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The Relation Among Essential Habitat, Ocean Acidification, and Calcification on the Nantucket Bay Scallop (Argopecten irradians)

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THE RELATION AMONG ESSENTIAL HABITAT, OCEAN ACIDIFICATION, AND CALCIFICATION ON THE NANTUCKET BAY SCALLOP (ARGOPECTEN IRRADIANS)

A Dissertation Presented

by

BRYANNA JOY BROADAWAY

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ABSTRACT

THE RELATION AMONG ESSENTIAL HABITAT, OCEAN ACIDIFICATION, AND CALCIFICATION ON THE NANTUCKET BAY SCALLOPS (ARGOPECTEN IRRADIANS)

June 2012

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The relation among essential habitat, ocean acidification, and calcification in *Argopecten irradians* (Lamarck 1819) was examined through field and laboratory research. Three major questions were addressed: 1) What habitat conditions are associated with abundant populations of bay scallops in Nantucket Harbor? 2) How might future predictions of ocean pH decline affect the biomineralization of shell by bay scallop across juvenile and adult life stages? 3) Are there biochemical indicators that can inform our understanding on how the bay scallop may cope with ocean acidifying events?

Elemental fingerprinting of adult and juvenile Nantucket bay scallop shells, *A. irradians,* revealed distinct element/Ca ratios that can be used to distinguish source habitat of bay scallops in Nantucket Harbor. These ratios were associated with proximity to the harbor mouth with elemental differences attributed to variation in salinity and pH.

This study identified boundary salinity and pH conditions that support large abundances of *A. irradians* within Nantucket Harbor.

Impacts of ocean acidification on calcification in both juvenile and adult life stages of bay scallop were examined, specifically, the relation between life stage and net calcification rate across varying levels of pCO_2-induced low pH. Net calcification, estimated by buoyant weight, was lower in adults compared to juveniles raised under the same conditions (e.g. pH = 7.2, Net Calcification $Rate_{adult}$ = -0.0058 d^{-1}, Net Calcification $Rate_{juvenile}$ = -0.0036 d^{-1}). The overall impacts across life stages suggest that juvenile bay scallops, as compared to adults, may budget more energy toward calcification of shell.

Stress induced by energy demands for shell loss under ocean acidification events was hypothesized to cause an increase in plasma taurine/glycine ratios. High performance liquid chromatography (HPLC) with fluorescence detection was used to measure the abundance of taurine and glycine in plasma (pre-and post-treatment) and the ratio of taurine/glycine was used as an indicator of environmental stress induced by high CO_2-induced reduction in pH. A nested general linear model revealed that the taurine/glycine ratio in plasma may be used in future studies as an indicator of pH induced stress for *A. irradians*.

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CHAPTER 1

INTRODUCTION AND STATEMENT OF THE RESEARCH PROBLEM

1.1 Overview of Dissertation

This dissertation is organized into five chapters. It presents a field and laboratory approach to answering fundamental questions concerning the essential habitats of the bay scallop (*Argopecten irradians*) and the impact of climate change, specifically ocean acidification, on net calcification. This dissertation also identifies a potential biochemical proxy for stress induced by increased pCO_2. The field component of the research was performed in Nantucket Harbor (Nantucket, MA) where the bay scallop is commercially harvested.

Here we present an overview of the research and a general discussion of ocean acidification. We define terms and set the context for the importance of this research. In the second chapter, to be submitted for publication, we take a close look at the bay scallop population in Nantucket and identify important essential habitat characteristics. In the third chapter, to be submitted for publication, we examine the effect of ocean acidification on the bay scallop, specifically on net calcification in adults and juveniles. In the fourth chapter, to be submitted for publication, the plasma taurine/glycine ratio is evaluated as a biomarker for ocean acidification-induced stress. In the fifth and final

chapter, we summarize the research findings and recommend directions for future research.

1.2 General Introduction

Commercial populations of Massachusetts bay scallop (*Argopecten irradians*) occur in the waters of Cape Cod and the Islands. Landings of bay scallop have declined precipitously over the past two decades from a mean of 399 metric tons ($1.4M) in the 1980's to a mean of 47 metric tons ($1.1M) from 2003-2010 (NMFS data; no data 1997-2002). Though landings have declined, the market value of the MA bay scallop has increased. The market price and import pressure (especially from China) have led to a significant increase in coastal aquaculture research centered on the bay scallop, particularly in Nantucket Harbor. The shellfish market has been facing lower catch numbers over the past years, which have been attributed to hypoxia, harmful algal blooms, habitat loss, and overfishing (Gobler 2005; Jackson 2001). More recently, ocean acidification has been identified as a contributor to population declines (Talmage 2010).

Several years of research performed at Mauna Loa, Hawaii by Keeling provided substantial information about the rate of atmospheric carbon dioxide increase (CO_2) and the $\delta^{13}C$ of atmospheric CO_2 that identified the contributions from fossil fuel burning (Keeling 1960). Keeling concluded that CO_2 was increasing in the atmosphere as a direct result of human activity. Recently, the relation between recent increases in atmospheric CO_2 and the coincident decline in ocean pH was more clearly established. pH of the global ocean has declined by 0.1 pH units since preindustrial times (Orr 2005) such that

current ocean surface water has an average pH of 8.08 (Potera 2010). This relation between atmospheric [CO_2] and oceanic pH is based on the fact that atmospheric CO_2 diffuses into the surface waters of the ocean due to partial pressure differences (Henry's Law). As atmospheric CO_2 increases so too does the dissolved CO_2 in the surface water. Through this air-sea gas exchange, atmospheric CO_2 enters the ocean where it either becomes dissolved or escapes, through evaporation, back into the atmosphere. Carbon dioxide, once dissolved in the surface waters, can form carbonic acid (H_2CO_3) as a first step in a series of several reversible dissociation reactions that can release free hydrogen ions (H^+) that form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions (Equation 1). The resultant increase in the dissolved H^+ leads to a decline in pH (pH = -log [H^+]).

$$CO_2 + H_2O \leftrightarrows H_2CO_3 \leftrightarrows H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-} \qquad \text{Equation 1}$$

The ability of the ocean to absorb atmospheric CO_2, regardless of whether the CO_2 is from natural (e.g., volcanic) or anthropogenic (e.g., fossil fuel burning) sources, depends largely on the extent of dissolution of calcium carbonate ($CaCO_3$(s)) (Equation 2).

$$CaCO_3(s) \leftrightarrows CO_3^{2-}(aq) + Ca^{2+}(aq) \qquad \text{Equation 2}$$

The formation and dissolution of $CaCO_3$(s) varies with the concentrations of both the calcium [Ca^{2+}] and carbonate [CO_3^{2-}] ions through what is known as the saturation state (Ω) (Equation 3).

$$\Omega = \frac{[Ca^{2+}][CO_3^{2-}]}{K_{sp}} \qquad \text{Equation 3}$$

The solubility product, K_{sp}, depends on pressure, temperature, salinity, and the crystal form (polymorph) of the calcium carbonate. Calcium carbonate occurs in a number of polymorphs (same chemical composition, different crystal structure): calcite, aragonite, and vaterite. However, aragonite, under atmospheric pressure and temperature, is 50% more soluble than calcite (Mucci 1983). Calcite can also occur as low-magnesium (Mg) calcite (rare in today's ocean) and high-magnesium calcite (common in today's ocean) with high-Mg calcite having similar solubility to aragonite. $\Omega_{aragonite} < 1$ correlates to dissolution of aragonite shells.

The increase in dissolved $[CO_2]$ and $[H^+]$ in the oceans coupled with the resultant decrease in pH and $[CO_3^{2-}]$, causes a change in the saturation state of $CaCO_3(s)$ due to shifts in thermodynamic equilibria. For ocean surface waters at pH~ 8.1, the carbonate reactions are reversible and near equilibrium (Millero 2002) such that approximately 90% of the inorganic carbon is bicarbonate, 9% is carbonate, and 1% is dissolved carbon dioxide. However, increased atmospheric CO_2 and the commensurate increase in dissolved CO_2 leads to an increased $[H^+]$ followed by a decrease in $[CO_3^{2-}]$. Thus, the 0.3 to 0.4 pH drop from today's ocean pH levels projected by future models for the 21st century, due to continued anthropogenic CO_2 inputs to the atmosphere, will result from an increase of $[H^+]$ by 150% and a decrease in the $[CO_3^{2-}]$ by 50%. The shift in ocean pH to less alkaline conditions is called ocean acidification (Borges 2010).

Ries et al. (2009) investigated the impact of changes in pCO_2 on calcifiers, including the bay scallop. They found that bay scallops showed a net calcification

decrease as the partial pressure of CO_2 was experimentally increased to 2856 ppm (pH = 7.45) from current ocean conditions of pH = 8.0. Ries' study did not discuss impacts on survival or reproductive success, but the results indicated that reduction in pH causes a negative response in net calcification. The research presented here on the bay scallop verifies the results of Ries et al (2009) and extends the work to include juveniles and adults providing vital data for the management of ocean acidification impacts as issues of survivorship are addressed. It is important to note that this research isolates CO_2-induced pH change effects from other climate change related factors, such as warming in the surface ocean, pH decline induced by water freshening, and hypoxia. The rate of pH decline in today's ocean is unprecedented and exceeds the rate of change experienced over the past 300 million years (Honisch 2012). This dramatic change forms the impetus for the research presented here (NRC, 2010).

1.3 Dissertation Objectives and Rationale

The goal of this dissertation was to enhance our understanding of the possible impact(s) of ocean acidification on the current populations of the Nantucket bay scallop. As the coastal estuarine habitat of the bay scallop is subject to variations in pH due to tide and freshwater discharge, it is important to note that they are already exposed to slightly lower pH values (pH = 7.8 than open ocean (pH = 8.01). The objectives of this research were to (1) identify the relative importance of pH to essential habitats for juvenile and adult bay scallop in Nantucket Harbor, (2) examine how net calcification changes due to ocean acidification in juvenile and adult bay scallop, and (3) explore the impact of ocean acidification on plasma taurine (organic acid) and glycine (amino acid).

