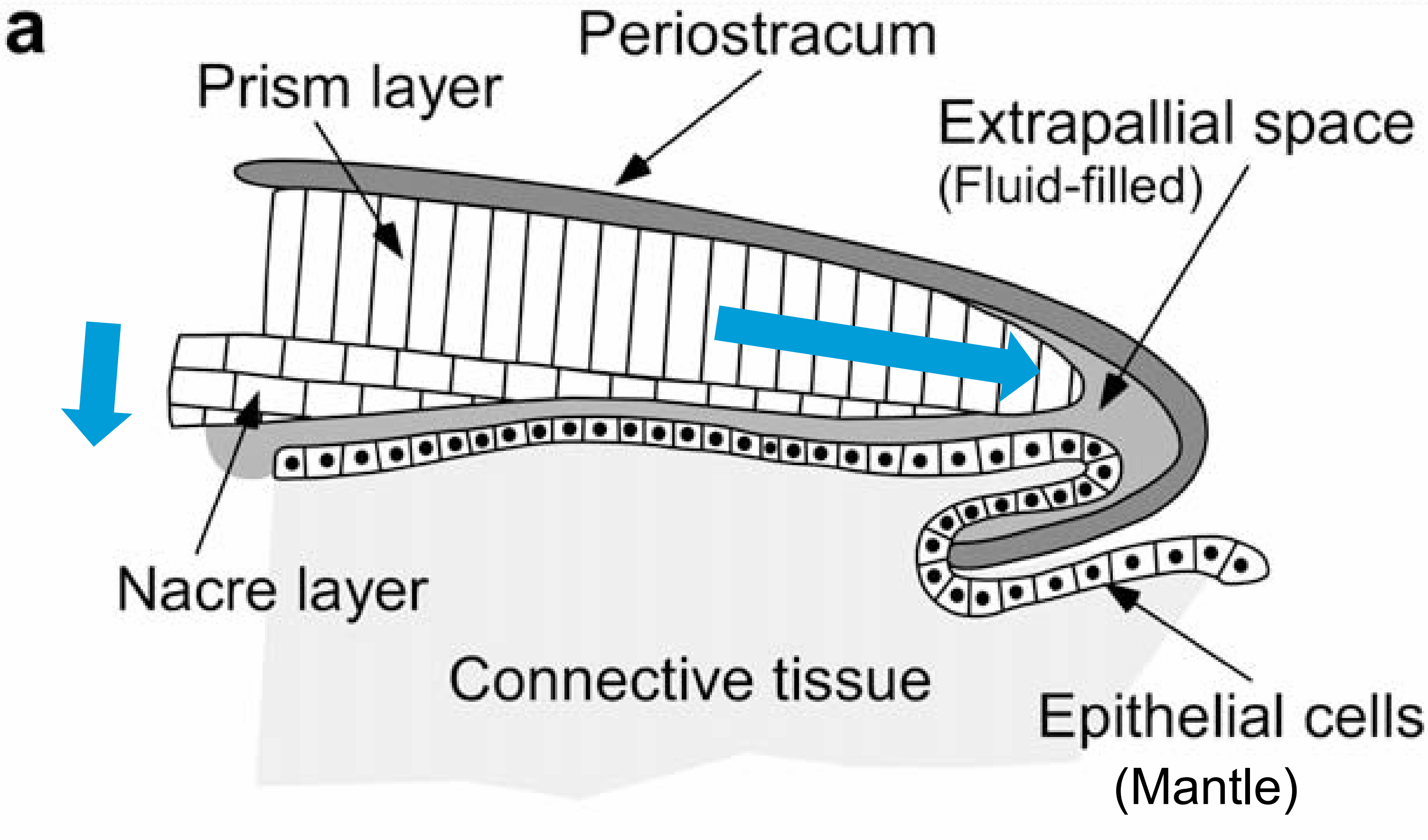
Calcification in Mollusks - Bivalves

Bivalve anatomy



www.marlin.ac.uk/.../Bivalvia.jpg

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)

Weiss et al. 2002, Weiner and Dove 2003

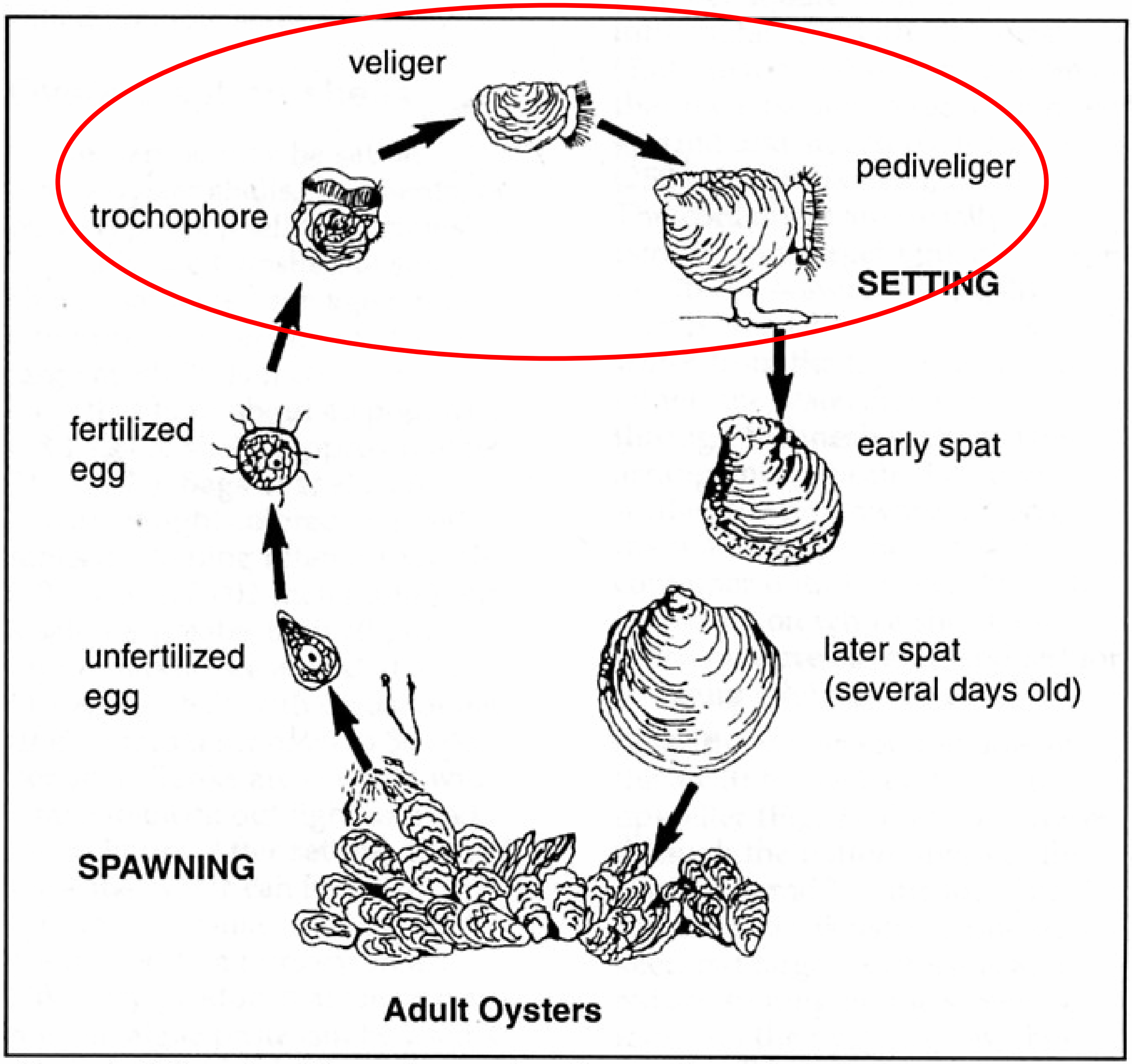
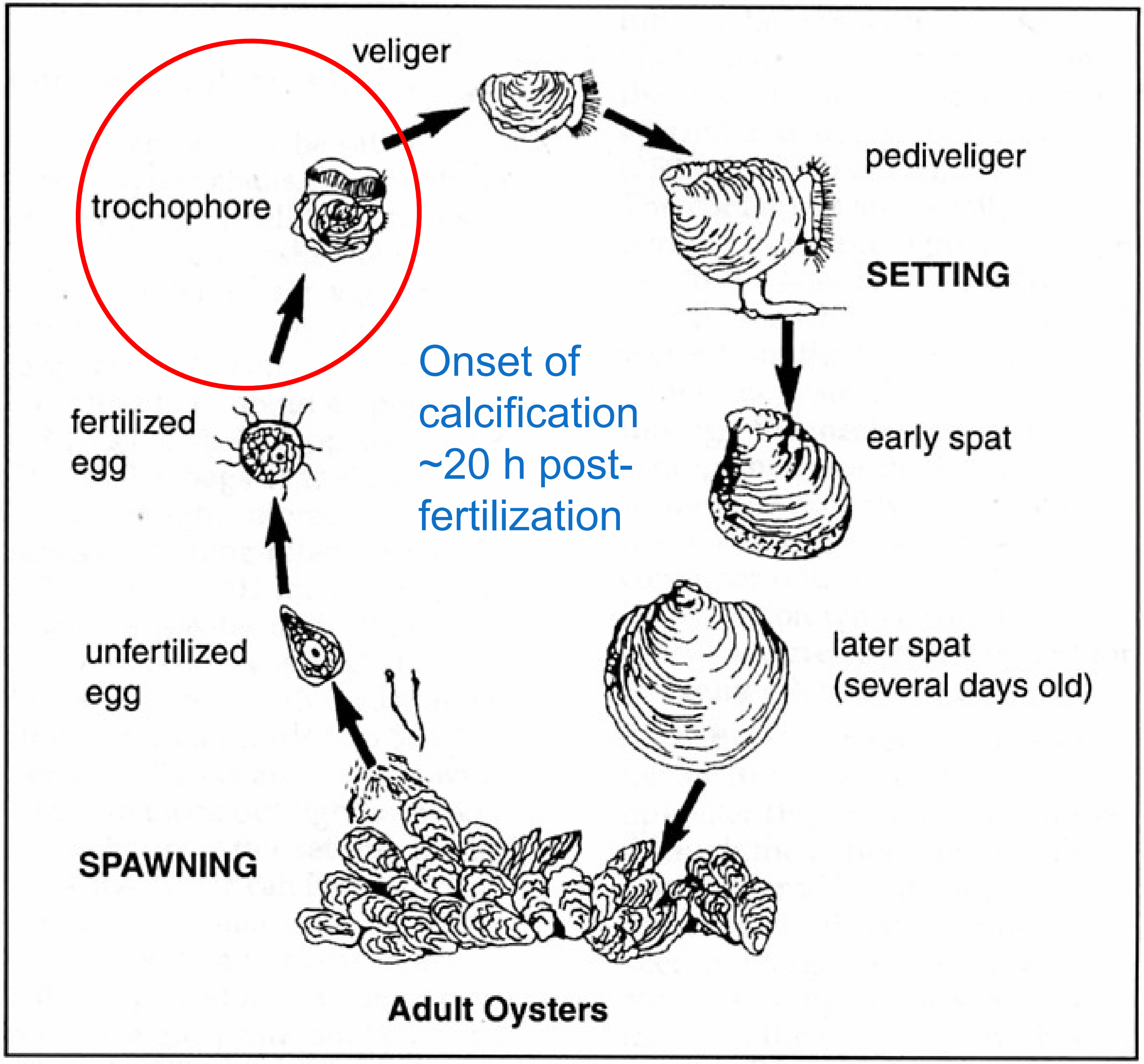


Figure 1. Life cycle of the eastern oyster, Crassostrea virginica.

Wallace 2001



Onset of calcification ~20 h post-fertilization

Figure 1. Life cycle of the eastern oyster, Crassostrea virginica.

Wallace 2001

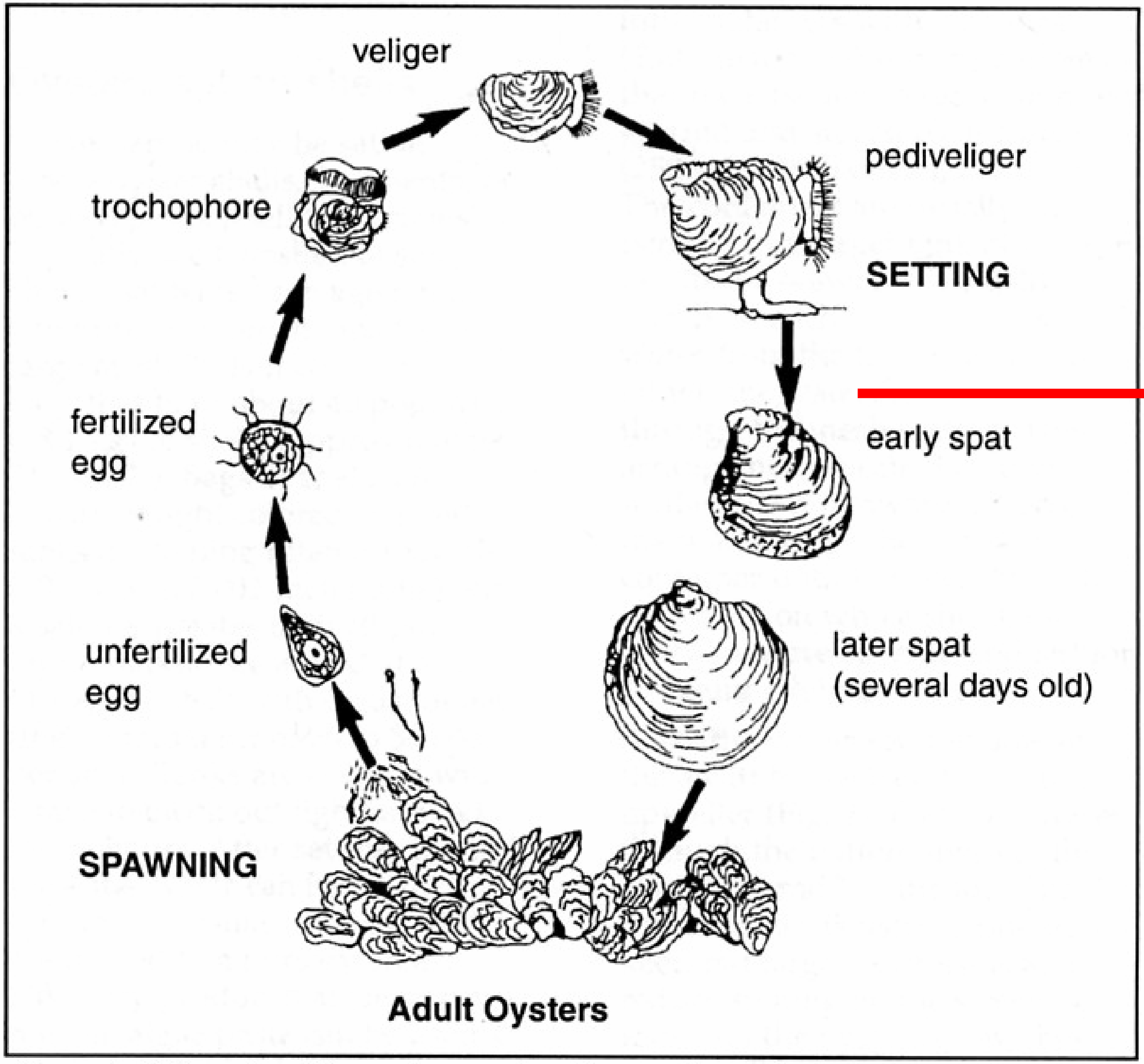


Figure 1. Life cycle of the eastern oyster, Crassostrea virginica.