More specifically, this research:

1) Assesses the distribution of the bay scallop population in Nantucket Harbor and evaluates the potentials of low abundance and barren habitats to support bay scallops by linking water quality, specifically pH, to adult and juvenile abundances in an effort to identify and characterize essential habitats.
2) Examines the controls on net calcification from exposure to pH changes induced by increases in atmospheric pCO_2 during short term exposures of adult and juvenile bay scallops (3 weeks).
3) Identifies increases in plasma taurine/glycine ratios of juvenile bay scallops exposed to pCO_2-induced low pH and evaluates the potential of this ratio as a biomarker of ocean acidification-induced stress.

Using elemental composition of juvenile and adult shells from individuals collected at sites within Nantucket Harbor and relating statistically modeled essential habitat to water conditions and population estimates is the first step in assessing population potentials in the Nantucket Harbor. Throughout we define "population" as the estimated number of bay scallops at a given geographical area based on the number of individuals touching the tape of a physical 50 meter transect. Using shell chemistry, statistical models were developed that reduced variance to identify element/Ca ratios that predicatively classify individuals to high abundance and low abundance habitats within the Harbor. Based on the statistical models of chemical characteristics associated with high abundance habitats, we identify pH and salinity as water quality influencing bay scallop elemental/Ca ratios in shells. Indeed low pH sites were most often barren of bay

scallops suggesting that ocean acidification has the potential to negatively impact bay scallop populations in Nantucket Harbor.

In the U.S., shellfisheries have shown lower catches over the past decade or more with these declines attributed to hypoxia, harmful algal blooms, habitat loss, overfishing, and, most recently, ocean acidification (Gobler 2005; Jackson 2001; Talmage 2010). With atmospheric CO_2 increasing more rapidly than has been experienced in hundreds of millions of years (Sabine 2004; Honish et al. 2012), the bay scallop and other calcifiers may be negatively impacted. The bay scallop is highly vulnerable to ocean acidification as its shell is primarily composed of a low – Mg calcite which is less stable than calcite and more likely to dissolve at lower pH (Mann 2001). Indeed the mineralogical composition as well as the shell microstructure itself is strongly influenced by environmental conditions (e.g., temperature, salinity, pH) (Kennedy et al. 1969; Harper et al. 1997; Harper 2000).

Bivalve Shell Microstructure and Mineralogy

Calcium carbonate shell microstructure evolved polyphyletically (Jackson et al. 2010; Giribet and Wheeler 2002) in bivalves and gastropods. Bivalve shells are multi-layered, consisting of 2 layers: organic and crystalline (calcite and/or aragonite). The layers occur in a number of patterns and the patterns are used to reconstruct bivalve phylogenies (e.g., Taylor et al. 1969; Giribet and Wheeler 2002). Aragonite occurs as prismatic, nacreous, crossed-lamellar, complex crossed-lamellar, and homogeneous structures (Turgeon et al. 1998) (Figure 1-1). Calcite occurs as prismatic or foliated structures (Turgeon et al. 1998). Myostracal layers (calcium carbonate laid down below

sites of muscle attachment) are always aragonitic. Modern bivalve phylogeny is based on genetics (e.g., rRNA, mtDNA) and shell microstructural analysis with the latter classified based on the crystallographic alignment and homogeneity of mineralogy.

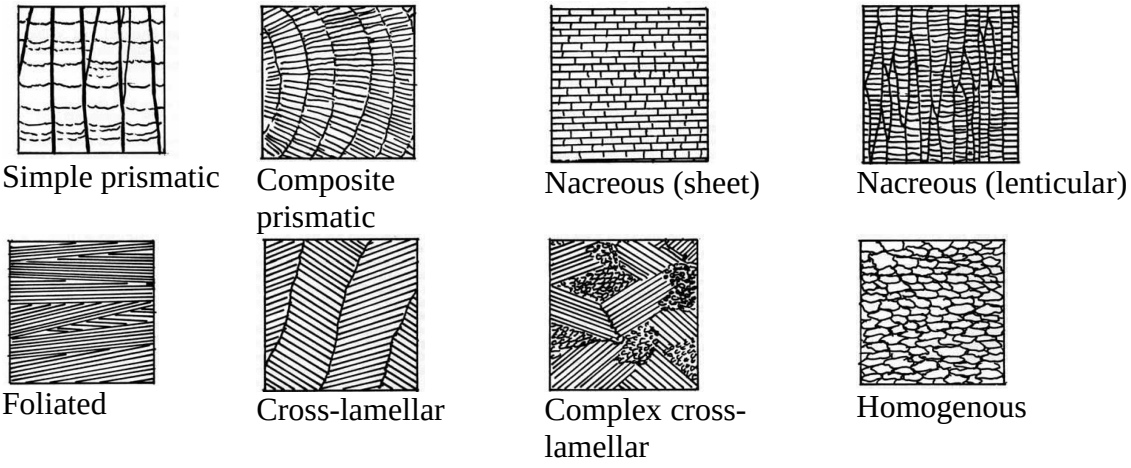

Figure 1-1. From Taylor and Layman (1972). Textures of bivalve microstructures seen in transverse sections of shells. These structures are used to describe the phylogeny of bivalves and to demonstrate polyphyletic or convergent evolution of shell components, such as nacre (Taylor et al., 2001).

It has been proposed that calcite, less soluble than other polymorphs of calcium carbonate (e.g., aragonite, vaterite) at low temperatures, offers a physiological advantage to bivalves (Harper 2000). No bivalve taxa are completely calcitic with most having at least aragonitic nacre and/or hinge ligaments (Giribet 2008) (Table 1). Family Pectinidae, of which *Argopecten irradians* (Bay Scallop) is a member, have cross-lamellar shells with low-Mg calcite over aragonite lamellae. In bivalves with calcitic shell layers, the calcite is added first in the outermost shell layer suggesting some adaptive advantage (Harper 2000). Other adaptations to slow dissolution induced by declines in pH include thickened periostracum (Vermeij 1993). In addition to mineralogy and microstructure, the content of magnesium in the calcite also increases solubility, such

that calcites with high magnesium contents have higher solubilities than aragonite (Walter and Morse 1984; Bischoff et al. 1987).

Table 1-1. Bivalve phylogeny (after Plazzi et al. 2011) including family-level shell structure and mineralogical composition of marine bivalves (non-marine bivalves not shown). Numbered references shown in Appendix 1.

Bivalve phylogeny	**Family**	**Shell Microstructure**	**Shell Mineralogy**
Protobranchia/Opponobranchia	Nuculoidea	Nacreous	Simple prismatic/aragonite[1]
Protobranchia/Opponobranchia	Solemyoidea	Homogenous	Simple prismatic/aragonite[1]
Autolamellibranchiata/Heterodonta	Pandoroidea	Cross-lamellar	Composite Prismatic, aragonite[7]
Autolamellibranchiata/Heterodonta	Poromyoidea	Cross-lamellar	Composite Prismatic, aragonite[7]
Autolamellibranchiata/Heterodonta	Myoidea	Cross-lamellar	Prismatic/aragonite-calcite[8]
Autolamellibranchiata/Heterodonta	Glossoidea	Cross-lamellar	Aragonite[10]
Autolamellibranchiata/Heterodonta	Veneroidea	Cross-lamellar	Aragonite; acicular aragonite in periostracum [11] Calcite outer layer occurs in chamoidean veneroids[12]
Autolamellibranchiata/Heterodonta	Hiatelloidea	Cross-lamellar	Calcite, aragonite/calcite, aragonite inner layer[8]
Autolamellibranchiata/Heterodonta	Cardioidea	Cross-lamellar	Aragonite[2]
Autolamellibranchiata/Heterodonta	Tellinoidea	Cross-lamellar	Aragonite[2]
Autolamellibranchiata/Heterodonta	Lucinoidea	Cross-lamellar	Aragonite[2]
Autolamellibranchiata/Heterodonta	Astaroidea	Cross-lamellar	Aragonite[2]
Protobranchia/Nuculanoida	Nuculanoidea	Homogenous	Simple prismatic[1]
Autolamellibranchiata/Pteriomorphia	Anomioidea	Foliate	Calcite[14]
Autolamellibranchiata/Pteriomorphia	Limoidea	Cross-lamellar	Calcite over aragonite lamellae[5,6]
Autolamellibranchiata/Pteriomorphia	Pectinoidea	Cross-lamellar	Calcite over aragonite lamellae, some fully calcitic[5,6,10]
Autolamellibranchiata/Pteriomorphia	Ostreoidea	Foliate	Calcite[14]
Autolamellibranchiata/Pteriomorphia	Pinnoidea	Nacreous	Calcite[15]
Autolamellibranchiata/Pteriomorphia	Pterioidea	Nacreous & Cross-lamellar	Aragonite[4]
Autolamellibranchiata/Pteriomorphia	Arcoidea	Cross-lamellar	Aragonite[2] (some simple prismatic)
Autolamellibranchiata/Pteriomorphia	Mytiloidea	Nacreous	Aragonite/Calcite[3]

Generally, within Superfamilies, shell structure and mineralogy are very constant (Kennedy et al. 1969). Calcitic outer layers only occur in Superfamilies of Pteriomorphia (of which *A. irradians* is a member) with the exception of 2 species of Heterodonta from Superfamily Chamacea (Table 1). Pteriomorphia are also the only subclass in which calcitic foliated shell structure is known to have developed (MacKinnon 1982).

Environmental factors modify the basic mineralogy/shell structure within a Superfamily. Often there is an inverse relation between percentage calcite and temperature. Only Superfamily Mytilacea has been shown to have a relation between temperature and percentage calcite, and only *Mytilus californianus* has an inner calcite layer as well as an outer calcite layer (Ford et al. 2008). In a study of the solubility of different calcitic and aragonitic bivalve shell microstructures (shell only), Harper (2000) found that changes in seawater chemistry, from that favoring high-Mg calcite and aragonite (today's ocean) precipitation to that favoring low-Mg calcite (early Paleozoic/Mesozoic) precipitation, led to shell dissolution. However, microstructure and crystal size, not polymorph type alone, were the controlling factors for shell dissolution. Bay scallop shells are comprised primarily of low-Mg calcite which is not common among today's bivalves.

Biomineralization and Calcification of the Prismatic layer in *Argopecten irradians*

Given that *A. irradians* shell microstructure and mineralogy are factors that will respond to pH, it is important to briefly review the steps involved in shell formation. Calcification, a type of biomineralization, involves biologically controlled extracellular crystallization of $CaCO_3$(s) through the interaction of organic and inorganic constituents

found within extrapallial fluid (Weiner and Dove 2003) (Figure 1-2) located between the mantle and shell. The organic periostracum is secreted by mantle epithelial cells at the base of the periostracal groove, between the outer and middle mantle folds, and may serve as a site of deposition for the $CaCO_3$(s) (Checa 2000; Kennedy et al. 1969). This process means that all shell forming constituents must be present within the extrapallial fluid prior to polymerization and precipitation. This aspect of shell formation is central to the work done here and will be revisited later in this dissertation. Shell deposition begins with the formation of the periostracum at the margin of the mantle (Figure 1-2). The periostracum also acts as a protective layer**,** and is especially thick in freshwater bivalves and burrowing mytiloids (Weiner and Dove 2003). Calcium carbonate crystals are induced to form within the matrix voids with the core of the voids composed of a layer of β-chitin sandwiched between two layers of glycine and alanine rich proteins (Fallini et al. 1996). Initially spherules of $CaCO_3$(s) form inside the chitin scaffold with the polymorph (calcite/aragonite) type controlled by the composition of the proteins within the periostracum (Fallini et al. 1996). Microstructure and crystal growth are maintained by the extrapallial fluid over the duration of shell growth.

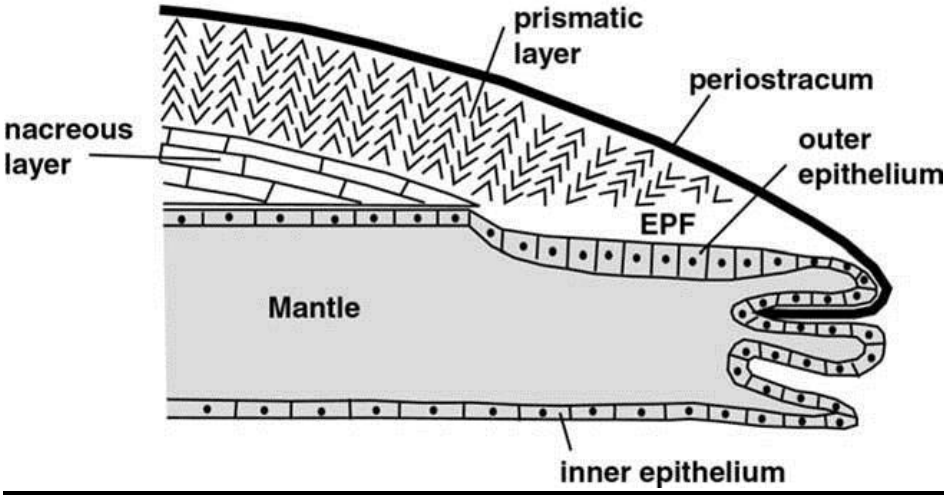

Figure 1-2: Transect from umbo to edge of a bivalve shell. Extrapallial fluid (EPF) is located between the calcifying mantle and the prismatic layer. This extrapallial space is where calcification occurs. (Carré 2006)

Calcification in Argopecten irradians – implications of ocean acidification

The shell of *Argopecten irradians* is predominantly low-Mg calcite. As discussed above this mineral is more soluble than calcite, and as such more likely to dissolve under low pH conditions. These broadcast spawners typically show high larval mortality until settlement within seagrass beds (Guinotte 2008) where they metamorphose into the juvenile stage. Once gonads are developed, the scallop enters the adult life stage. Should ocean acidification lead to loss of shell integrity, transitions between life stages may be negatively impacted further increasing mortality. Bay scallops have adapted strategies for overcoming larval mortality (e.g., functional hermaphroditism and rapid juvenile growth). However, they remain vulnerable to environmental change as juveniles (e.g., low pH). This research shows definitive evidence that bay scallop habitat quality is defined by a combination of physical characteristics (e.g. pH, salinity, depth, dissolved oxygen, and temperature), and that CO_2-induced pH changes profoundly impact net

calcification in both juveniles and adults. Therefore, managers of bay scallop populations must develop sustainable fishery practices in response to ocean acidification.

The calcification of shell material ($CaCO_3$) in *A. irradians* for the prismatic layer occurs in the extrapallial space between the mantle and shell at the leading edge where the periostracum is excreted to protect new deposition from seawater. $CaCO_3$ is deposited from carbonate ions from external dissolved inorganic carbon or metabolic CO_2 that leads to the production of H^+ ions in the extrapallial fluid (Decoursey, 2003). To avoid acidification, of the extrapallial fluid, H^+ ions are evacuated from the extrapallial fluid through proton channels which prevents denaturing of proteins and/or dissolution of newly deposited shell (Decoursey, 2003). The organic matrix involved in the facilitation of calcium carbonate deposition is still an active area of research. Previous research had indicated that calcification may be enhanced by secretion of sulfur-bearing organics (e.g. cysteine, methionine, chondroitin) (Freitas 2009). Taurine (2-aminoethansulfonic acid) which may be present in the organic matrix found in the extrapallial fluid space to promote calcium carbonate deposition, has been identified to increase calcification up to 96% (Malkaj 2006). Taurine has been identified in osmoregulation activities (Yancey 2002). It is proposed here that taurine production (increases calcification growth rates) may increase with an increasing demand to maintain shell deposition We hypothesize that, under ocean acidification conditions, taurine concentration may increase in the extrapallial compartment of the animal where the organic matrix is synthesized for incorporation into the shell (Rosenberg 1991; Addadi et al. 2006).

In summary, this dissertation serves to enhance our understanding of the impact of ocean acidification on net calcification and biochemical changes in the bay scallop. It utilizes a field and laboratory approach to obtain empirical data that compares to published studies on other commercially relevant bivalve species. Understanding how the bay scallop may respond to ocean acidification will help manage essential habitats and bay scallop populations.

1.4 Study Site

Nantucket Harbor (Massachusetts, USA; Figure 1-3) is approximately 10 km by 1 km; much smaller than other locations where shell-based trace element records have been used to reconstruct life histories of bivalves (Becker 2007). The Harbor is considered to be well-mixed due to tidal influences on the shallow water and protected by strict environmental regulations controlling waste management (Wilkin 2006). Site locations and number of scallops retrieved in this study followed the sampling protocols of the Nantucket Shellfish Management Plan (http://www.nantucketharborplan.com/). We evaluated 32 sites, beginning in June 2009, for the presence/absence of bay scallops (*Argopecten irradians*). Where more than 30 adults were present, (21 sites) we collected 30 specimens. At the 11 sites where fewer than than 30 adults along a 50 m transect were present, no specimens were collected due to management restrictions on harvesting. Juveniles were visible later in the sample season (August) at 4 sites where greater than 30 adults were also found and at these sites a maximum of 10 specimens were collected.

Characterization of essential habitats in this study relied on elemental analysis of bay scallop shells from individuals collected at multiple locations within Nantucket Harbor.

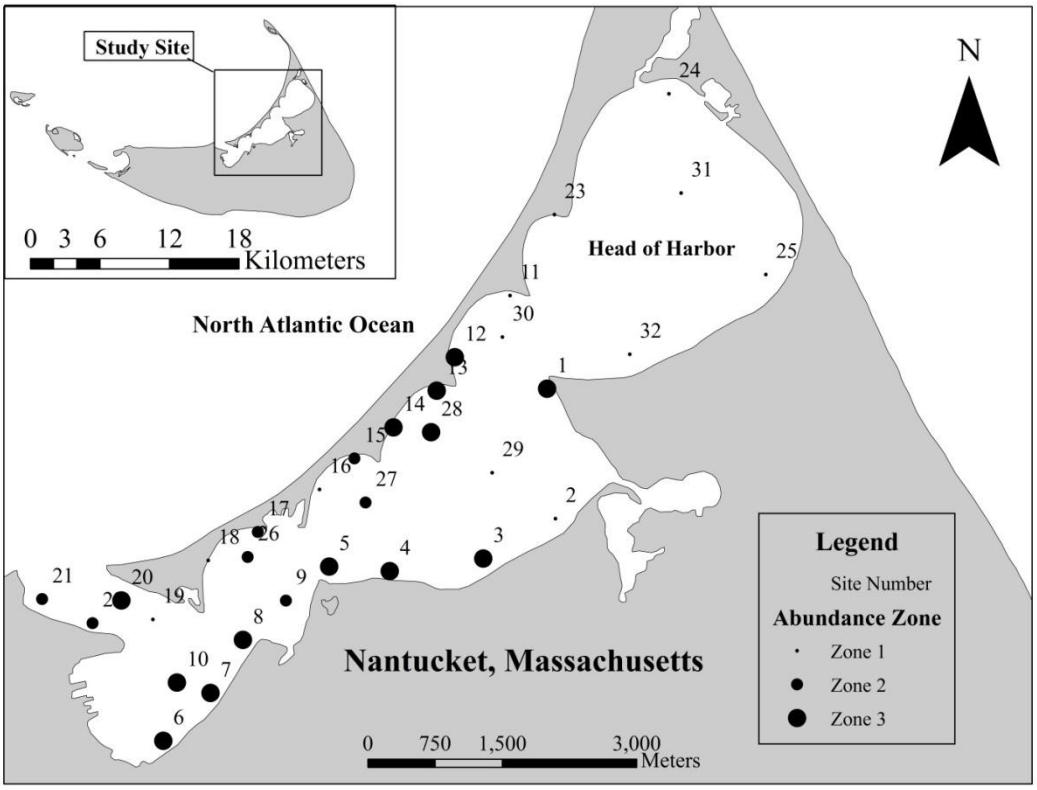

Figure 1-3: Collection sites within Nantucket Harbor (July 21st – August 12th, 2009). Abundance zones shown by circle diameter where the most abundant sites are indicated by the largest circle, and sites where no scallops were found are indicated by dots.