Aragonite

Calcite

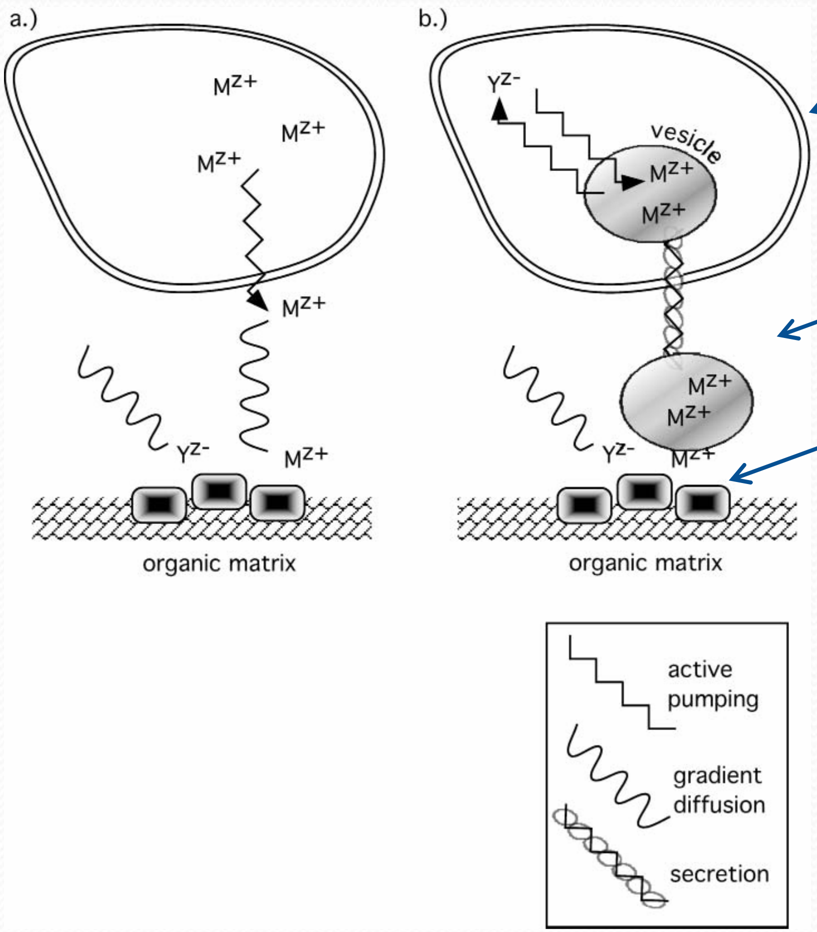
Wallace 2001

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



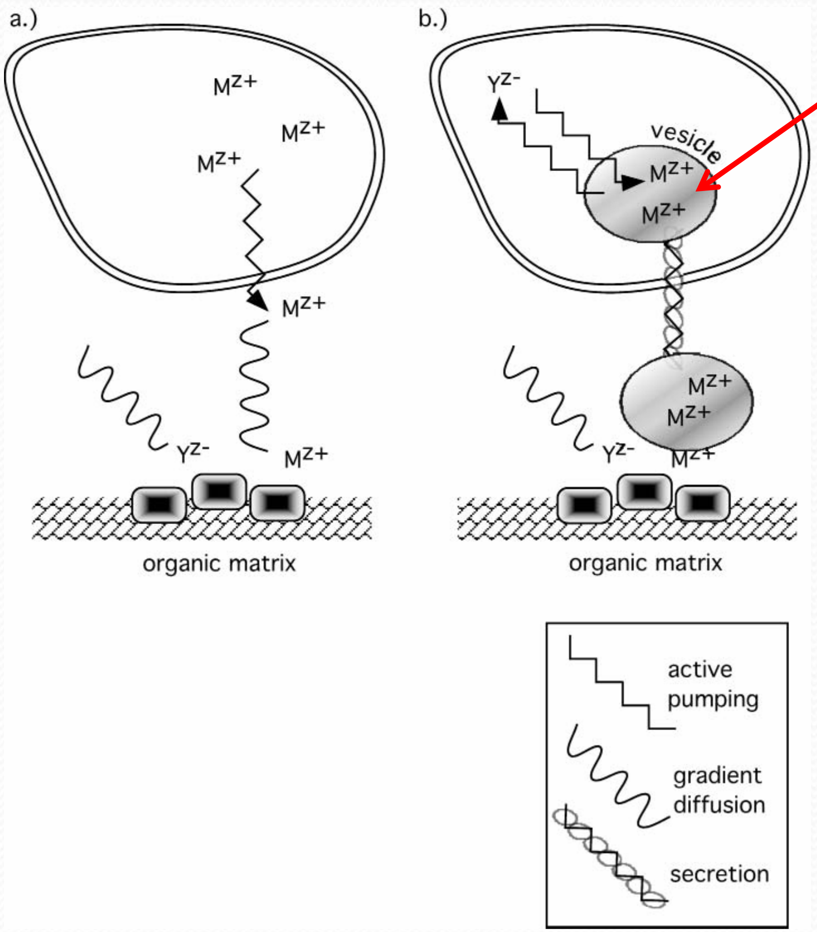
Mantle epithelial cells

Extrapallial space

Site of mineralization (outside cell)

Weiner and Dove 2003

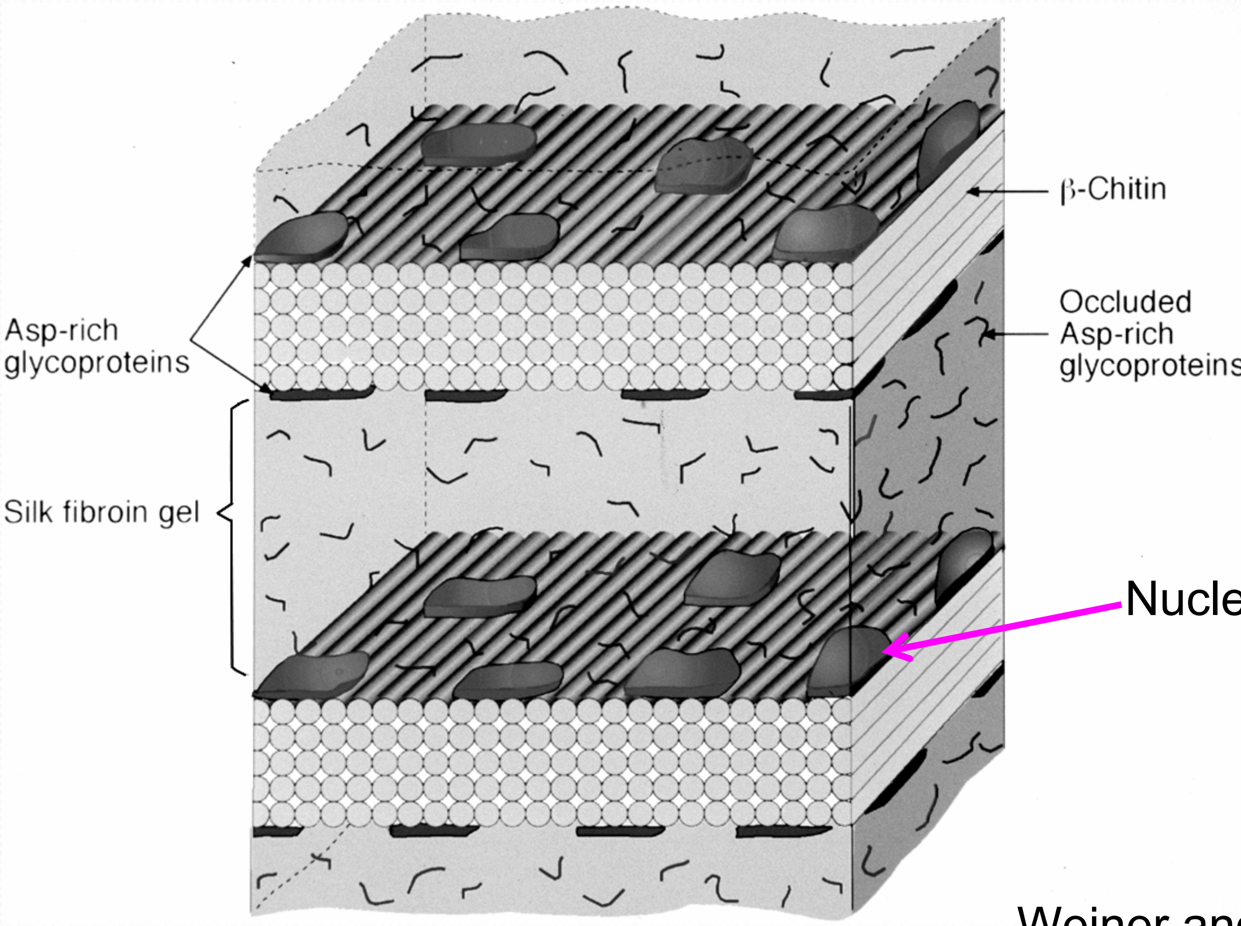
Biologically controlled, extracellular mineralization



Amorphous $CaCO_3$??