1.5 Experimental Methods

Juvenile and adult, *Argopecten irradians*, were maintained for 3 weeks in 5 gallon plexiglass aquaria filled with 5 μm-filtered seawater collected from Boston Harbor at the Fox Point dock in Boston, MA. Each aquarium was equipped with a saltwater fan to induce water movement and promote feeding, and covered with a plexiglass lid to minimize evaporative water-loss. Seventy-five percent of seawater was replaced twice a

week throughout the duration of the experiment. Organisms were fed aliquots of Shellfish Diet 1800 (Reed Mariculture Inc.) twice a day (~ $1.0x10^6$ cells per mL of seawater per day), which is a commercially produced mix of four marine microalgae – *Isochrysis sp, Pavlova sp, Thalassiosira weissflogii,* and *Tetraselmis sp*.

Experimental air-CO_2 mixtures were mixed prior to bubbling into aquaria (Figure 1-4). CO_2 tanks were mixed with air provided from aquaria air pumps where mixing was encouraged by securing air-stones to the base of the aquaria directly below saltwater fans. To control the pH of each tank, Allied Electronic gas solenoids (Part # VX2330-02T-3CR1) were triggered using a feedback system attached to a Digital Aquatics pH probe causing CO_2 to be introduced to the tank using a pH = 0.01 threshold. The pH of each tank was logged using a ReefKeeper Control (Digital Aquatics) set consisting of: 8 RKM – SL1 units connected to a ReefKeeper Elite Head Unit and retrieved from a Net box. pH, calibrated to NBS standards, was logged at 10 minute intervals using this DigiAquatics data management system over the entire duration of the experiment. Using an Orion 5-Star Plus benchtop meter (Thermo Scientific), pH, calibrated to NBS standards, was confirmed externally to account for any drifts in the Digital Aquatics pH probe influenced by algal growth or electronic noise.

Figure 1-4: Experimental aquaria used for juvenile and adult pCO_2 treatment exposures.

Salinity, temperature, dissolved oxygen, and conductivity were measured daily using a YSI Professional Plus hand meter. Alkalinity (ALK) and dissolved inorganic carbon values (DIC) were analytically determined in parallel twice a week at each saltwater change. ALK was determined following the EPA 5.10 total alkalinity method, which involved a titration with 1.6 N sulfuric acid using a digital titrator. DIC was determined using a Tekmar Dohrmann Phoenix 8000 UV-Persulfate analyzer. Aragonite/calcite saturation states (Ω), $pCO_{2(aq)}$, and experimentally modeled DIC values were calculated from directly measured parameters using CO2SYS (Pierrot 2006) (Appendix 2).

The buoyant weight method used to calculate net calcification has been published previously (Ries, 2010). Briefly, specimens were suspended in their dosed aquarium by a basket attached to an aluminum wire hanging on a Mettler Toledo scale (Figure 1-5). Net

calcification rates were calculated as the weight difference between the specimens' buoyant weight at the beginning and end of the 21-day experiment divided by the beginning weight.

Figure 1-5: Apparatus used to obtain buoyant weight. Specimens were placed in the basket which was suspended within the treatment tank to ensure minimal stress due to handling.

1.6 Analytical Methods

To evaluate the relation between shell elemental composition and habitat, and to use these relations to identify essential habitat characteristics for the Nantucket bay scallop population, field and laboratory data must be collected. In the field, notation of site coordinates, time of collection (start/stop), depth, and water quality parameters are required as these data will be used in conjunction with laboratory measurements. Also, the number of specimens collected and the estimated population along transects must be noted. Water quality measurements were taken both in the surface waters and at the sediment-water interface. We used, for our statistical models, measurements taken at depth as these waters are in closest contact with the *A. irradians*. Collected specimens

were transported back to the University of Massachusetts Boston where they were cleaned, and the shells were prepared for analysis (Appendix 3). Analytical details for these measurements are presented in Chapter 2 of this dissertation.

The experimental aquaria design is described above and also in Chapter 3. Here, we provide additional details regarding steps taken to improve the quality of the carbonate and pH measurements. In my experiments, the pH in the aquaria was being monitored by the ReefKeeper system every 10 minutes. This logging information was used to provide the standard deviation of each pH treatment (pH ± s.d.). When preparing seawater filtered from Boston Harbor, an YSI probe was used to measure salinity. Batches of filtered water were mixed directly in carboys and manually rolled on the floor for mixing. Since the water in the aquaria must be changed regularly, we found it best, since scallops are sensitive to handling, to do partial water changes using a syphoning system that allowed the ReefKeeper system to maintain a steady pH and to ensure minimal variation in salinity. Water quality data (temperature, salinity, dissolved oxygen, pH) were collected using an YSI Professional Plus. Aquaria water was collected for nutrient concentration determination by flow injection analysis (FIA) to monitor ammonia where high ammonia concentrations signal a need for water change. However, the time delay associated with sample preparation and analysis by FIA is significant and future research should monitor ammonia by spectroscopic methods which will minimize the lag time between measurement and water change. Since the CO2SYS model includes silicate and phosphate concentrations, which can shift saturation states of calcite and aragonite at

high concentrations, we measured dissolved silicate and phosphate concentrations at water change throughout the experiment. CO2SYS model results showed no influence on modeled saturation states for calcite or aragonite as a result of dissolved silicate and phosphate concentrations. Therefore, it is unnecessary, in experiments as described here to monitor these two nutrients.

To ensure that the aquaria pH probes are calibrated, , measurements of alkalinity and dissolved inorganic carbon (DIC) were performed. DIC was measured by UV-persulfate reduction (Appendix 4). We used EPA Method 5.10 for measurement of total alkalinity for this study. When performing alkalinity measurements, it is important to record the volume of seawater being used for the measurement (100 mL in a graduated cylinder) and the associated weight (measured in a glass beaker used for the titration) for density calculations. We recorded total number of clicks from a digital titrator along with time, endpoint pH values, and temperature. Total alkalinity (μmol/kg) was calculated as (Equation 4):

$$Total\ alkalinity = \frac{2a-b}{60g} x 1000 x \frac{0.1L}{mass\ of\ seawater\ (g)} x \frac{1000g}{1kg} \quad \text{Equation 4}$$

Where "a" is the number of clicks to reach a pH of 4.5 and "b" is the number of clicks to reach a pH of 4.2.

Lastly, methods for the measurement of plasma taurine and glycine are presented in Chapter 4 with the Standard Operating Procedure presented in Appendix 5.

1.7 Statistical Methods

Statistical analyses were performed using SPSS 18. Kolmorov-Smirnov normality test was used to confirm distributions. Two-way ANOVAs were initially utilized to examine differences in net calcification and T/G ratio with a Tukey's HSD *post hoc* test ($\alpha \leq 0.05$) to determine between and within treatment effects. Relations between T/G, pH, and net calcification were examined using Pearson correlation. A general linear model was used when investigating relationship of T/G and net calcification with pH. For example, T/G data were log normalized to achieve equality of variance. Two-way ANOVAs were performed to confirm no interaction effects or variance in y-intercept. A nested general linear model was used to determine tank position within treatment pH effects (Appendix 6). By using this method, a general linear model was created that confirmed that no effect of pseudoreplication was present.

For the research centered on essential habitat identification in Nantucket Harbor, all shell trace elements were normalized to Ca concentration, except carbon and nitrogen. Multivariate statistics (factor analysis with principal component extraction and binomial logistics regression) were used to determine if the shell elemental composition could be used to distinguish zones within the Harbor. Binomial logistics regression was used to preserve as much of the class discriminatory information as possible while only classifying low abundance (Zone 2) and high abundance (Zone 3) sites to a defined zone, while factor analysis was used to identify relations between physical water parameters, elemental composition of the shells, and abundance of *A. irradians*.

CHAPTER 2

ELEMENTAL FINGERPRINTS USED TO IDENTIFY ESSENTIAL HABITATS: NANTUCKET BAY SCALLOP

2.1 Abstract

Elemental fingerprinting of adult and juvenile Nantucket bay scallop shells, *Argopecten irradians* (Lamarck 1819) revealed distinct element/Ca ratios that can be used to distinguish provenance of bay scallops in Nantucket Harbor. Within this small Harbor (~10 km^2), we identified three distinct habitat zones defined by the abundances of scallops at a location: Zone 1 (barren), Zone 2 (low abundance), and Zone 3 (high abundance). Element/Ca ratios were associated with proximity to the harbor mouth with elemental differences attributed to variation in salinity and pH. Using binary logistic regression, we identified the source zones based on shell elemental ratios (i.e. various elemental concentrations divided by [Ca]). We also identified boundary salinity and pH conditions that support large abundances of *A. irradians* within Nantucket Harbor. This information is crucial to seeding projects and the management of the Nantucket Harbor *A. irradians* population.

2.2 Introduction

Marine planktotrophic larvae, such as the bay scallop (*Argopecten irradians*), have the ability to travel great distances from their natal habitat. However, in some instances, larvae may be retained in a defined, local environment (Arnold 1998). Elemental fingerprinting of shells has been used to reconstruct environmental life histories (variability in environmental exposures that lead to distinct elemental signatures from different locations) of organisms (Becker 2005; Thorrold 2002) and identify essential habitats (e.g., Dorval et al., 2005). Essential habitat, in this study, is defined as a combination of physical structure, availability of food, substrates, hydrodynamics, and hydrology, which makes the area ideal habitat for *A. irradians* (Minello 1999). If there is sufficient variation in elemental signatures in shells between sites, and sufficient stability in water chemistry within sites, then it is possible to retrospectively determine the location in which the organism lived prior to capture. Since these elemental signatures are unique to the environment and linked to individuals, they can serve as a natural tag for tracking movement patterns, eliminating the need for artificial tagging (Thorrold 2002; Becker 2005). Retrospective identification of habitats in populations that are geographically restricted is complicated given potentially low variation in water chemistry between sites, which may preclude identification of unique fingerprints. In this study, essential habitat is determined by population estimates, or abundance, of *A. irradians* along a 50 m transect.

Often studies that focus on habitat identification using natural tags, such as shell chemistry, are conducted in areas where sample sites are separated by twenty kilometers

or more to insure chemical and physical heterogeneity between sites (e.g., Becker 2005). However, there is a growing need for methods that determine the smallest spatial scale at which essential habitats can be accurately distinguished using shell chemistry. Identification of essential habitats is useful for ecological studies that assess impacts of environmental change on recruitment and survivorship, for management of critical habitats, and for geographic provenancing of seafood (Petraitis 2004; Brown 2006; Hastein 2001). Here, we use elemental fingerprinting of shells to identify optimal habitat for *A. irradians*.

In bivalve shells, mechanisms for incorporation of trace elements into biogenic carbonates during shell formation differ across species and life stage due to physiological factors and chemical and physical characteristics of the environment (Dodd 1967; de Paula and Silveira 2009). Temperature, pressure, salinity, dissolved oxygen, pH, and other environmental parameters can affect trace element speciation and bioavailability and so will influence the trace element composition of the shell. Despite the effects environmental parameters have on element incorporation, elemental concentrations have been used for many purposes including quantification of shell growth rates (Carré et al. 2006) as well as identification of natal habitat, water chemistry variation over time, recruitment success, stock structure, and movement patterns (Becker 2005). A recent study by Becker (2005) assessed spatial variability in the elemental composition of larval mussel shells (*Mytilus californianus* and *M. galloprovincialis*), and showed that spatial resolution of geographic source was possible at a scale of ~20 km. Although mussels

disperse as pelagic larvae, mussels are sessile as adults. To date, no one has examined whether it is possible to use elemental fingerprinting to resolve the location of essential habitats for bay scallops particularly at small (<10 km) spatial scales.

The goal of this research was to evaluate whether elemental chemistry of *A. irradians* shells, a rapidly growing nektonic species, could be used to identify zones defined by scallop abundances. Scallops are the only bivalves that have the ability to swim in the adult stage making reconstruction of their life histories more complicated than for sessile species. We developed an approach for identifying environmental correlates of essential *A. irradians* habitat characteristics at a meaningful spatial resolution. The environmental characteristics identified under-utilized habitat which could be leveraged into the current management strategy of this commercially important species.

Nantucket Harbor

We selected Nantucket Harbor (Figure 2-1) primarily due to its significant commercially fished bay scallop (*A. irradians*) population. Commercial populations of *A. irradians*, in Massachusetts (MA), occur in the waters of Cape Cod and the Islands. Landings of *A. irradians* in MA have declined precipitously over the past two decades from a mean of 399 metric tons ($1.4M) in the 1980's to a mean of 47 metric tons ($1.1M) from 2003-2010 (NMFS data; no data 1997-2002). As landings have declined, the market value of the MA *A. irradians* has increased. The market price and import pressure (especially from China) have led to a significant increase in coastal aquaculture research centered on the *A. irradians*. Despite significant investments in aquaculture and

seeding programs to increase recruitment and age class yields, we know comparatively little about the movement patterns of *A. irradians* within Nantucket Harbor.

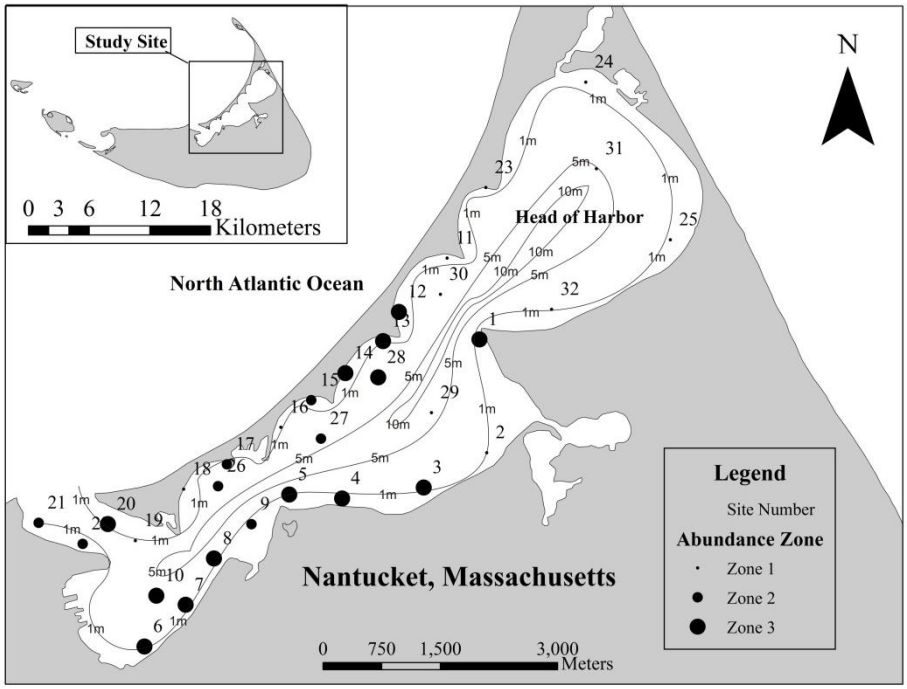

Figure 2-1: Collection sites within Nantucket Harbor (July 21st – August 12th, 2009). Abundance zones shown by circle diameter where the most abundant sites are indicated by the largest circle, and sites where no scallops were found are indicated by dots. (C.I. 2m)

Nantucket Harbor (Massachusetts, USA; Figure 2-1) is approximately 10 km^2, much smaller than other locations where shell-based trace element records have been used to reconstruct life histories of bivalves (Becker 2005). The Harbor is considered to be well-mixed due to tidal influences on the shallow basin and protected by strict environmental regulations controlling waste management (Wilkin 2006). Characterization of habitats relied on elemental analysis of *A. irradians* shells from

individuals collected at multiple locations within Nantucket Harbor. Using these data, we constructed a binary logistic regression model that enabled identification of source habitats and revealed a range of salinity and pH conditions that support high abundances of scallops.

2.3 Materials and Methods

Sampling and sampling sites

Site locations and number of scallops retrieved followed the sampling protocols of the Nantucket Shellfish Management Plan (http://www.nantucketharborplan.com/). In this experiment water sampling occurred between 9 a.m. and 11 a.m. to reduce the number of factors influencing pH, which is known to change over a daily cycle. We evaluated 32 sites (Figure 2-1) from mid-July to mid-August in 2009 for the presence/absence of bay scallops (*Argopecten irradians*). Twenty two sites had adult scallops present (Zones 2 & 3, Figure 2-1). Four of these sites also had juveniles (Sites 8, 13, 19, and 30) and ten were barren (Zone 1, Figure 2-1). *In situ* measurements of bottom water quality (e.g. pH (NBS standard scale) and salinity; Table 5-1; Appendix 7) were also made at the time of collection. All sites were well oxygenated (> 6.0 mg/L dissolved O_2) with a temperature range from 22.4°C to 26.5°C. Thirty adults were collected by divers from each of the Zone 2 & 3 sites. When juveniles (spat; average shell height of 10.5 mm) were visible we collected a small number (maximum of 10 from a site, 19 juveniles in total). Divers also recorded total scallop abundances on the 50 meter physically taped transect line at each site. The critical threshold for zone identification was defined by dividing zones of reported abundances, n = 71, where this

value divided three zones: Zone 1 (no scallops), Zone 2 (between 1 and 70 scallops), and Zone 3 (> 70 scallops) (Figure 2-1). The median value of individuals was 71 and the median routinely differentiated between low and high abundant sites as determined by the shellfish managers at Nantucket. Using this median value we define zones with scallops as low abundance (Zone 2, < 71) and high (Zone 3, 71).

Analytical procedure

Soft tissues were separated from the shells using a Teflon coated spatula. The shells were then cleaned by initially scrubbing off loosely attached particles in 18.2 M-Ohm water with a non-metal brush (adapted from Barats 2007). Shells were then rinsed in glacial acetic acid for 60 seconds, followed by 2 consecutive - 1 minute soaks in 18.2 M-Ohm water. Samples were allowed to dry overnight under laminar air flow at room temperature.

The right valve was used for LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry). A section of the shell, approximately 2 mm in thickness, was prepared, from the umbo to growing edge, using an IsoMet® diamond saw blade (Buehler Diamond Wafering Blade Series 15 LC blade) on an IsoMet® low speed saw. The section was secured to a glass petrographic slide using Crystal Bond™ adhesive (Electron Microscopy Sciences), and hand polished before analysis. A total of 5 thin sections of adult shells per site were prepared.

The LA-ICP-MS instrumentation consisted of a laser ablation system (213 nm Nd:YAG; Cetac Technologies, Omaha, Nebraska, USA) connected to a Perkin Elmer

ELAN DRC II ICP-MS (Perkin Elmer, Norwalk, CT, USA). Thirteen isotopes were monitored ^{43}Ca, ^{46}Ca, ^{55}Mn, ^{88}Sr, ^{138}Ba, ^{139}La, ^{208}Pb, ^{24}Mg, ^{57}Fe, ^{63}Cu, and ^{66}Zn. Analyte signal was collected using a line scan along the edge of most recent deposition only with a spot size of 200 μm at 25 μm/sec. Signal processing was performed using GeoPro2010 Software (Cetac Technologies). The mean signal along the edge (~500 μm; newest area of shell deposition) recorded at each isotopic mass for each scan was quantified as micromole per mole of Ca using USGS carbonate standard reference materials, MACS-1 and MACS-3. The calibration standards were analyzed 3 times each at the beginning of an analysis, and again every 10 samples. ^{43}Ca was monitored as an internal standard, and ^{46}Ca was used for quantification. Elemental ratios (element/Ca) were used for statistical analysis to account for heterogeneity in shell matrix. We also measured total carbon and nitrogen in duplicate on a Costech 4010 Elemental Analyzer from powdered shell material collected along the edge (~ 1 mg per sample).