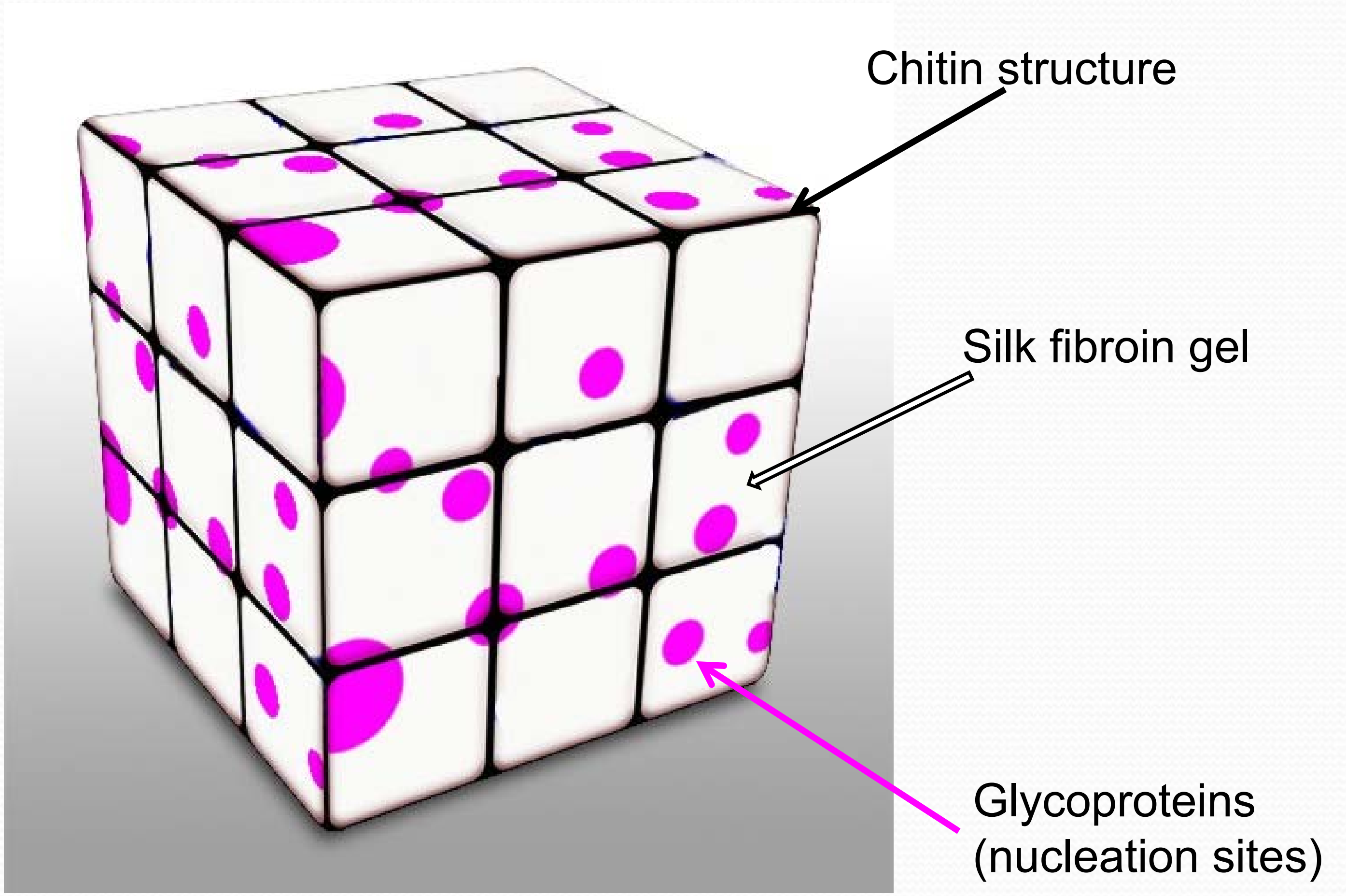
Addadi et al. 2006

Model of shell forming organic matrix

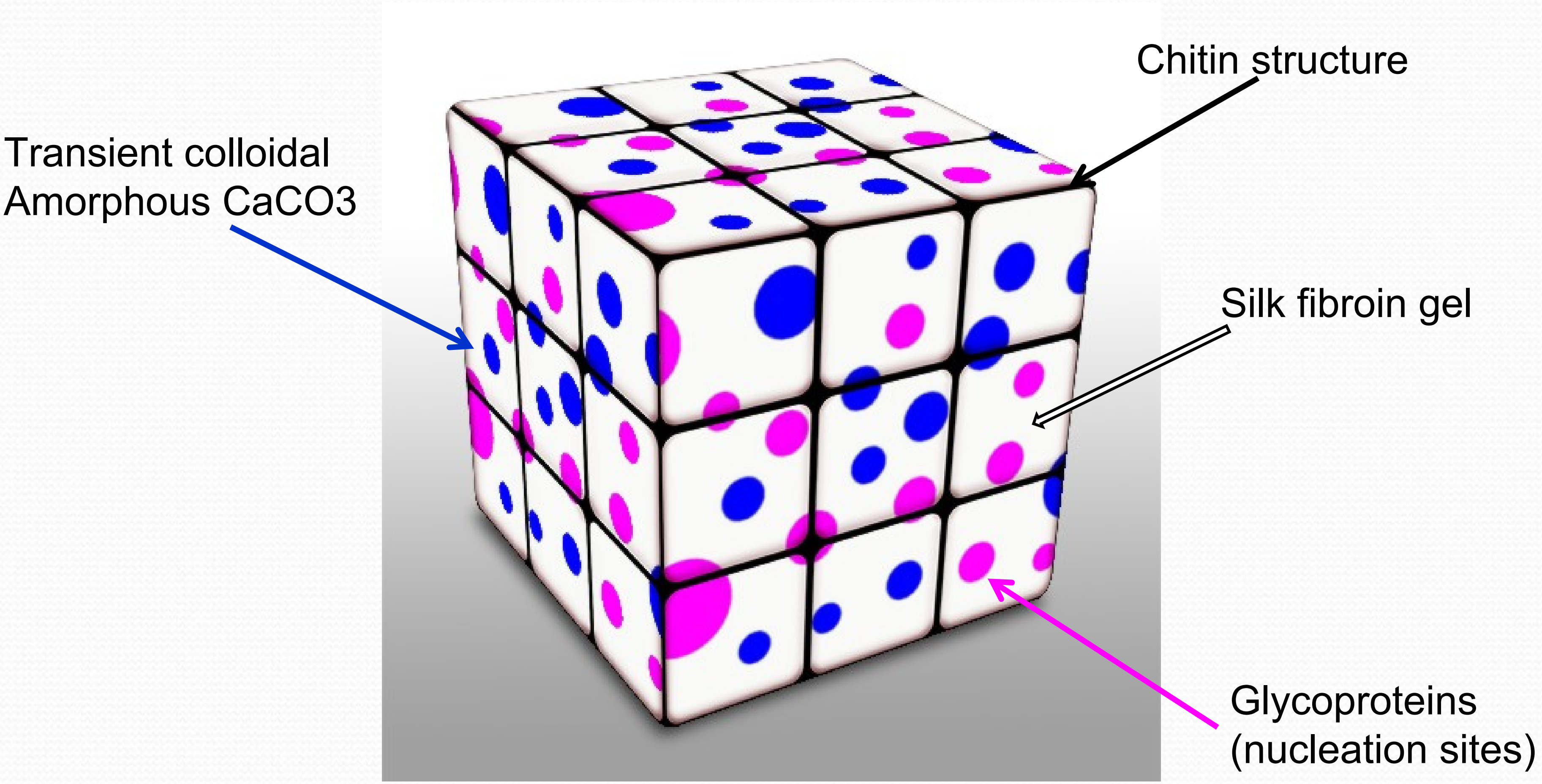


Weiner and Dove 2003,
Levi-Kalisman et al. 2001

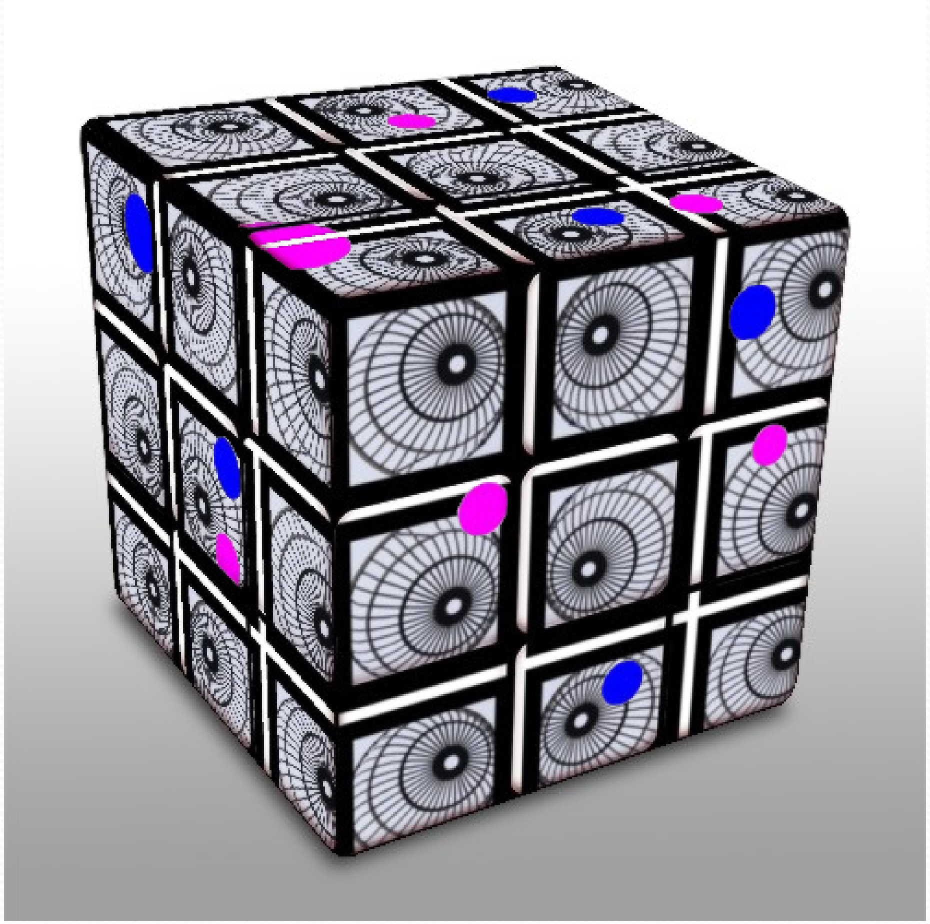
Organic Matrix cartoon



Organic Matrix cartoon



Nucleation and aragonite crystallization occur at expense of ACC ⟶ Biomineral



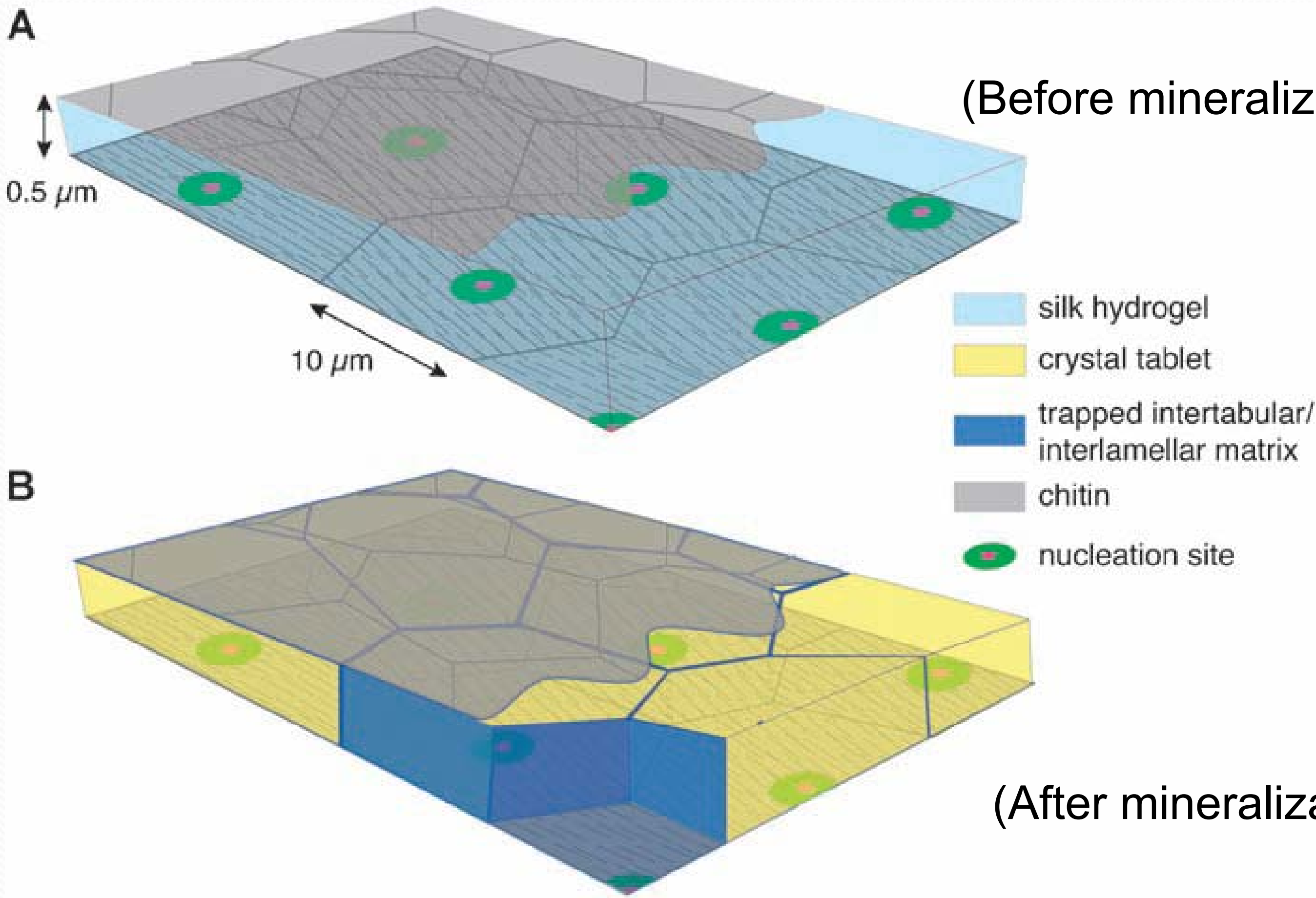
Biomineralized aragonite with ACC and glycoprotein occlusions

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

Levi-Kalisman et al. 2001, Weiss et al. 2002, Weiner and Dove 2003

Schematic model of nacre formation

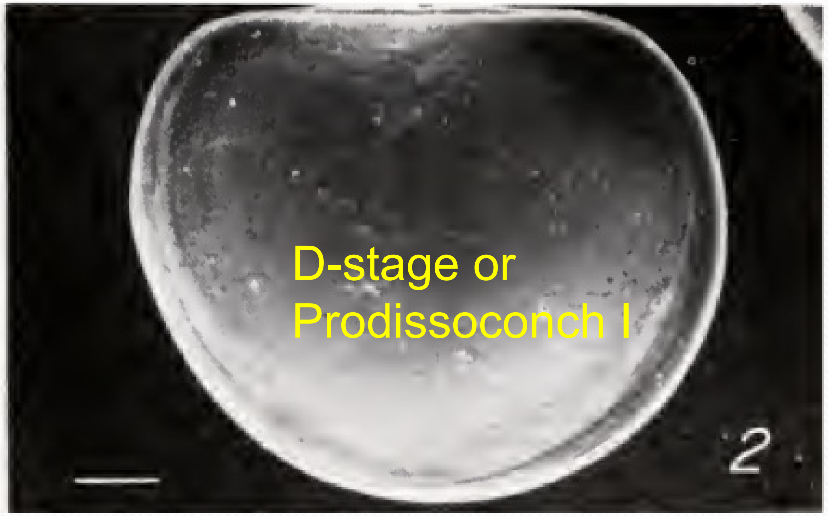


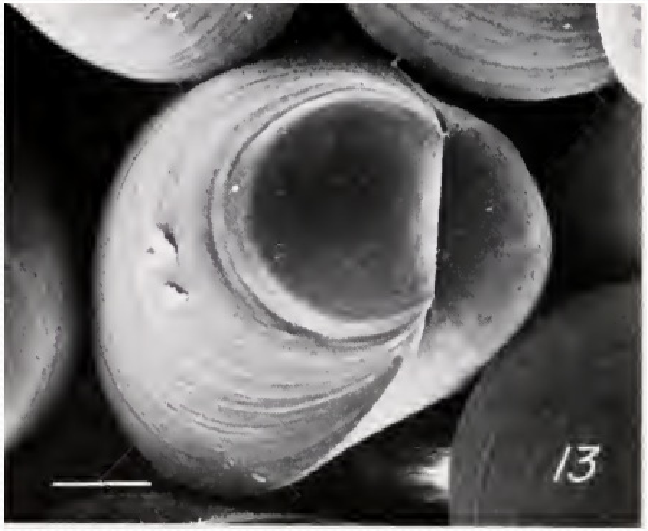
(Before mineralization)

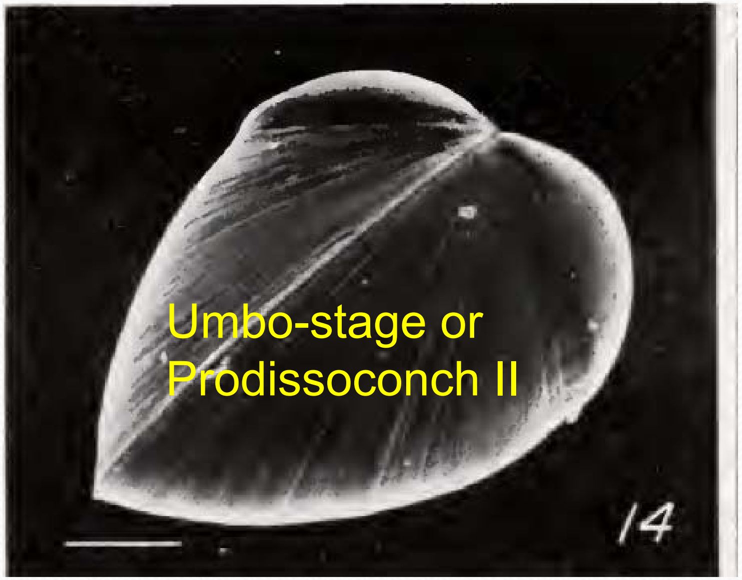
(After mineralization)

Addadi et al. 2006

Larval Shell Architecture







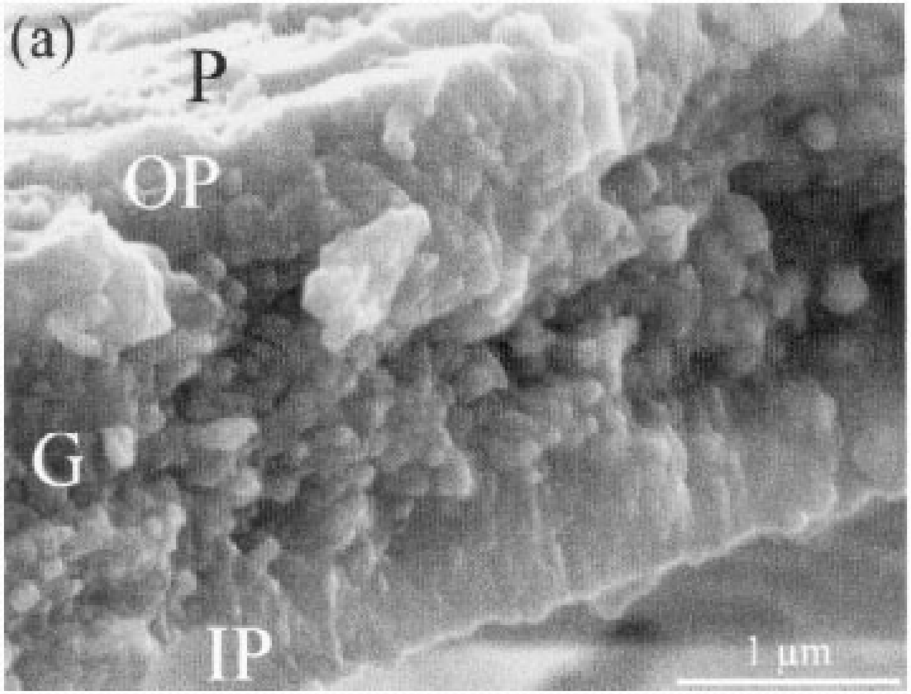
Carriker & Palmer 1979

Amorphous $CaCO_3$ precursor to aragonite

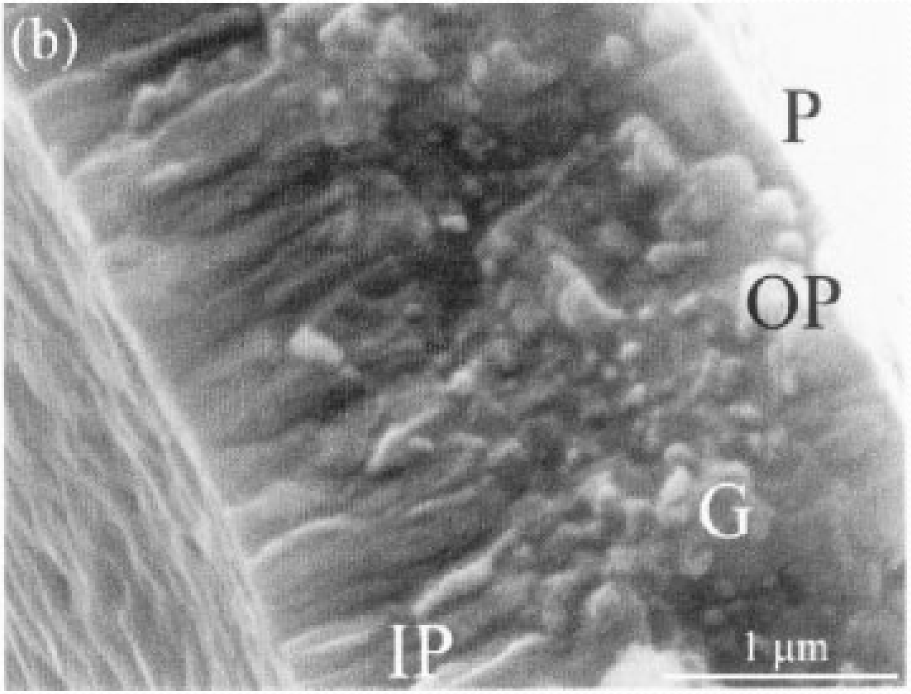
- Larval *Mercenaria mercenaria* and *Crassostrea gigas*
- Applied the multiple techniques to shell cross-sections to determine crystalline and amorphous $CaCO_3$ forms:
  - Polarized light microscopy
  - Infrared spectroscopy
  - Raman imaging spectroscopy
  - Scanning Electron microscopy
- Evidence of ACC to aragonite transformation

Weiss et al. (2002)

Ultrastructure of *Mercenaria* larval shell



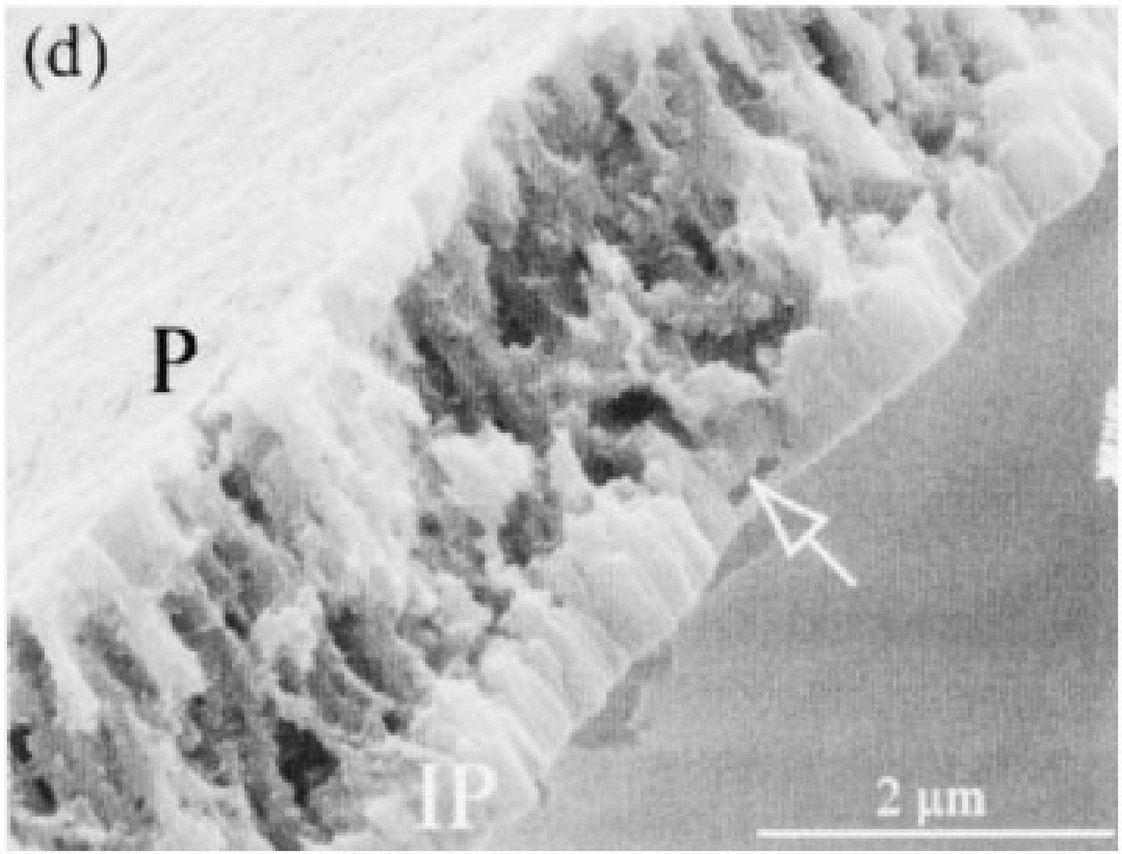
3 days old



9 days old

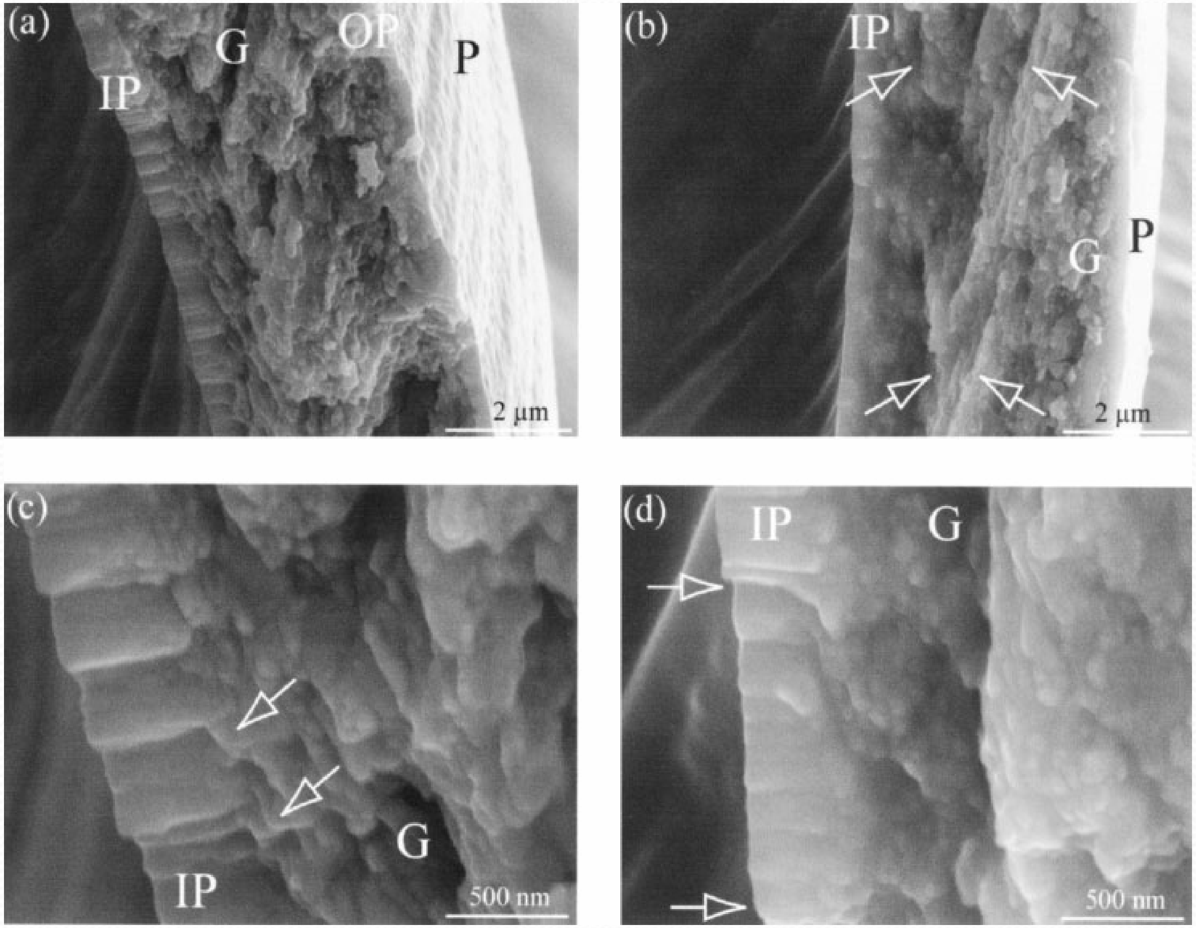
Weiss et al. (2002)