Statistical Analysis

All elements were normalized to Ca concentration, except carbon and nitrogen. Multivariate statistics were used to determine if the elemental ratios could be used to distinguish individual sites or regions within the Harbor. For the binary logistic model used to predict which zone, low or high abundance, that an organism came from, a total of 118 individuals were used, where 54 came from Zone 2 and 64 were from Zone 3. Data reduction using factor analysis (principal component extraction with varimax rotation) identified elemental signatures used to allocate individuals to the zone of collection. Data from adult and juvenile scallops (edge only) were used to evaluate the

ability of the binary logistic regression to correctly allocate individuals to "zone" across life stages.

All statistical analyses were performed using SPSS 18. Binary logistic regression was used to preserve as much of the zone (low/high abundance) discriminatory information as possible, while the factor analysis was to reduce the variables to the lowest potential number of uncorrelated variables

2.4 Results

Of the 32 sites, between 0 and 144 *A. irradians* individuals were surveyed along each of the 50 m transects. Based on relative abundances of scallops, we defined zones within the Harbor as Zone 1 – barren, Zone 2 – low abundance, and Zone 3 – high abundance. None of the elemental ratios were found to be statistically different in adult shell edges between sites ($p = 0.050$). La/Ca, Pb/Ca, and Zn/Ca are shown as representations of adult shell edge elemental ratios that are not statistically different ($p=0.050$; Figure 2-2). Zone 2 samples typically show lower extremes in La/Ca and higher variance in Zn/Ca when compared with Zone 3.

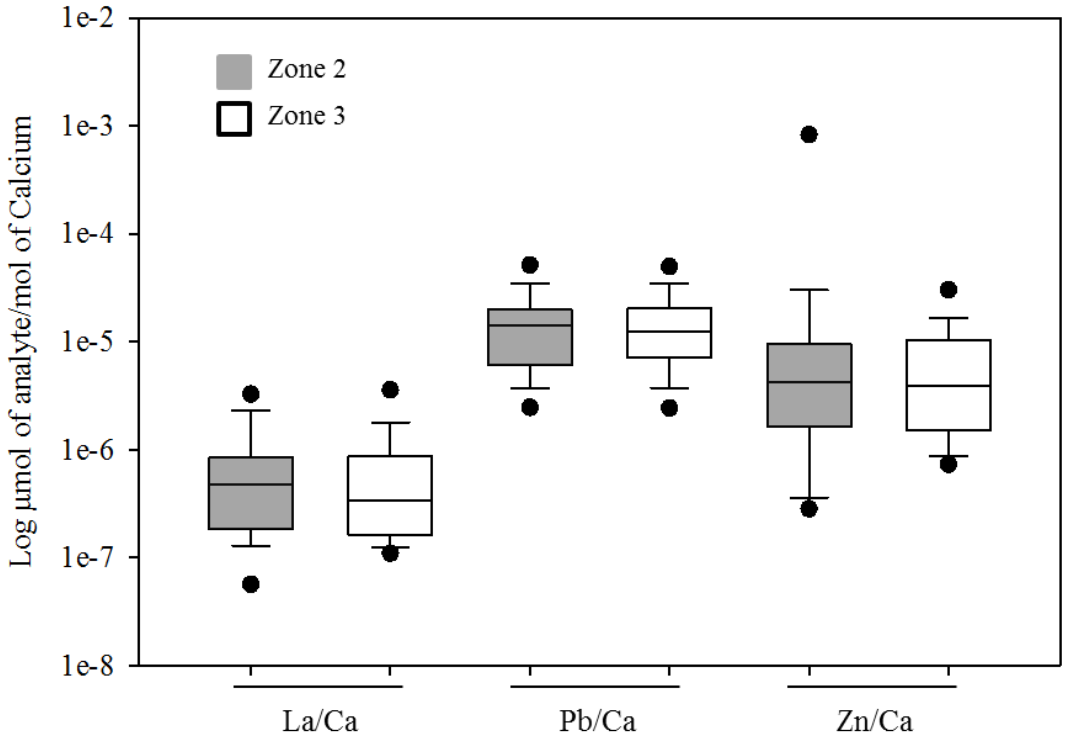

Figure 2-2**:** Log mean adult shell edge Zn/Ca, La/Ca, and Pb/Ca: Zone 2 (1-71 scallops) and Zone 3 (> 71 scallops). The 5th and 95th percentile are displayed as dots to indicate variance. A one-way ANOVA showed Zones were not statistically different at p = 0.05 with regards to La/Ca (p = 0.899), Pb/Ca (p = 0.771), or Zn/Ca (p = 0.756). Zone 1 was barren.

Next, ANOVA was used to test for differences in depth and bottom water quality (pH, temperature, salinity, dissolved oxygen) between scallop abundance zones. ANOVA indicated that there was a statistical relationship between depth and scallop abundance (F_{2,29} = 3.135, p = 0.059) and no statistical relations between scallop abundance and bottom water quality (Figure 2-3). Boxplots show Zone 1 often has higher variation in depth and bottom water quality than Zones 2 and 3 (Figure 2-3).

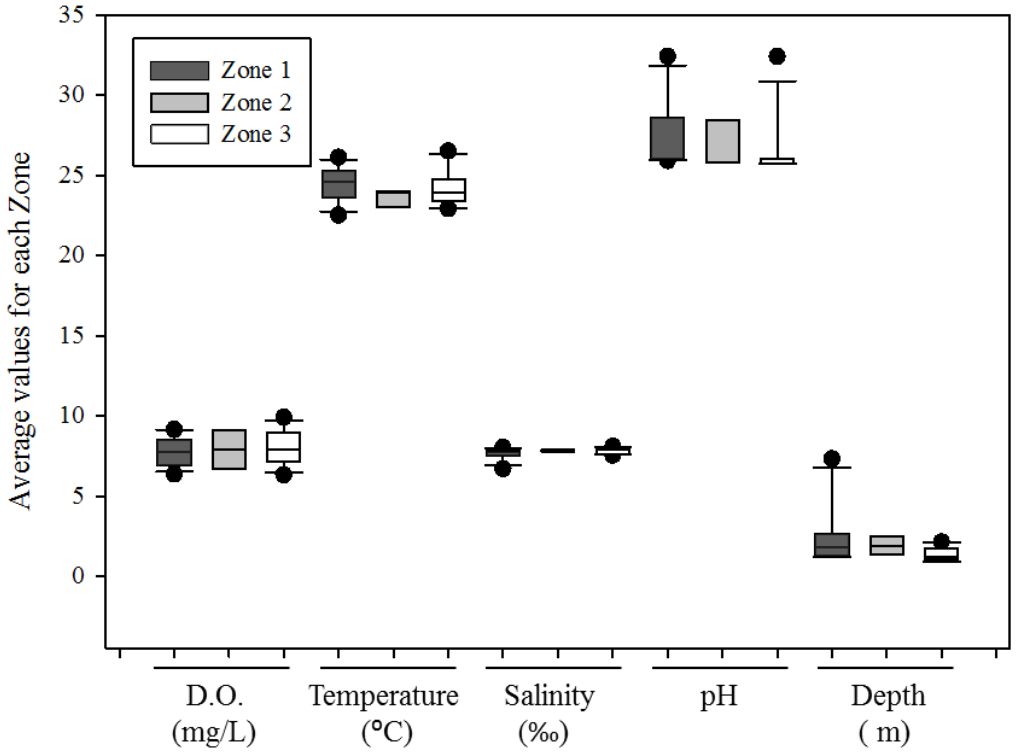

Figure 2-3: Mean physical bottom water quality parameters: Zone 1 (no scallops, n=12), Zone 2 (1-70 scallops, n=9), and Zone 3 (>71 scallops, n=13). There are no statistical differences between zones with regards to dissolved oxygen ($F_{2,29} = 0.742$, $p = 0.485$), temperature ($F_{2,29} = 1.204$, $p = 0.315$), salinity ($F_{2,29} = 0.673$, $p = 0.518$), pH ($F_{2,29} = 1.823$, $p = 0.180$), or depth ($F_{2,29} = 3.135$, $p = 0.059$).

Principal component analysis coupled with factor analysis was used to see how the variation in elemental ratios related to physical water parameters. Factor analysis was used to describe the variance in shell chemistry and water quality parameters from Zones 2 and 3 (e.g., pH and salinity) in terms of the lowest number of uncorrelated variables (factors). Using principal component extraction (Zones 2 and 3), 5 factors were identified that explain 74.4% of the variance in the dataset (Table 2-1). Factor 1 strongly influences La/Ca, Fe/Ca, Cu/Ca, Pb/Ca, and pH and accounts for the variance in trace

metal concentration and water column pH. Factor 2 accounts for variance in Sr/Ca, which is influenced by both temperature and salinity (Dorval et al. 2007). The first two factors explain a cumulative variance of 40.6%. Since ambient temperatures across the harbor are comparable within a given day, pH from Factor 1 and salinity from Factor 2 were identified as physical water quality parameters strongly influencing the shell elemental composition. Loading of samples on these factors, show that Zone 2 and Zone 3 cluster around an average salinity of 26 ± 2 ‰ (Figure 2-4). Some of the highest salinity values and lowest pH values are associated with the barren sites not included in the factor analysis.

Table 2-1: Eigenvalues and variance accounted for by factors extracted using principal component analysis (PCA).