Mercenaria larval shell etched in DI water



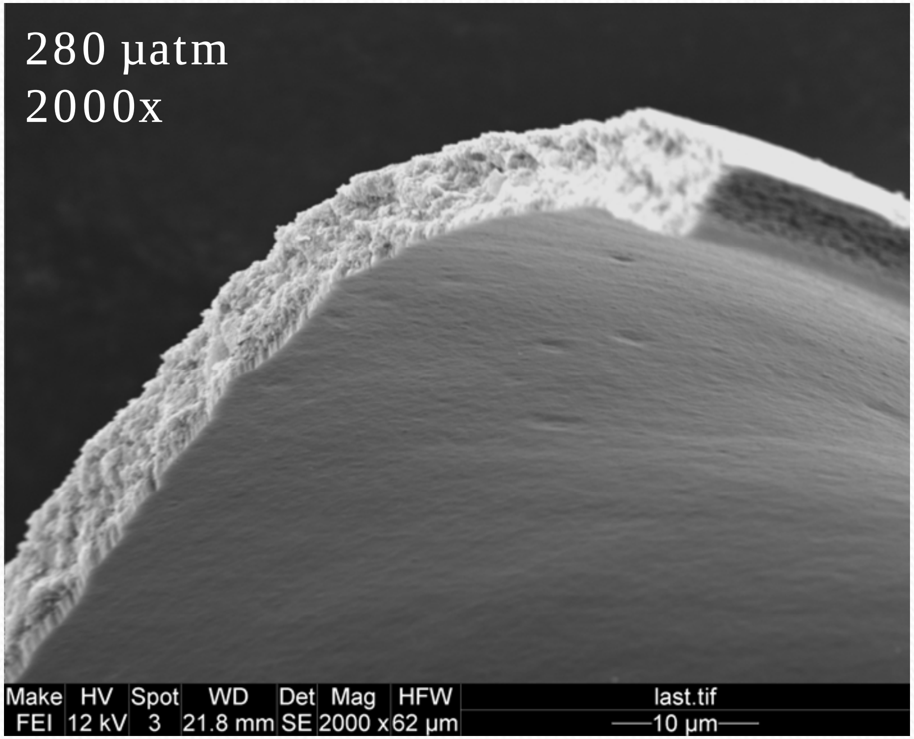
Weiss et al. (2002)

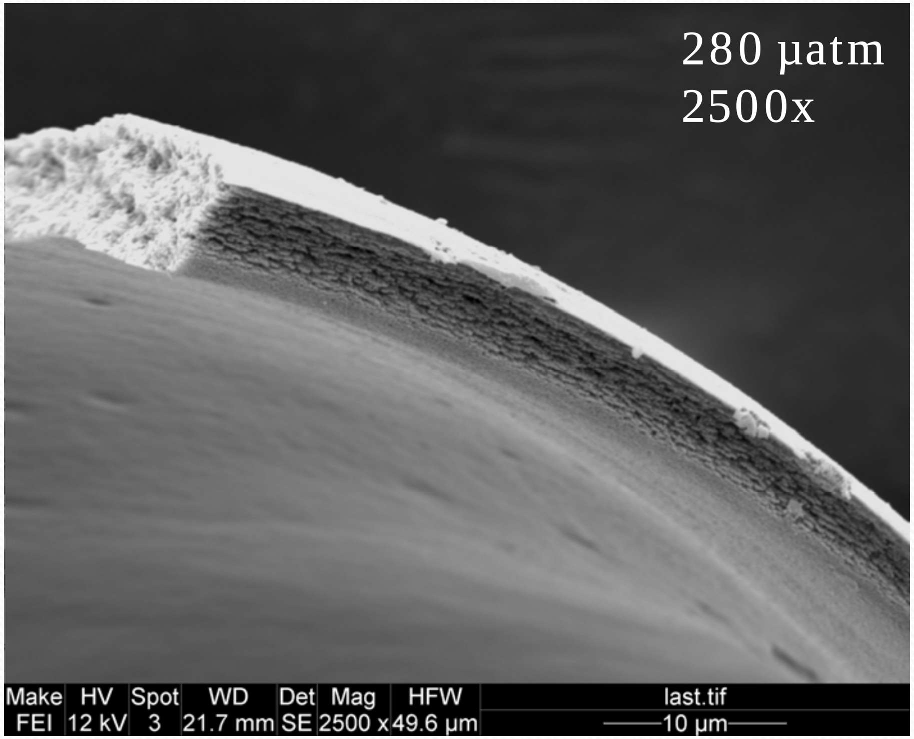
Crassostrea gigas – 9 days old



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)

Crassostrea ariakensis

Crassostrea 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)

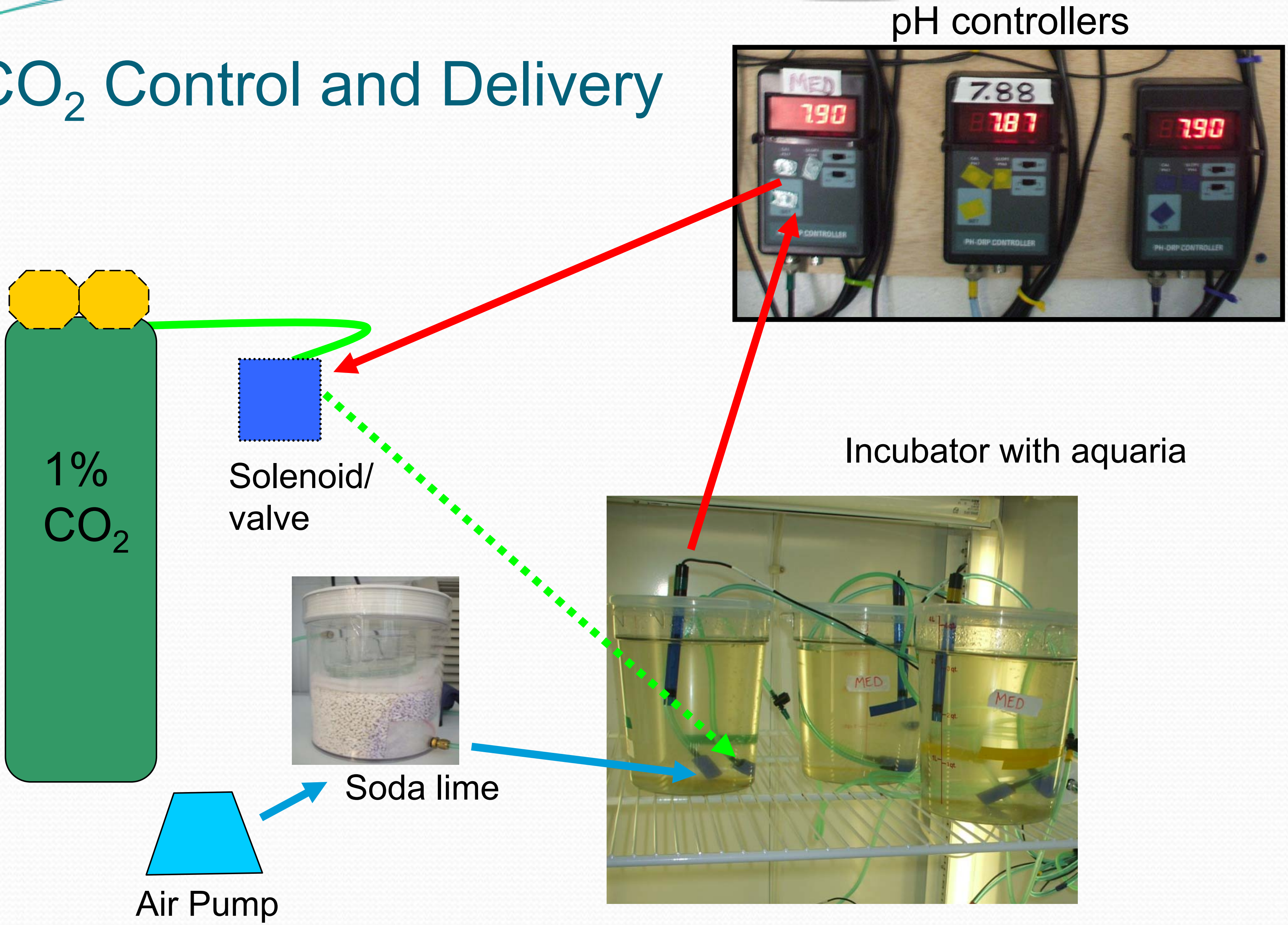
- Salinity = 18 ppt
- TA titration (1225 µmol/kg-SW) to set pH targets
- Temperature = 25°C
- Light/Dark cycle = 14hr/10hr
- Diet = *Isochrysis galbana* (controlled amount daily)
- Water changed every 48hrs
- pCO_2 adjusted continuously/ pH tracked hourly
- DIC measured every 2-3 days (pCO_2 tracking)
- Treatment targets: (280), 380, 560, 800 matm CO_2

Experimental Treatments

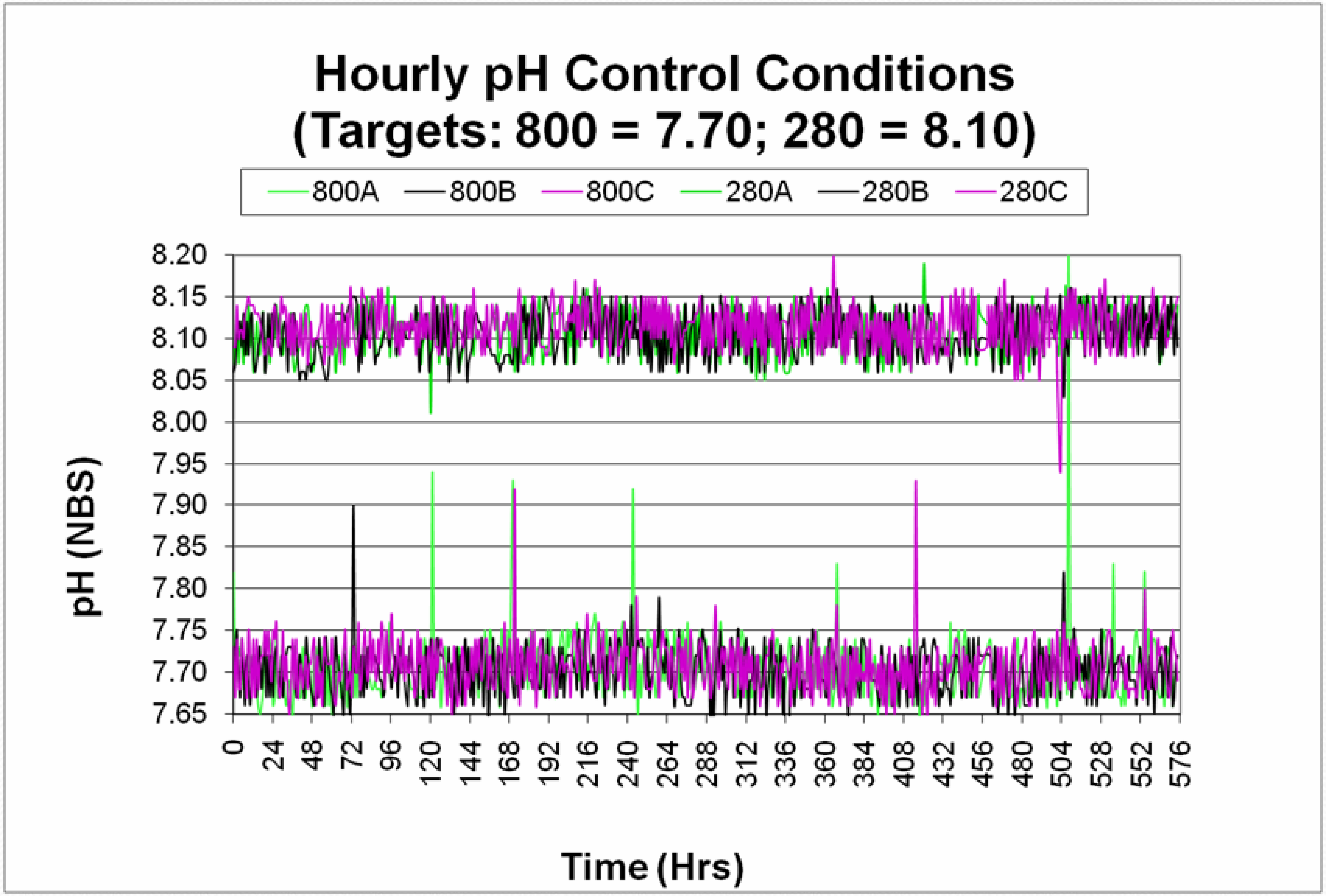
280 Low <1800	380 Ambient 2008	560 Mid 2050	800 High 2100
A	A	A	A
B	B	B	B
C	C	C	C

- Each aquarium inoculated with 15,000 three day old oyster larvae

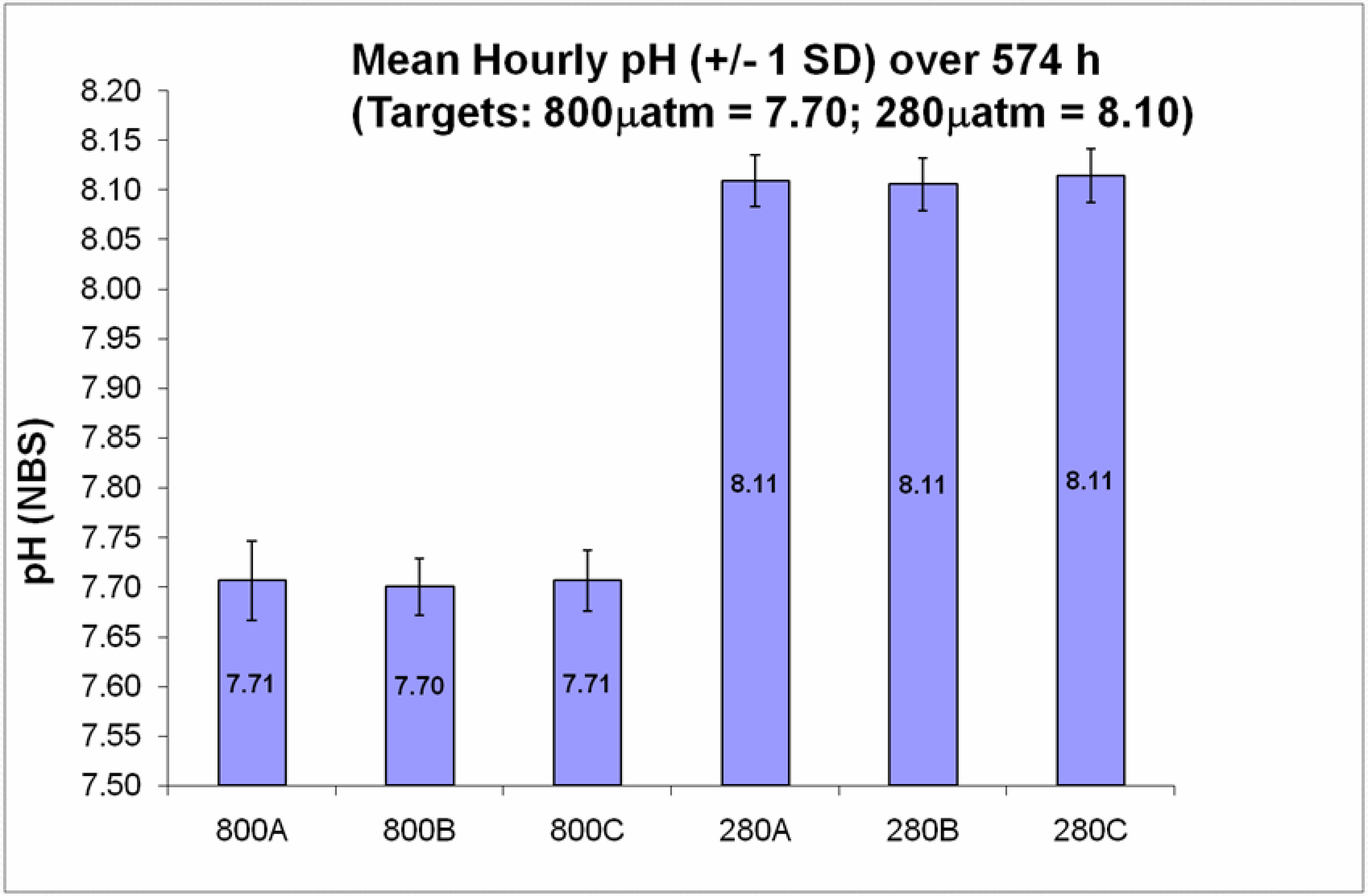
CO_2 Control and Delivery



Hourly pH readings/ continuous CO_2 control

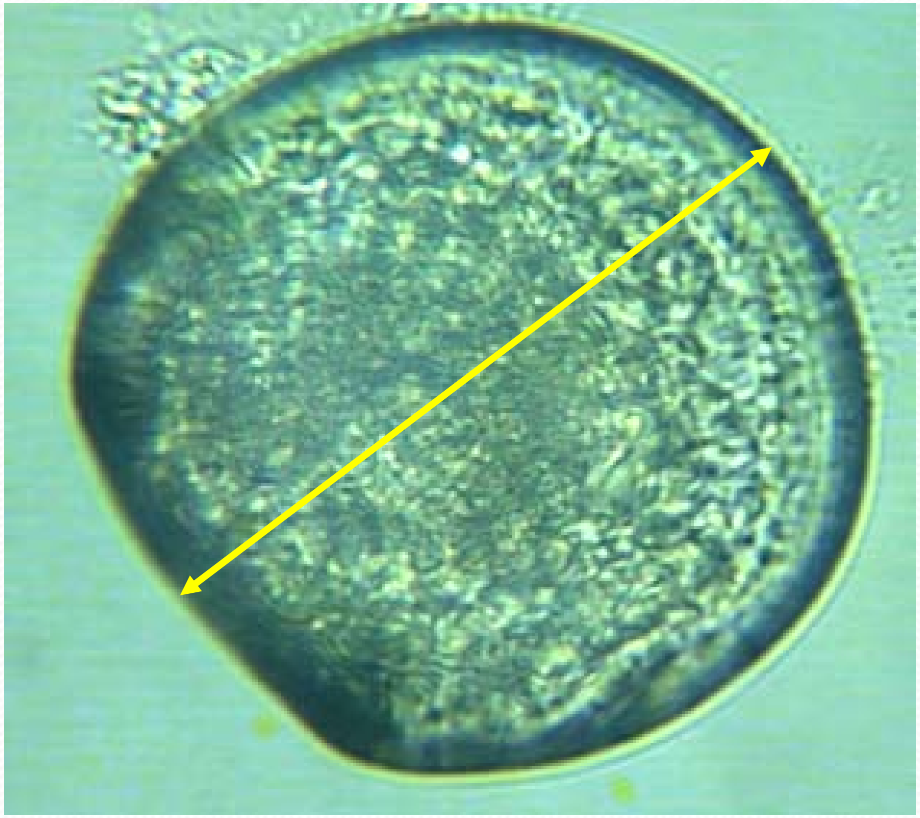


Hourly pH Control Conditions
(Targets: 800 = 7.70; 280 = 8.10)
800A
800B
800C
280A
280B
280C
8.20
8.15
8.10
8.05
8.00
7.95
7.90
7.85
7.80
7.75
7.70
7.65
pH (NBS)
0
24
48
72
96
120
144
168
192
216
240
264
288
312
336
360
384
408
432
456
480
504
528
552
576
Time (Hrs)

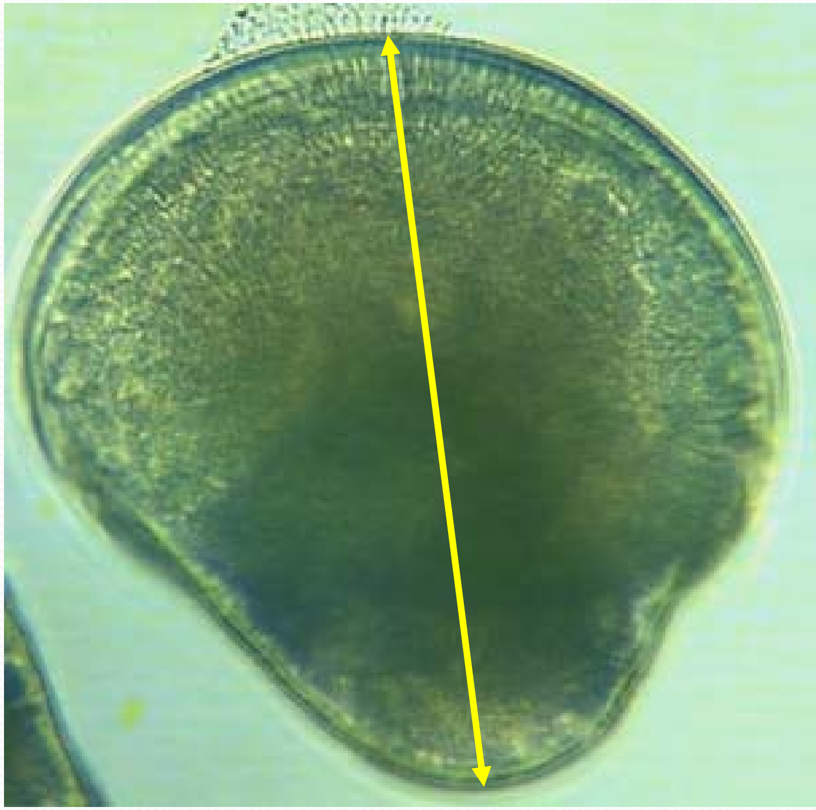


Mean Hourly pH (+/- 1 SD) over 574 h
(Targets: 800μatm = 7.70; 280μatm = 8.10)
pH (NBS)
8.20
8.15
8.10
8.05
8.00
7.95
7.90
7.85
7.80
7.75
7.70
7.65
7.60
7.55
7.50
7.71
7.70
7.71
8.11
8.11
8.11
800A
800B
800C
280A
280B
280C

C. virginica veligers

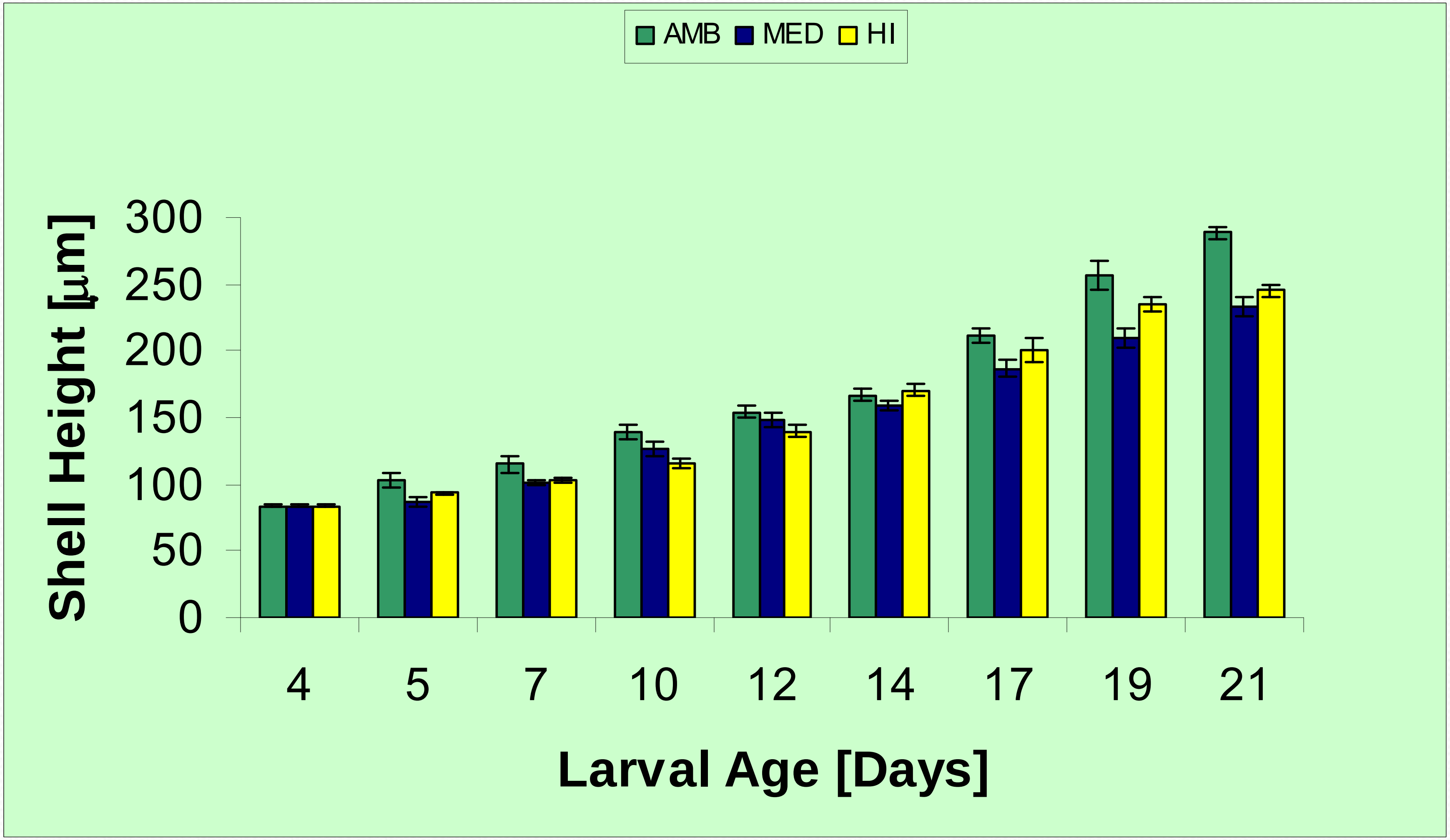


D-Stage Larva



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)

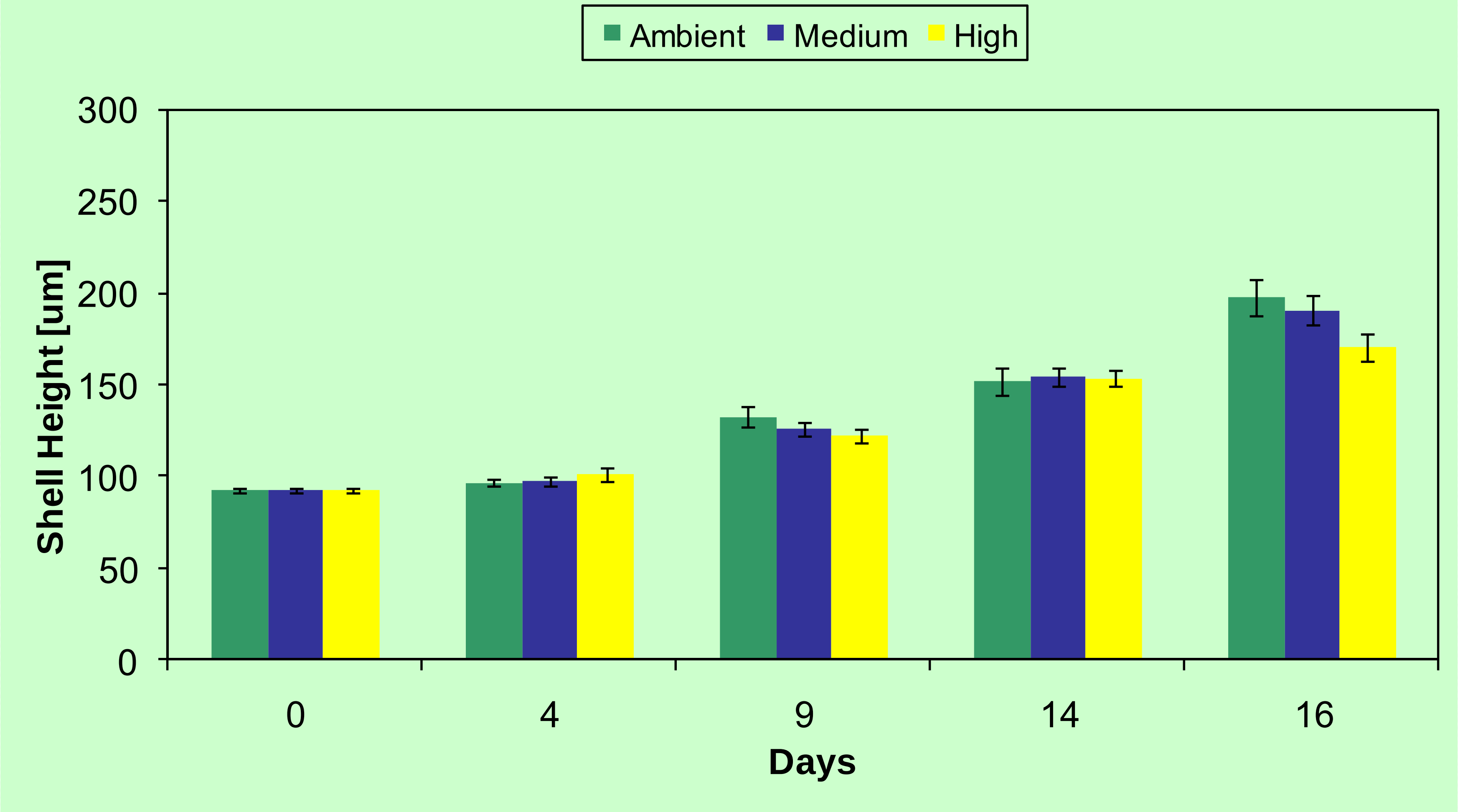
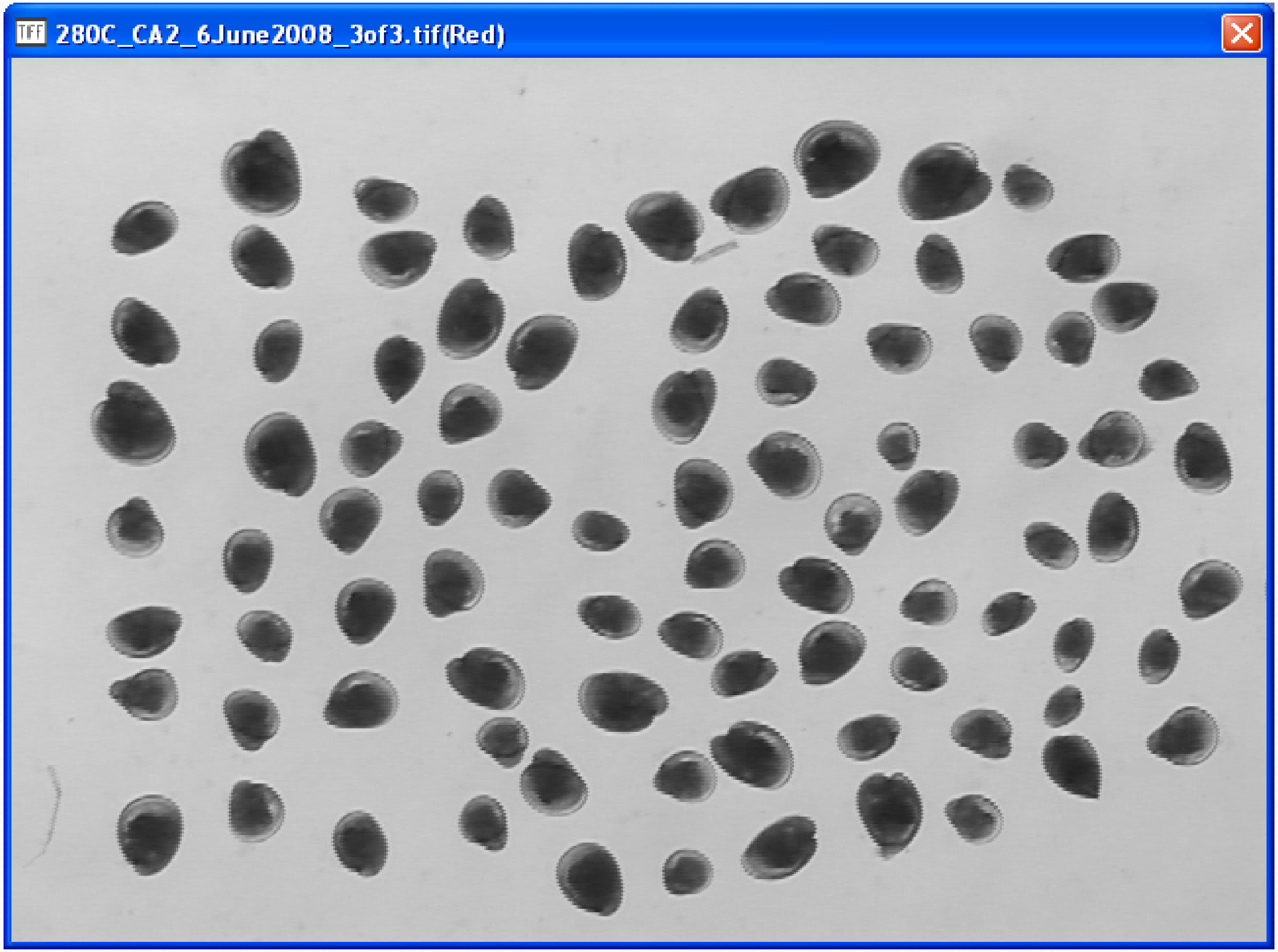
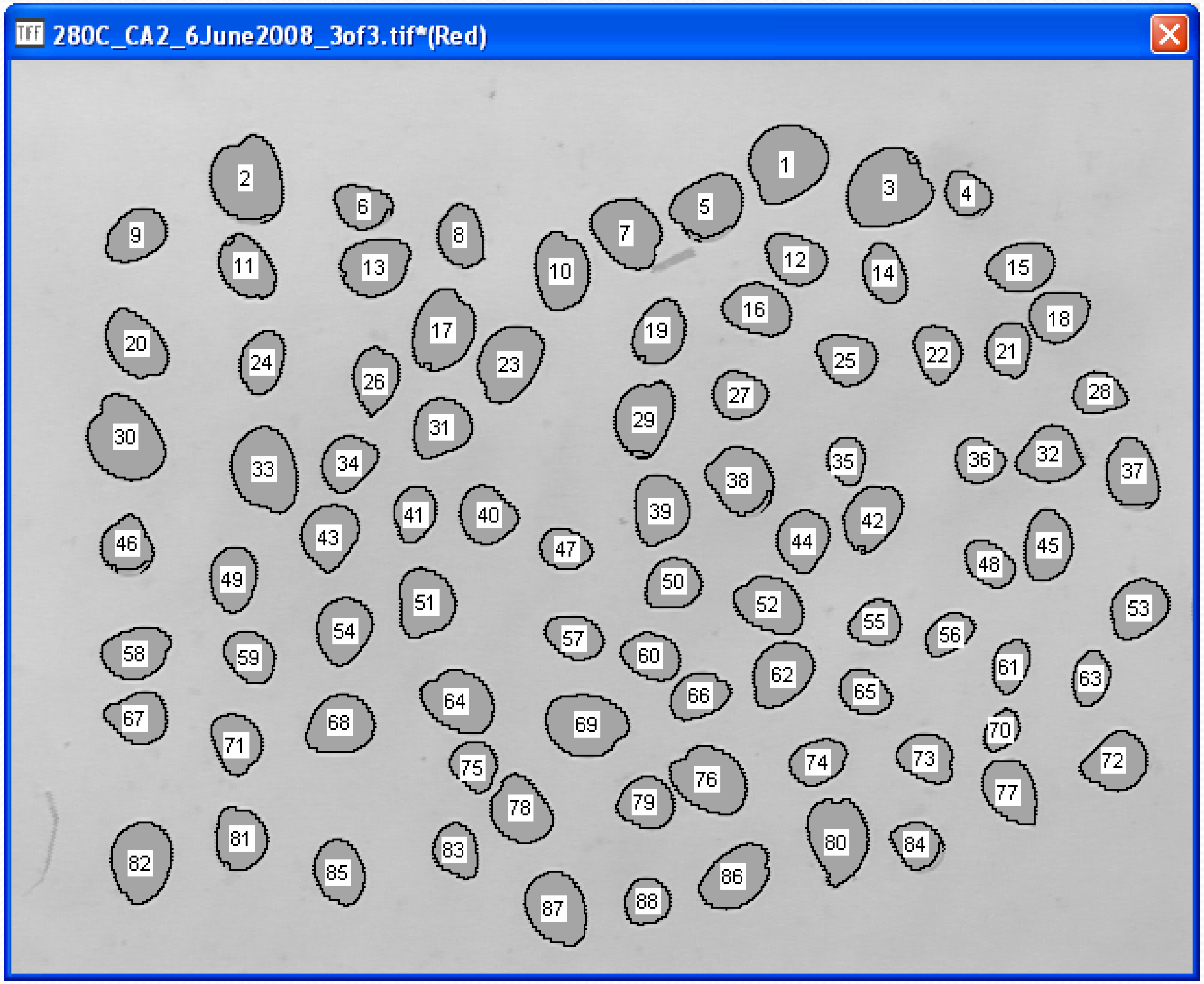