	Eigenvalue	Proportion (%)	Cumulative (%)
PC1	2.785	25.317	25.317
PC2	1.678	15.255	40.572
PC3	1.394	12.675	53.247
PC4	1.303	11.844	65.091
PC5	1.019	9.262	74.353

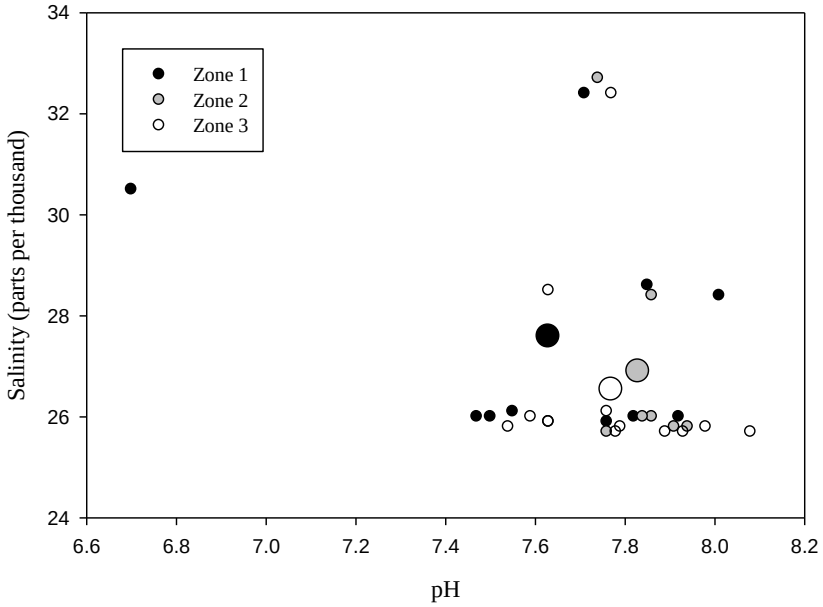

Figure 2-4: Salinity (‰) and pH of all sites by Zone. Zone 3 (> 70 scallops) defined by an average salinity of 26 ± 2 ‰ and pH of 7.80 ± 0.13. The large circles represented for each zone indicate the average salinity and pH for the given abundance.

Factor 3 influences total carbon, Mg/Ca, and also accounts for some of the variance in Mn/Ca, Ba/Ca and D.O. Since *A. irradians* shell is primarily composed as a low-Mg calcite, we attribute Factor 3 to variance associated with shell matrix composition. Factor 4 influences Zn/Ca and accounts for variance in protein composition and/or anthropogenic contamination. Factor 5 influences both nitrogen and depth and is associated with diet as nitrogen in the shell is likely provided by the algae which are most abundant at mid-depth sites such as in Zone 3. Based on principal component analysis, 5 elemental ratios (Mn/Ca, Sr/Ca, Fe/Ca, Cu/Ca, and Pb/Ca) were identified that account for variance across abundance zones. The model revealed the influence of pH and

salinity on shell elemental ratios and also the differences in influence between abundance zones.

A binary logistic regression was used (n-adults=100, n-juvenile=18) to allocate specimens to the zone of collection based on shell Mn/Ca, Sr/Ca, Fe/Ca, Cu/Ca, and Pb/Ca. The binary logistic regression predicted membership (n = 118) in either Zone 2 or Zone 3 with 72.9% accuracy. Predicted "zone" membership probabilities (Figure 2-5) show overlap between zones. Predicted probabilities for Zone 2 sites were generally more variable than those from Zone 3. The model presented here applies primarily to adults since juveniles were not collected in both zones. Binary logistic regression showed that individuals from Zone 3 were correctly classified 90.6%. The the ability to classify individuals from Zone 2 was barely better than chance (51.9%).

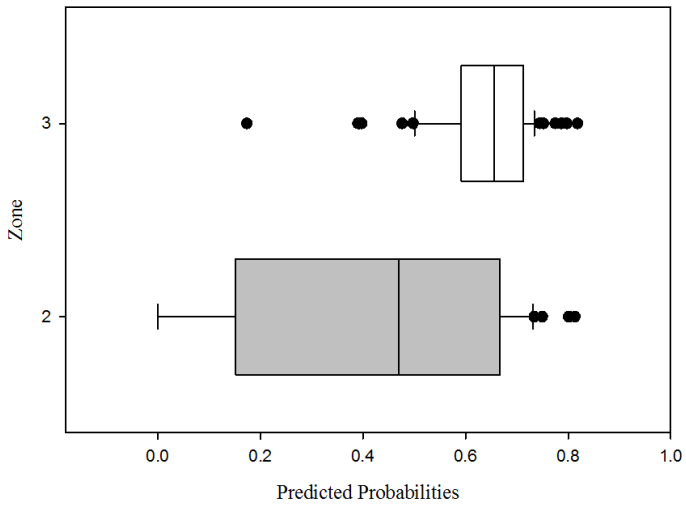

Figure 2-5: Predicted "zone" membership probabilities from a binary logistic model show an overlap between adult and juveniles samples between Zones 2 and 3, which are defined by abundance.

2.5 Discussion

Allocation of individuals to the zone of collection relied on the variance of elements that accumulate in shell (Lin and Liao 1999). Elemental ratios were not, individually, different between zones (e.g., Pb/Ca Zone 2 vs. Pb/Ca Zone 3). Given that free metal ion activity is strongly controlled by pH, which varies little across zones, the lack of significant differences in shell Fe/Ca, Cu/Ca, and La/Ca is not surprising. Though not statistically significant, Pb/Ca in shells tended to be higher in Zone 2 adults than in Zone 3 adults. In mussel shells, individuals collected from habitats located close to areas of elevated dissolved Pb (e.g., anthropogenic input) have increased concentration in their shells (Richardson et al 2001). Pb levels in whole shells of the New Zealand cockle, *Chione (Austrovenus) stutchburyi,* were similar to the soft tissues (Purchase and Ferguson 1986), while the lowest Pb levels are enriched in the oldest regions of a shell. Therefore it could be that Zone 2 habitats are more closely impacted by anthropogenic activity along the coast of the harbor.The purpose of this study was to build a robust model that characterizes elemental/Ca signatures and physical characteristics of sites that currently support large numbers of *A. irradians*. In order to characterize these habitats, sites were grouped based on overall abundance of the *A. irradians* along 50 meter transects at 32 sites. In this study, the abundance of the *A. irradians* is assumed to be reflective of optimal conditions for both juvenile and adult *A. irradians*. Since our data were collected over a short period of time they do not capture large-scale annual or seasonal variations in water quality.

Using elemental ratios in shells of adult and juvenile *A. irradians* we were able to allocate to abundance zones over a small spatial scale (< 10 km). The lack of scallops at the head of the harbor is attributed to lower tidal flushing and local variations in depth due to sediment accumulation (Wilkin 2006). Head of the harbor depths are greater than in other regions of the harbor (~ 7.3 m) that are closer to the shore and near the harbor mouth where scallops were abundant (average = 1.5 m). Also, in the head of the Harbor, the substrate needed to support eel grass beds, which are critical substrates for juvenile settlement, is often absent.

Based on factor analysis, shell chemistry was associated with scallop abundance indicating strong relations between the physical bottom water chemistry. Shell Mn/Ca, Fe/Ca, Cu/Ca, Pb/Ca, and Sr/Ca were used to allocate adults and juveniles to abundance zone. Previous research on other biogenic carbonate structures, such as fish otoliths (earstones), concluded that salinity changes (Δ = 4.84 ‰) strongly influenced carbonate Ba/Ca and La/Ca (Dorval 2007). This is a smaller change than we observed in Nantucket Harbor (Δ = 7.00 ‰) and so we can attribute some of the variance in shell Ba/Ca and La/Ca to differences in salinity between zones. The variability in shell Mg/Ca can be attributed to metabolically regulated partitioning of Mg within the low-Mg calcite shell of *A. irradians*. In the spotted seatrout, Mg/Ca incorporated into the otolith is expected to be regulated in blood plasma (Dorval 2007), which may mean that, in other biogenic calcites, such as the low-Mg calcite of *A. irradians* shells, the partition coefficient may be strongly influenced by biochemical processes (Campana 1999) or preferential release of

Mg from the lattice to the surrounding water as in corals (Amiel et al. 1973). Variations in shell Mn/Ca, Fe/Ca, Sr/Ca, Cu/Ca, and Pb/Ca are most likely due to changes in the surrounding water chemistry. We found individuals from Zones 2 and 3 are discriminated using these shell elemental chemistries. By utilizing the chemistry of the *A. irradians* shells collected at various ages, our model has provided evidence that there are areas, currently barren, within the harbor that could support scallops. Binary logistic regression modeling revealed differences in pH and salinity that are associated to high abundances of scallop. Based on average values for Zones 1, 2, and 3, the optimal pH was approximately 7.80 ± 0.13 with a salinity of 26 ± 2 ‰. Barren sites had greater variability. For example within Zone 1, site 19 has a high salinity (32.4 ‰) with a relatively normal pH (7.71). However, site 31 also in Zone 1, has a reduced salinity (25.9 ‰) and a low pH (6.97) reflective of the distance from the harbor mouth and water freshening that occurs from runoff. The lack of scallops in Zone 1 suggests that the pH or salinity extremes prevent scallops from residing at a given site. There were small differences in the water chemistry between Zones 2 and 3 associated with their position in the Harbor. This is reflected by small variations in elemental chemistry that is incorporated into the shell providing further evidence that the Harbor is well-mixed.