Image Analysis (Scion Image)



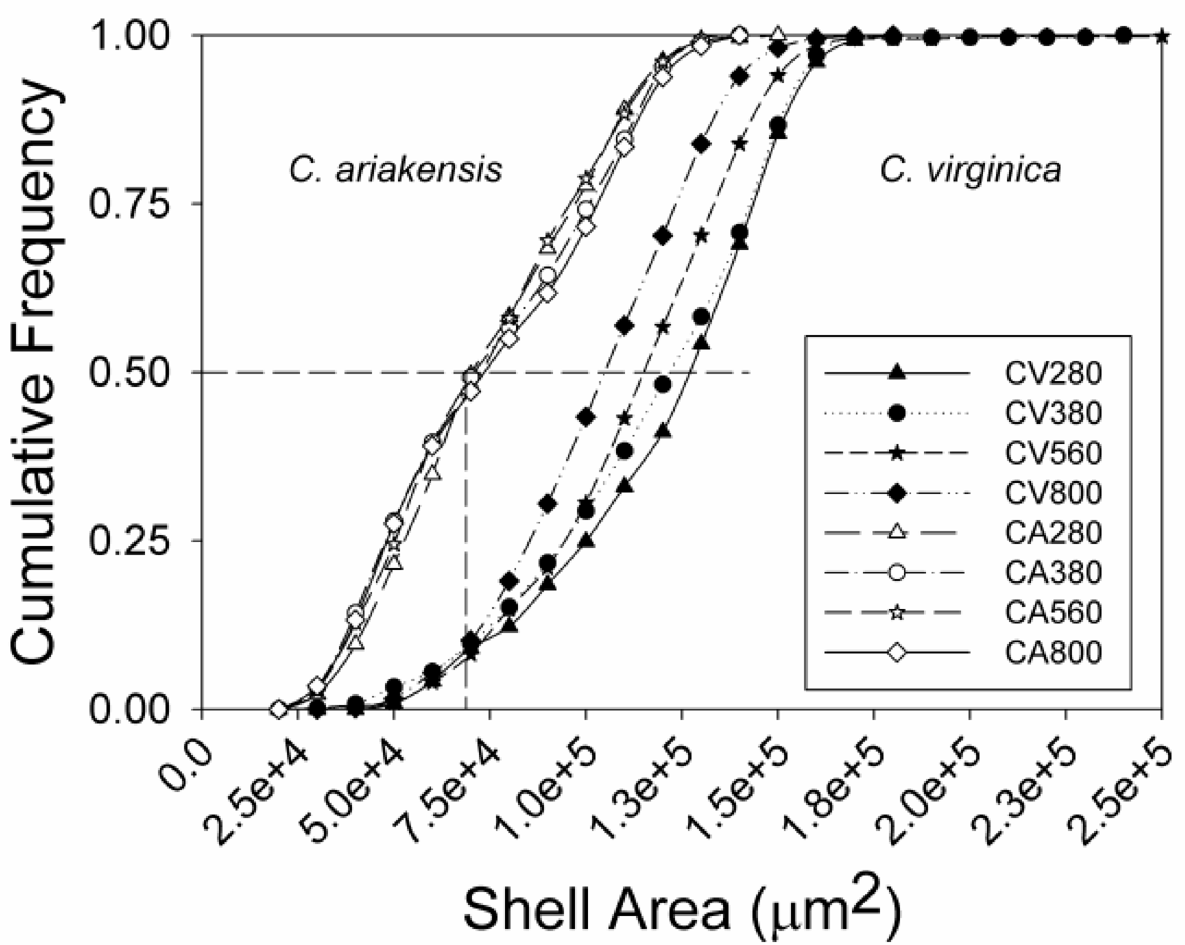
Area measurements



Results

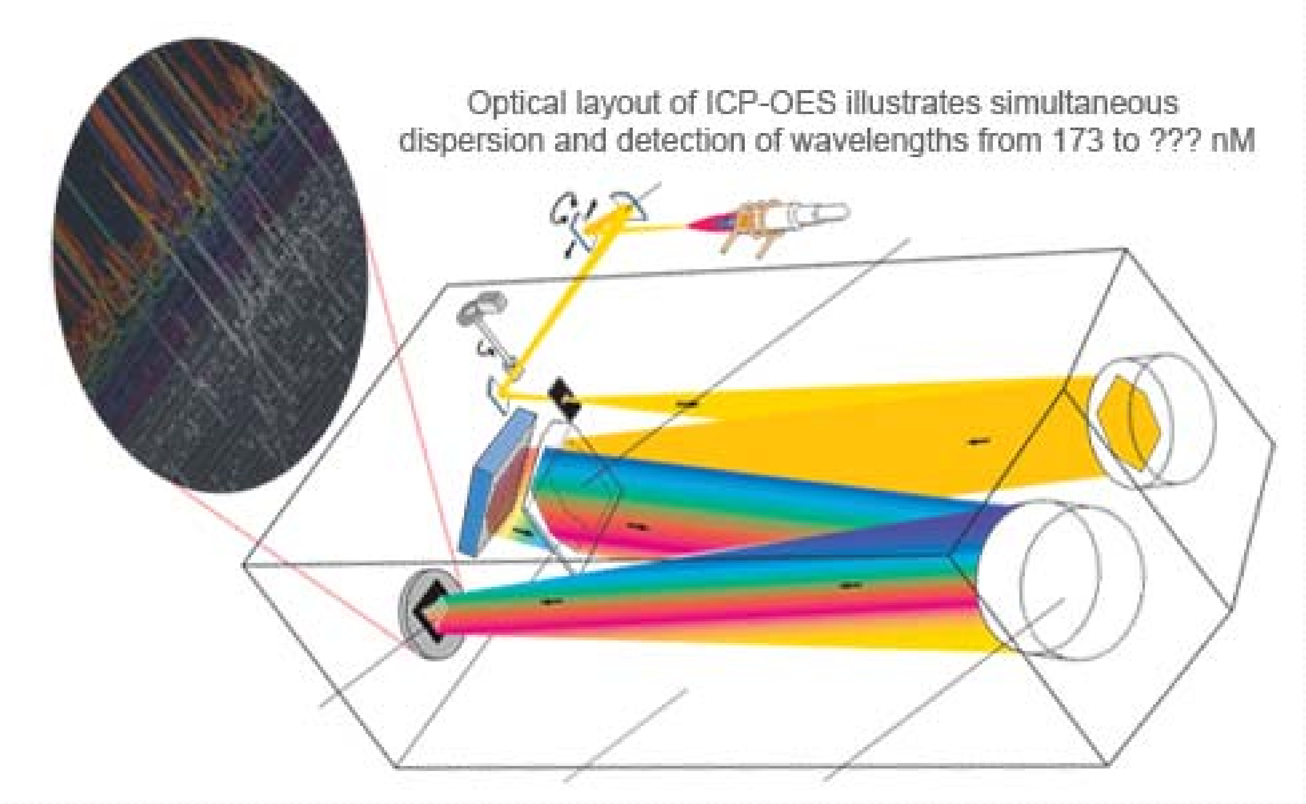
	Area
1.	54784.23
2.	56010.44
3.	59530.87
4.	19303.03
5.	40742.06
6.	23456.35
7.	43352.72
8.	26937.23
9.	29073.22
10.	39318.06
11.	31881.65
12.	28282.11
13.	37419.41
14.	24010.13
15.	30655.43
16.	33186.98
17.	45805.15
18.	29191.88
19.	31130.10
20.	36667.85
21.	23416.80
22.	24999.01
23.	44143.82
24.	26067.01
25.	28242.55
26.	27253.67
27.	24445.24
28.	20766.58
29.	40148.73
30.	56920.22
31.	32435.43
32.	32870.54
33.	51461.57
34.	27926.11
35.	17404.37
36.	21043.47
37.	33780.31
38.	39476.29
39.	36351.41
40.	30220.32
41.	22071.91
42.	34136.31
43.	33740.75
44.	28400.78
45.	32079.43
46.	26067.01
47.	20173.25
48.	20964.36
49.	28598.55
50.	26818.56
51.	35995.41
52.	35599.86
53.	31050.99
54.	34017.64
55.	21518.14
56.	18551.48
57.	23931.02
58.	32751.87
59.	24445.24
60.	25750.56

Cumulative size frequency of larval shells @ 30 d (μm^2/shell; n= 205/replicate)



Miller et al. 2009

Inductively Coupled Plasma/Optical Emission Spectrophotometry used for detection of trace metals - Ca

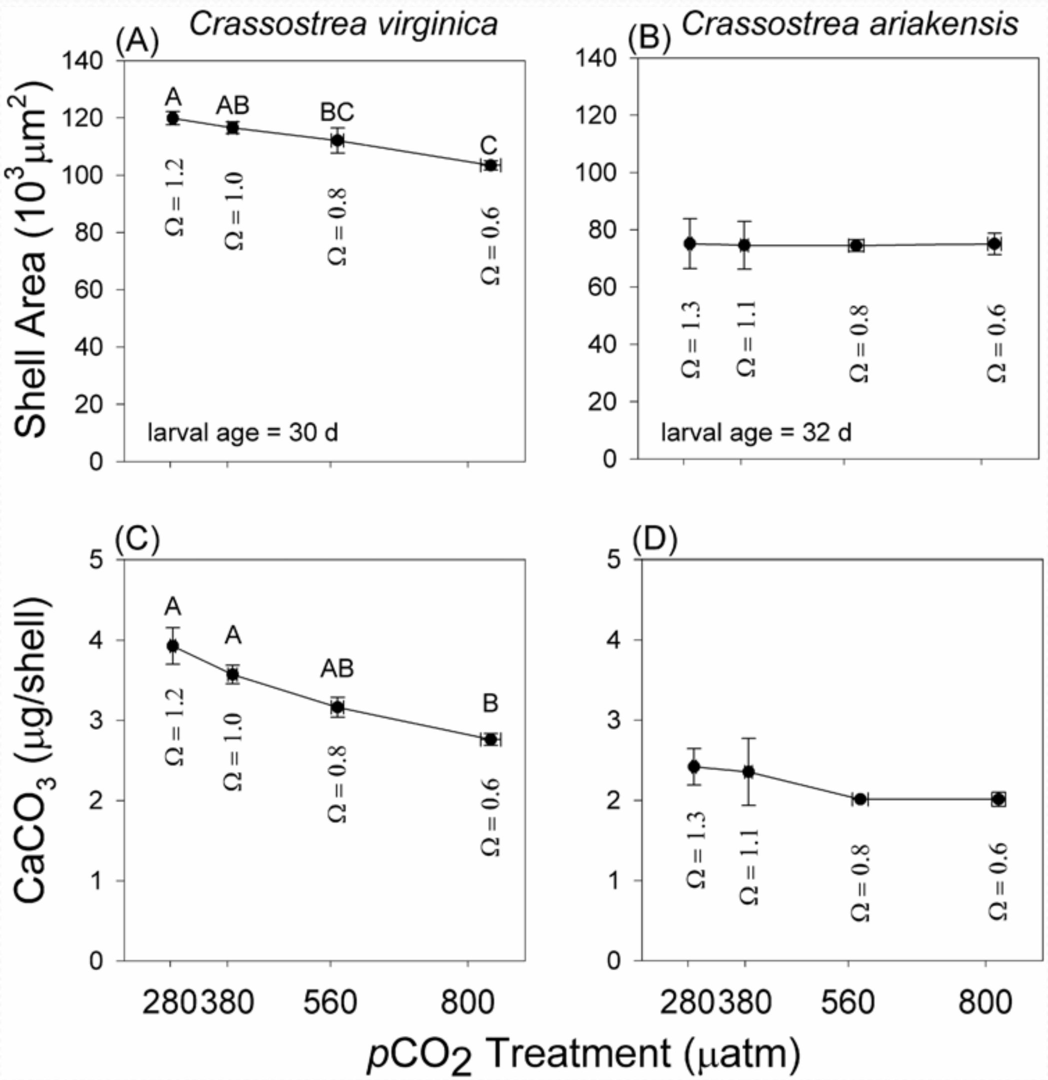


Rutgers Inorganic Analytical Laboratory 2009

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



Miller et al. 2009

Conclusions

- Eastern oysters showed differences in growth and calcification at varied CO_2
- Suminoe oysters showed no significant CO_2 effects
- When $\Omega_{arag} < 1.0$, both species have net growth and calcification, indicating some degree of biological resiliency to acidification

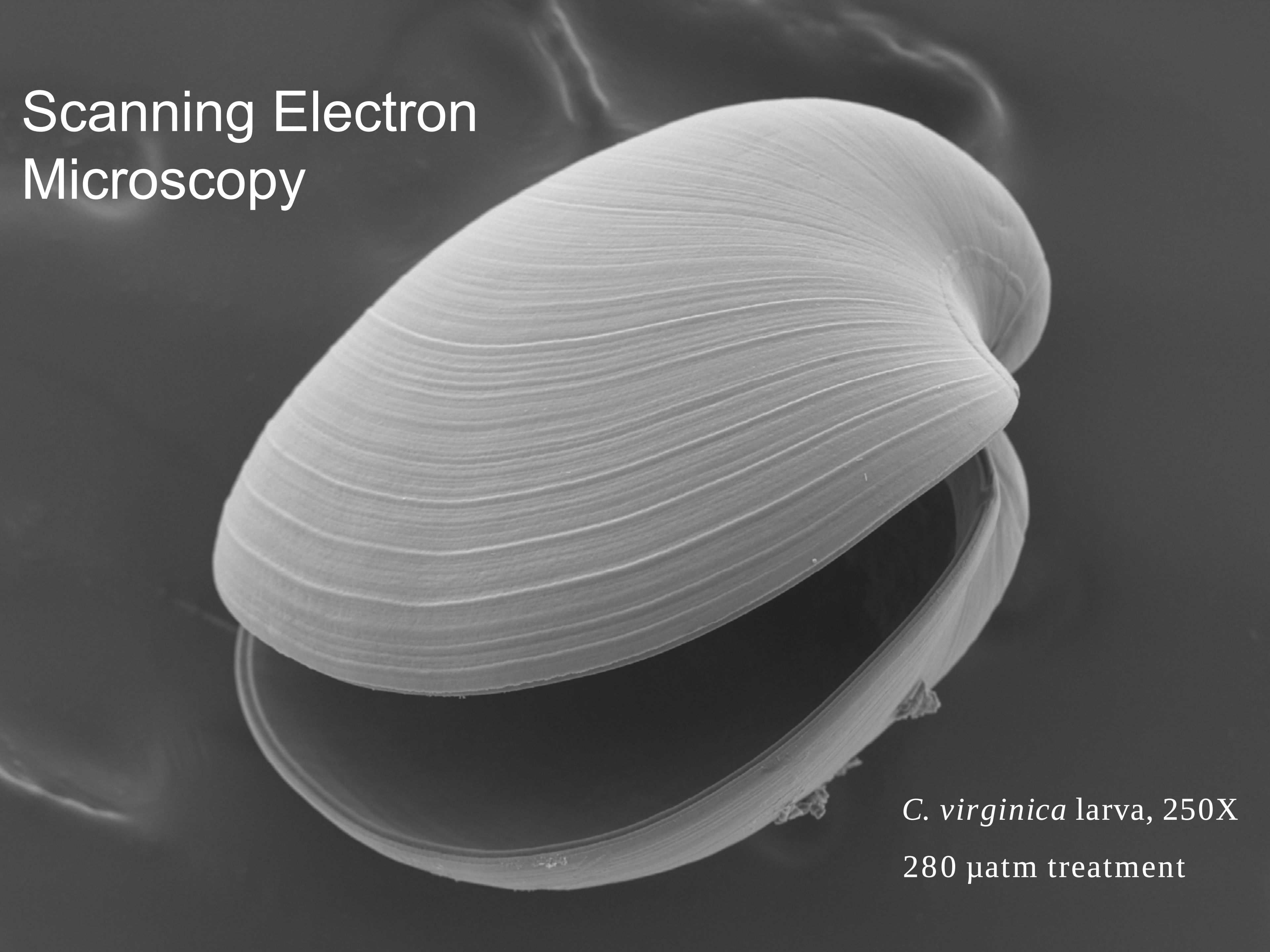
Alkalinity Anomaly Method (Smith and Key 1975)

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

$$G = -\frac{\Delta TA}{2}$$

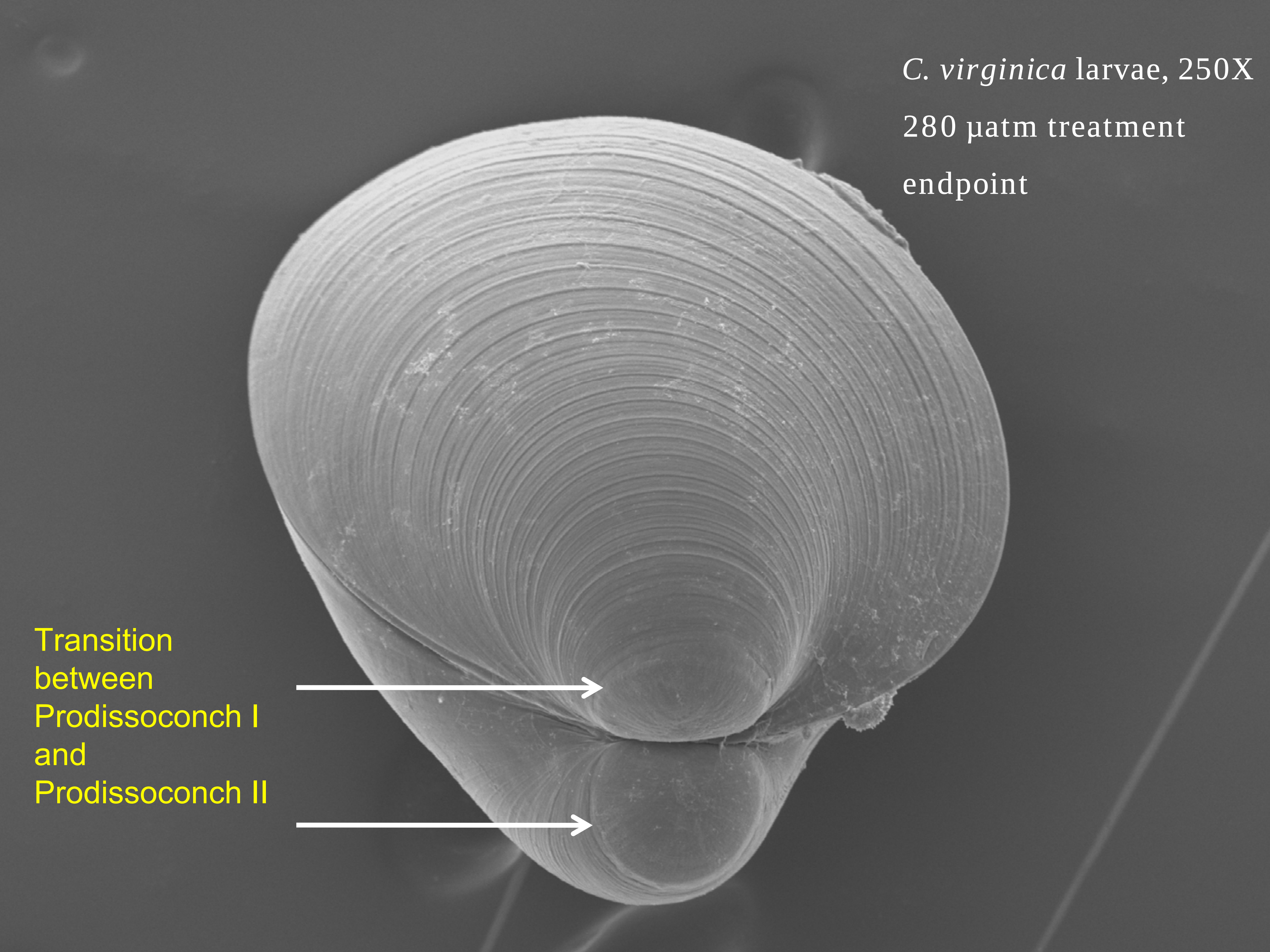
- Precipitation of 1 mole of $CaCO_3$ consumes 2 moles of HCO_3^-, decreasing TA by 2 equivalents
- In mollusks, respiration and calcification cause changes in pH and pCO_2
- Method is sensitive and can be applied in incubations that last only a few hours (e.g., Gazeau et al. 2007)

Scanning Electron Microscopy

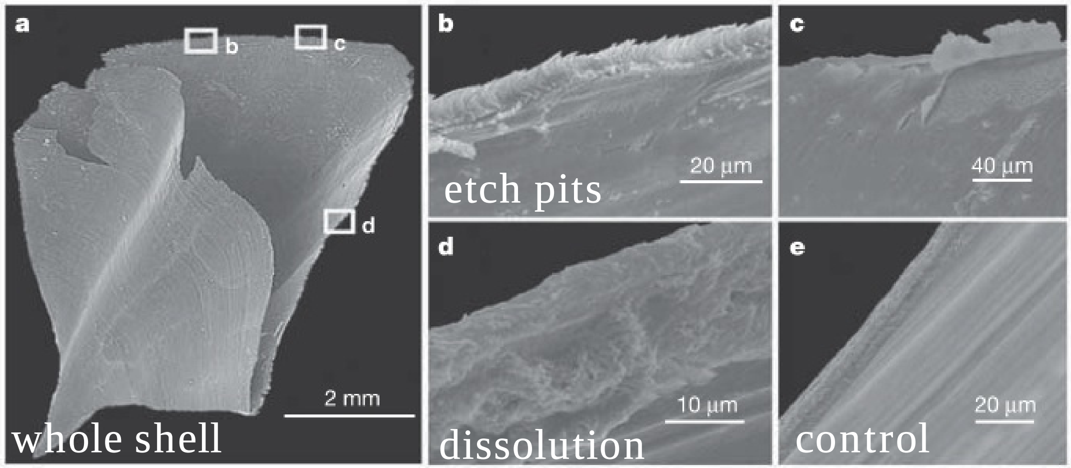


C. virginica larva, 250X

280 µatm treatment


C. virginica larvae, 250X
280 µatm treatment
endpoint
Transition
between
Prodissoconch I
and
Prodissoconch II

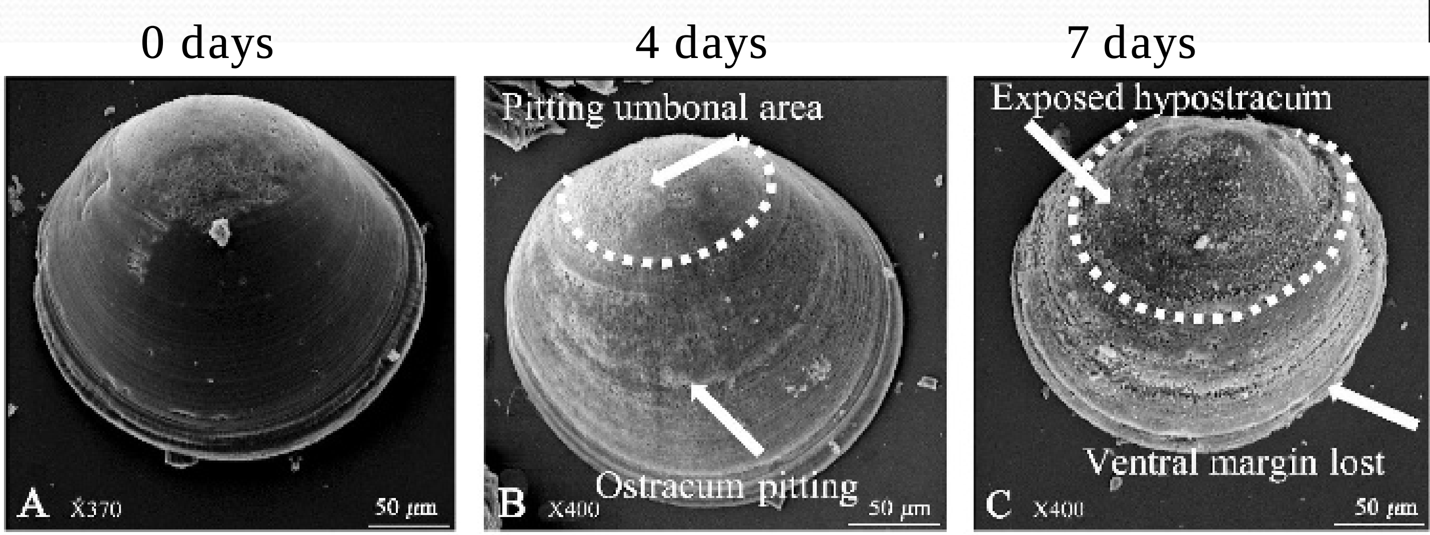
What does dissolution look like?



- *C. pyramidata*, pteropod
- Ω_{arag} <1.0 for 48 hrs

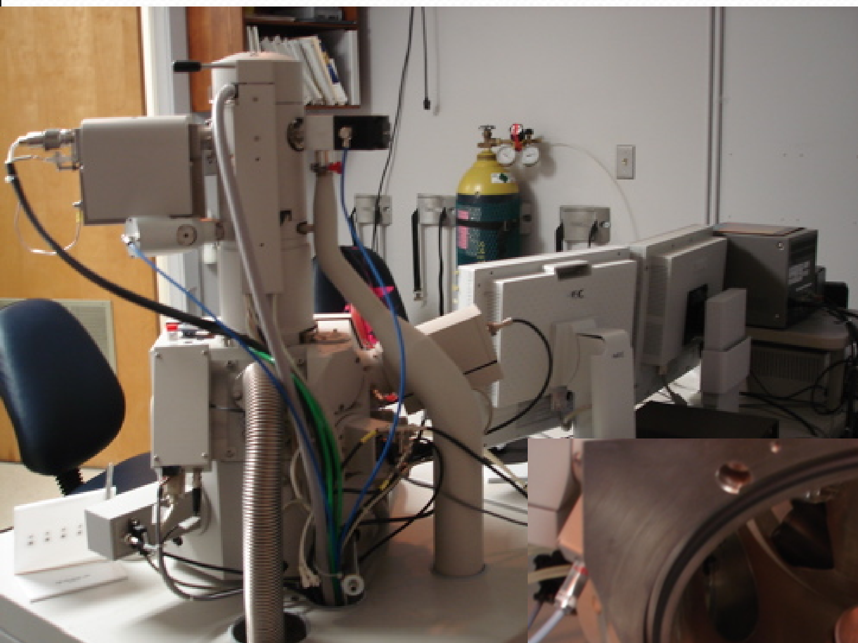
Orr et al. 2005

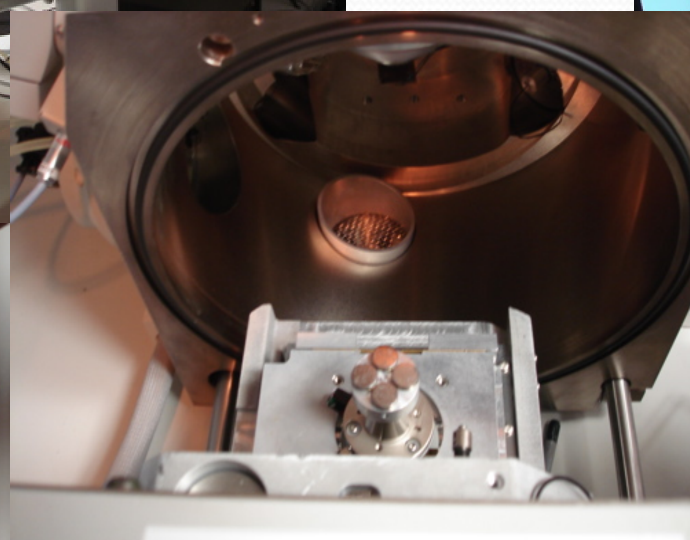
- $\Omega_{arag} = 0.6$
- *M. mercenaria*
- Juvenile infauna

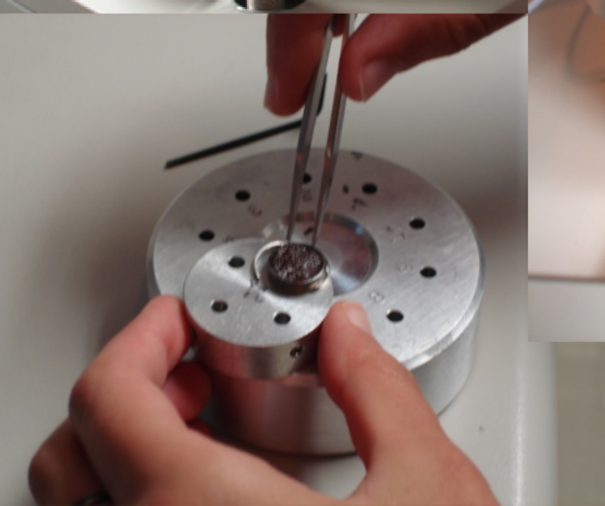


Green et al. 2009

SEM Lab at Smithsonian NMNH

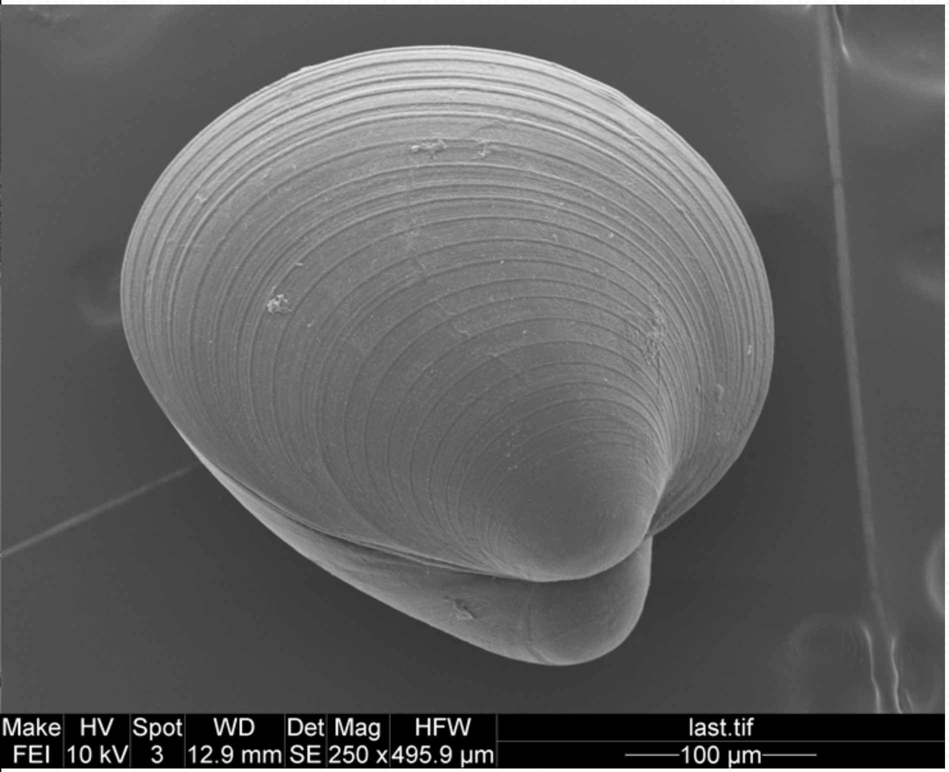




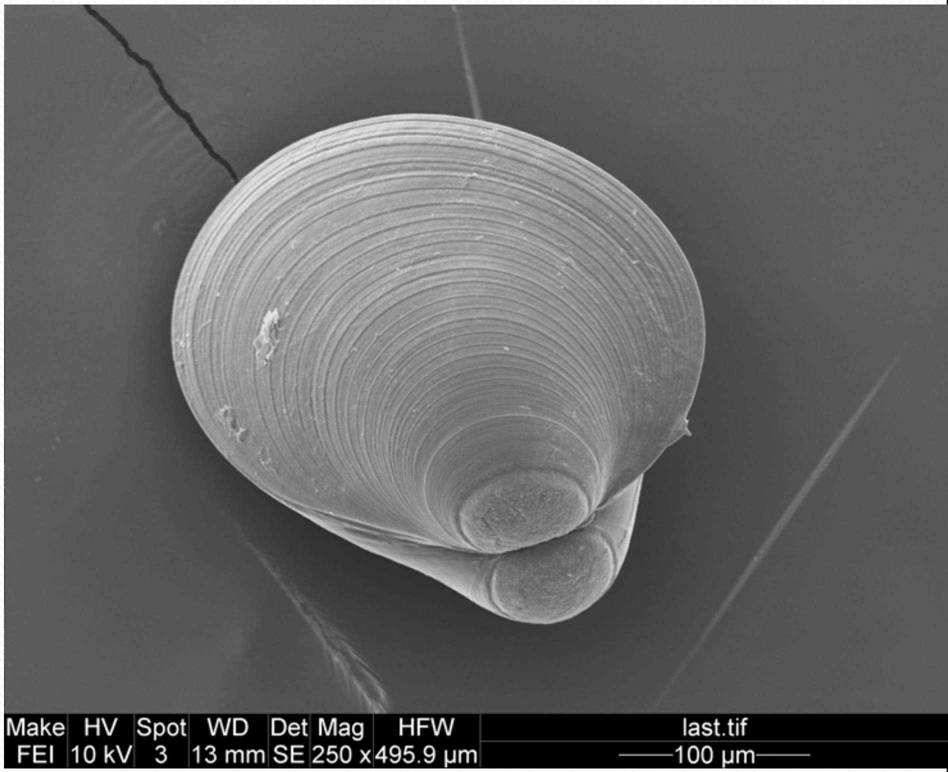


No obvious evidence of deformities or severe dissolution *(C. virginica)*

Preindustrial, 280 µatm



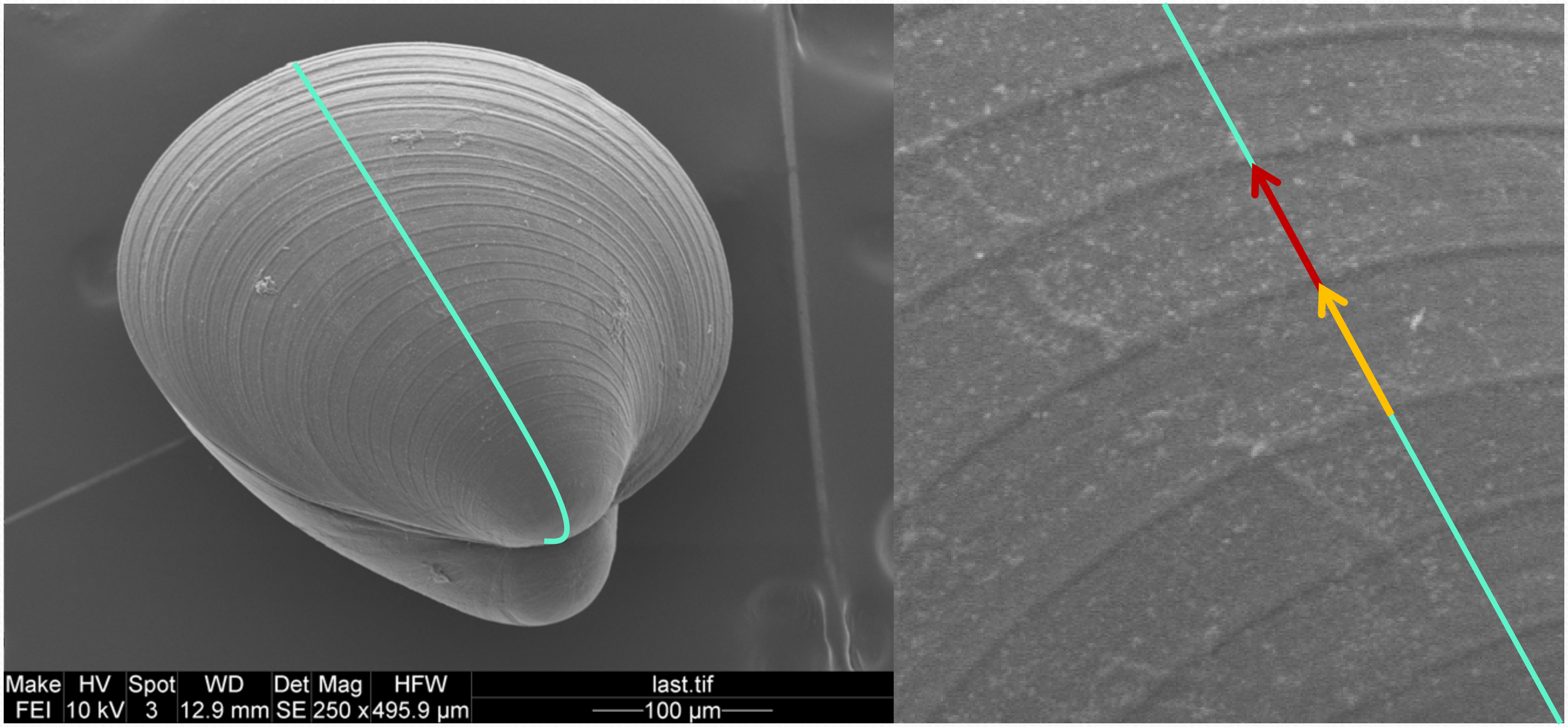
Year 2100, 800 µatm



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

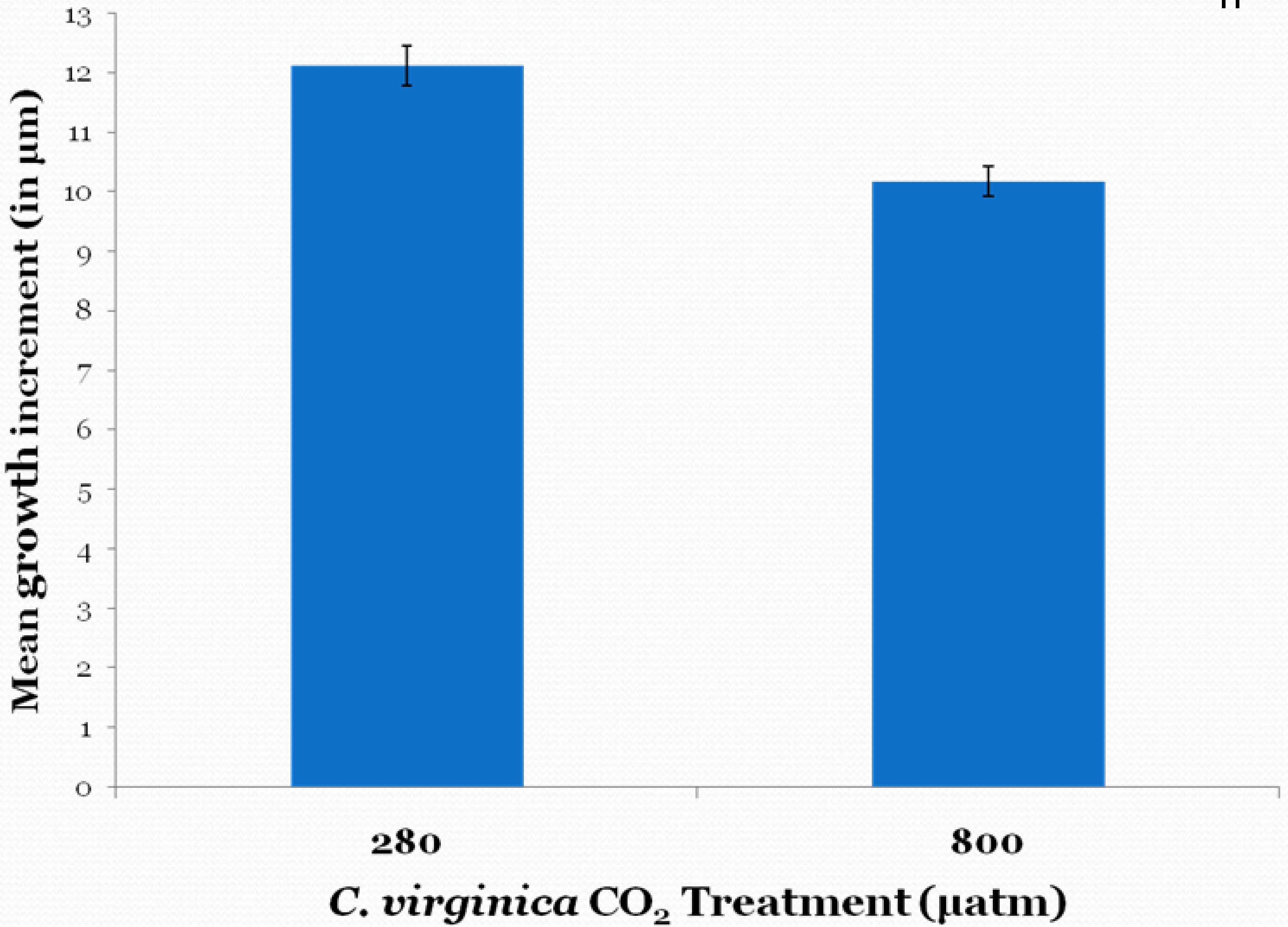
- No difference in number of growth rings between treatments

	280 µatm	800 µatm
Mean	22.879	23.933
Standard Deviation	2.318	2.544

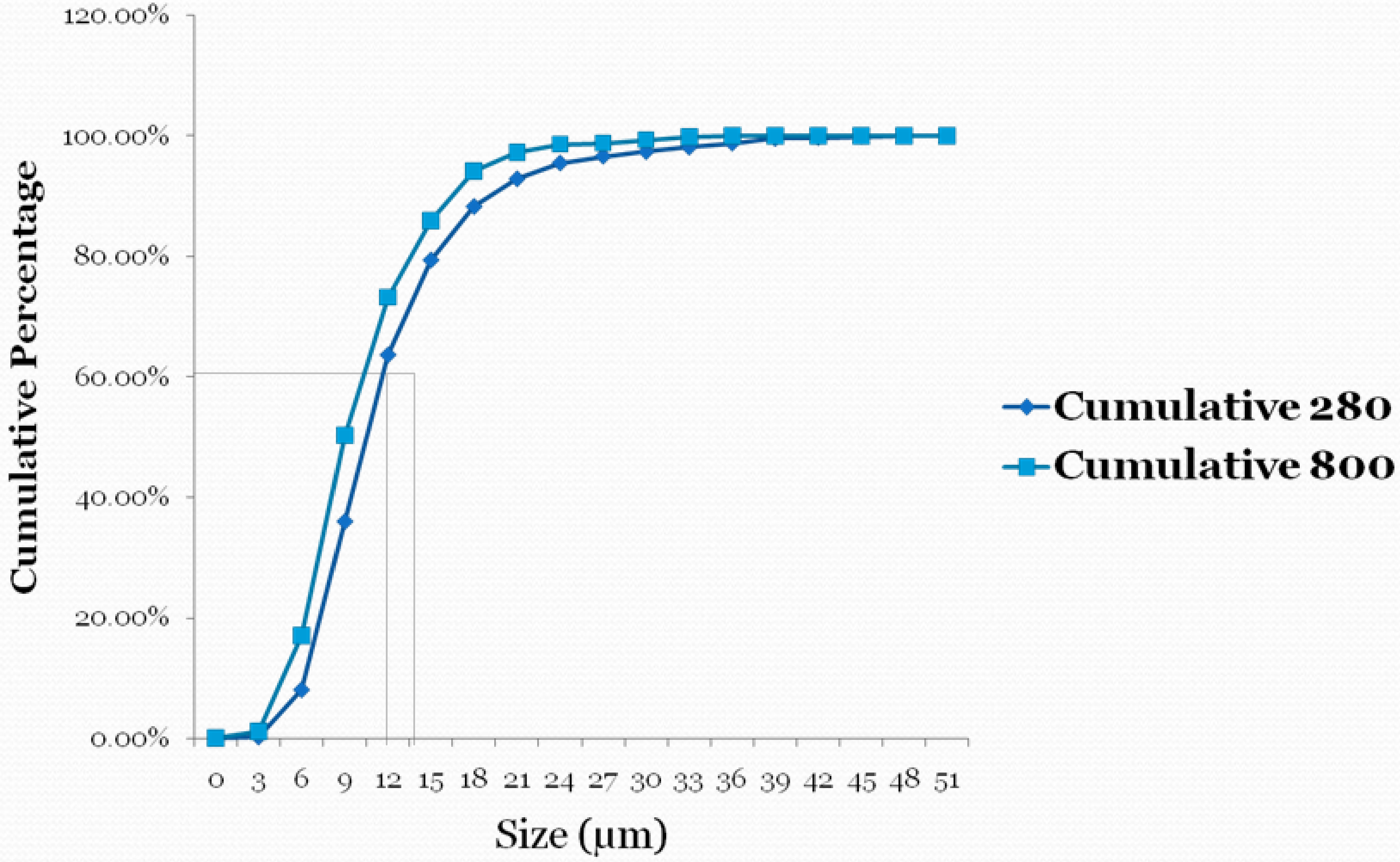
- Mean approximates the length of experiment, 28 days
  - Suggests daily growth rings
- Adult and larval daily growth lines in many bivalves
  - Growth rings correlate with tides, seasons, environmental conditions, diurnal changes

Mean Growth Increment

p = 0.0001
t = 4.753
n = 60

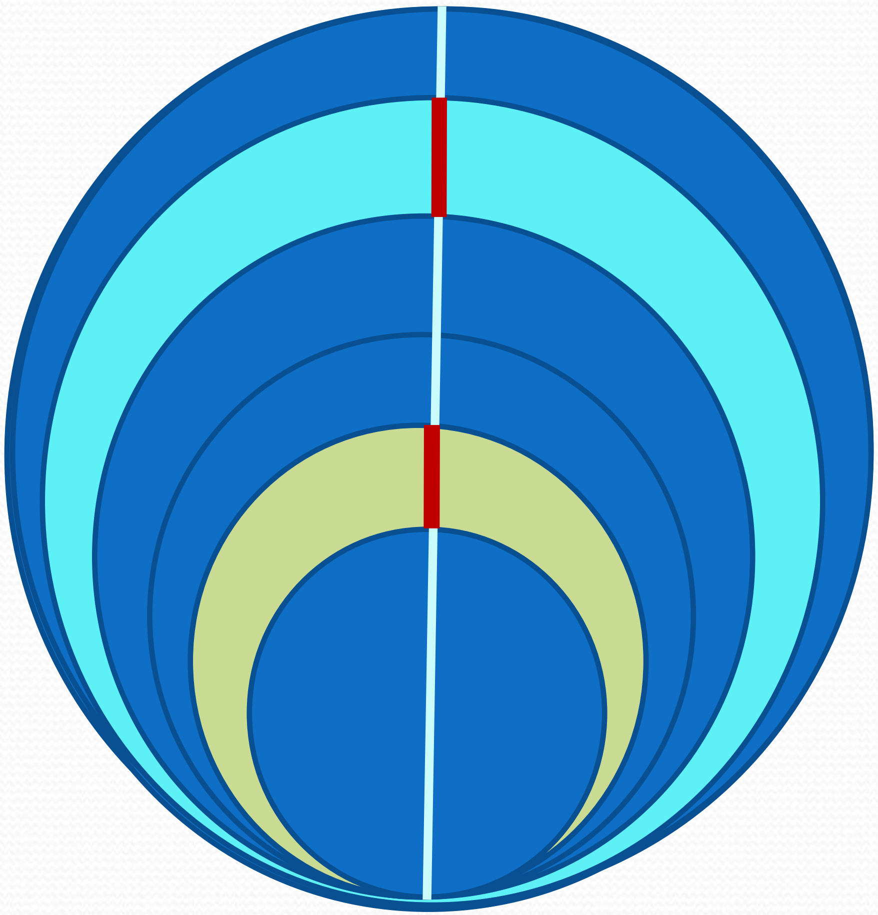


Cumulative growth increment distributions

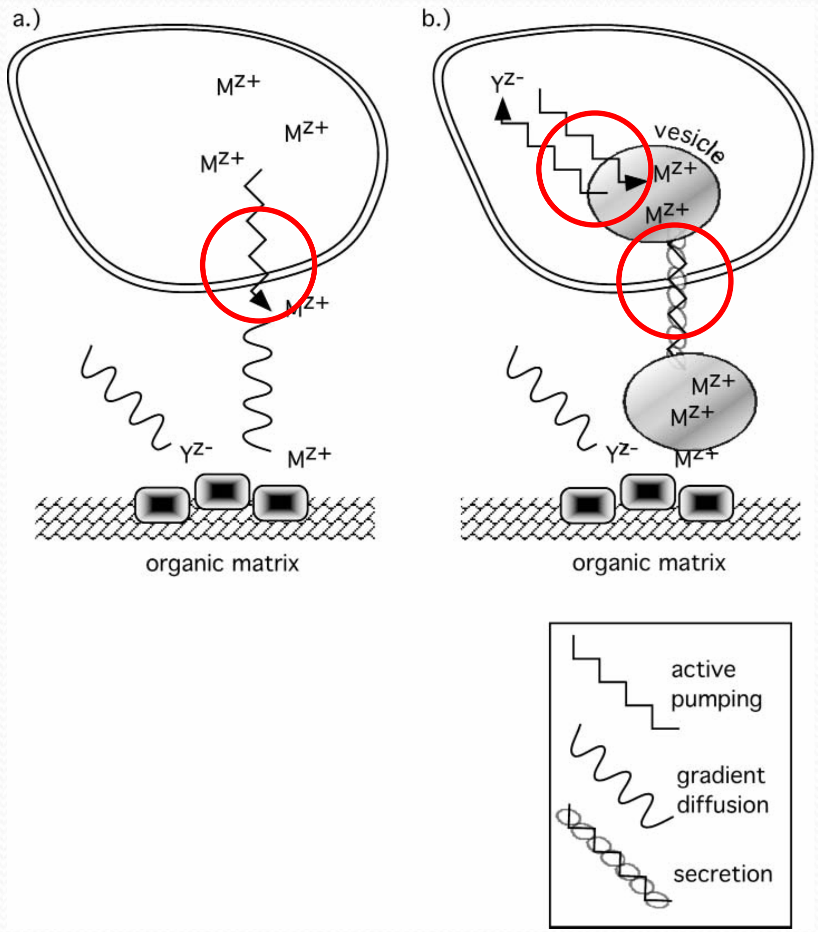


Importance of Incremental growth

- 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