In conclusion, based on binary factorial analysis shell chemistry (Mn/Ca, Fe/Ca, Cu/Ca, Sr/Ca and Pb/Ca) allocated individuals, with 72.9% overall accuracy, to either Zone 2 or 3. Variations in pH and salinity were directly associated with zonal differences in the trace elements incorporated into the shell. The ability of the shells to both

incorporate and maintain the relative concentrations of metals in the surrounding water suggest that shell chemistry is useful in identifying essential habitats in Nantucket Harbor. Moreover, since the elemental chemistry is associated with salinity and pH, trace element chemistry can be used to evaluate changes in salinity and pH away from optimal conditions. Using shell chemistry to identify habitat could also provide an estimate of the overall health of a population given that shell chemistry differences are associated, here, with pH and salinity. For example, if the harbor becomes more acidic, free metal Pb concentration is likely to increase and Pb/Ca ratios would be expected to increase. Thus, the incorporation of Pb to bay scallop shells may be dependent on ocean acidity.

The ability of binary logistic regression to distinguish between habitat Zones 2 and 3 also provides a management tool to assess habitat quality for conservation efforts. Based on the results presented here, combining water quality and population abundances, sites 2, 11, and 23 in Zone 1 (currently barren) have the water quality characteristics necessary to support *A. irradians* which could translate to an increase of 35,000 to 1,400,000 individuals. Thus, the information provided by our model could allow managers to make decisions on seeding habitats as well as identifying sites for conservation. In addition to providing crucial data for population management, this approach also provides insights into how *A. irradians* may respond to changes in pH associated with water freshening and ocean acidification.

2.6 Acknowledgments

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CHAPTER 3

OCEAN ACIDIFICATION IMPACTS NET CALCIFICATION IN ADULT AND JUVENILE *ARGOPECTEN IRRADIANS* (LARMARCK 1819)

3.1 Abstract

Predicting the impact of ocean acidification on calcification across life stages of invertebrates is essential in anticipating the consequences of future climate change on these populations. Our study focused on the impact of ocean acidification on net calcification in bay scallops (*Argopecten irradians*). We investigated the impacts on both the juvenile and adult life stages and explored the relation between life stage and net calcification rate across low pH induced by increased pCO_2. Scallops were maintained for three weeks in seawater bubbled with air-CO_2 mixtures yielding pH values of 7.86 ± 0.19 (Control, $pCO_{2(aq)}$=468 ± 191 μatm), 7.61 ± 0.16 (Treatment 1, $pCO_{2(aq)}$=1029 ± 130 μatm), 7.38 ± 0.09 (Treatment 2, $pCO_{2(aq)}$=1823 ± 220 μatm), and 7.19 ± 0.10 (Treatment 3, $pCO_{2(aq)}$=3437 ± 518 μatm). Net calcification, estimated by buoyant weight, was lower in adults compared to juveniles raised under the same conditions (e.g. pH = 7.2, Net Calcification $Rate_{adult}$ = -0.0058 d^{-1}, Net Calcification $Rate_{juvenile}$ = -0.0036 d^{-1}). No statistically significant differences were found in survivorship between treatments or life

stages. Our results indicate that ocean acidification impacts net calcification most strongly in adults and we attribute the differences in net calcification in adults compared to juveniles to the fact that adults, having fully developed gonads, are focusing energy on reproduction rather than shell growth.

3.2 Introduction

Monitoring of atmospheric CO_2 and $\delta^{13}C_{CO2}$ at the Mauna Loa Observatory (Keeling, 1960) revealed rapid increases in atmospheric CO_2 attributable to anthropogenic CO_2 inputs (e.g., fossil fuel burning). A decline in ocean pH by 0.1 units since pre-industrial times is directly associated with increased atmospheric CO_2 (Orr et al., 2005), such that the mean pH of today's ocean averages 8.08 (Potera, 2010). The shift in ocean pH to less alkaline conditions due to increased atmospheric CO_2 is referred to as **"**ocean acidification**"** (Borges, 2010). The relation between atmospheric CO_2 and ocean pH results from CO_2 diffusion into surface waters based on partial pressure differences (Henry's Law) between the atmosphere and ocean. CO_2, once dissolved in the surface waters, can form carbonic acid (H_2CO_3) as the first step in a series of several reversible dissociation reactions that release free hydrogen ions (H^+) to form bicarbonate ion (HCO_3^-) and also lead to dissociative formation of carbonate ion (CO_3^{2-}) (Eq.4). The resultant increase in dissolved free hydrogen ion (H^+) due to the formation of carbonate ion leads to a decline in pH, where pH = -log[H^+].

$$CO_2(g) + H_2O(l) \rightleftarrows H_2CO_3(aq) \rightleftarrows H^+(aq) + HCO_3^- \rightleftarrows 2H^+(aq) + CO_3^{2-}(aq) \quad \text{Eq.4}$$

The ability of the ocean to absorb atmospheric CO_2, whether from a natural or anthropogenic source, depends largely on the extent of dissolution of calcium carbonate ($CaCO_3$(s)) and the resultant production of dissolved calcium (Ca^{2+}) and carbonate (CO_3^{2-}) ions. Whether $CaCO_3$(s) is formed or dissolved depends upon the concentrations of both the calcium and carbonate ions, and the solubility product (K_{sp}) of the mineral. The solubility product of a mineral is itself, dependent upon the pressure, temperature, and salinity of the environment in which the mineral forms/dissolves, and the crystal structure (e.g., polymorph) of the mineral. These relations of concentration and solubility define the saturation state (Ω, Eq. 5).

$$\Omega = \frac{\left[Ca^{2+}\right]\left[CO_3^{2-}\right]}{K_{sp}} \qquad \text{Eq. 5}$$

For ocean surface waters at a pH ~ 8.1, the carbonate reactions (Eq.4) are reversible and concentrations are near equilibrium (Millero, 2002) such that approximately 90% of the inorganic carbon is HCO_3^-, 9% is CO_3^{2-}, and 1% is dissolved CO_2. However, the increase of aqueous CO_2 leads to increasing $[H^+]$, which causes a decrease in carbonate ion concentration, $[CO_3^{2-}]$ (Eq. 5). Thus the projected 0.3 to 0.4 pH drop from today's oceanic pH (increased hydrogen ion concentrations by up to 150 %) projected for the future ocean under conditions of continued anthropogenic CO_2 input to the atmosphere will result in a decrease in dissolved $[CO_3^{2-}]$ by 50%.

Calcium carbonate can occur in a variety of crystal structures in shell material, such as calcite (trigonal; Ω = 5 - 6), aragonite (orthorhombic, Ω = 3 - 4), vaterite

(hexagonal, Ω = 2 - 3), and high-magnesium (Mg) calcite (trigonal, Ω = 2 - 3). Aragonite and calcite containing more than 4 mole % $MgCO_3$ (high-Mg calcite) in solid solution with $CaCO_3$ are metastable and are the most common calcium carbonate skeletal materials (Chave et al. 1962). Because of their low Ω values, aragonite and high-Mg calcite are the dominant forms precipitating in today's ocean (Tynan and Opdyke, 2011, and references therein). All living pectinoids (e.g., *A. irradians*) have calcite in the outer shell layer (Carter et al. 1998). In *A. irradians*, low-Mg calcite occurs in most, if not all, of the middle and inner shell layers, with only the prodissoconch, ligament, and muscle attachment sites (myostracum) containing aragonite (Taylor et al. 1969, 1973; Carter 1990). Incorporation of Mg within the calcite crystal lattice enhances the solubility of the mineral making it more soluble than pure calcite (Berner 1974). The saturation state is dependent, to a large extent, on the amount of $MgCO_3$ in solid solution, and the exact value of Ω for the calcite present in *A. irradians* shell which is not known.

Changes in the saturation state of the ocean with respect to $CaCO_3$ is related directly to changes in carbonate ion concentration which has varied throughout the Phanerozoic in response to changes in atmospheric CO_2, carbon sequestration, continental weathering, temperature, and sea level (Palmer and Wilson 2004). Throughout the Phanerozoic, seawater chemistry has oscillated between 'aragonite' and 'calcite' seas. During periods of 'aragonite' seas, such as today, aragonite and high-Mg calcite are stable (Harper et al. 1997). Interestingly, there is a clear link between the evolution of bivalves and shell mineralogy attributable to oscillations in carbonate

saturation states of the ocean, and the paleontogical record indicates that low-Mg calcite shells, such as found in *A. irradians,* first appeared during the middle Paleozoic when oceans were predominantly 'calcite' seas. Therefore, Ω for low-Mg calcite must be higher than that of aragonite and lower than that of pure calcite. In fact, it is widely accepted that bivalves with entirely aragonitic shells were at a selective disadvantage during periods of 'calcite' seas compared to those with calcitic outer shell layers. However, today's ocean is aragonitic so it is reasonable to consider that *A. irradians,* and other bivalves with shells comprised almost entirely of low-Mg calcite as well as bivalves with aragonite shells, may be at a selective disadvantage as ocean pH declines.

Here we evaluate the effects of ocean acidification on net calcification in two life stages, adult and juvenile, of the bay scallop, *Argopecten irradians* [Lamarck 1819]. Previous work by Ries (2009) found that adult *A. irradians* showed a net calcification decrease as the partial pCO_2 was experimentally increased to 2856 μatm (pH ~ 7.45). The decline in pH led to a decrease in the $[CO_3^{2-}]$ from 158.2 μmol/kg SW (seawater) to 41.0 μmol/kg SW. To date no experiments have explored impacts in juveniles. Loss of shell or slowed rates of shell growth due to low pH induced by increased atmospheric CO_2 could lead to juveniles being unable to metamorphose into their adult stage and to stunted growth in adults (Talmage, 2009). Both of these outcomes could have dramatic effects on *A. irradians* populations. Landings of *A. irradians* in Massachusetts alone have declined precipitously over the past two decades from a mean of 399 metric tons ($1.4M) in the 1980's to a mean of 47 metric tons ($1.1M) from 2003-2010 (NMFS data; no data 1997-2002).

3.3 Methods

Specimen Collection and Experimental Conditions

Juvenile and adult *A. irradians* were collected from Nantucket Sound, MA, pursuant to local, state, and federal regulations, by the Martha's Vineyard Shellfish Group, Inc. After collection, specimens were transported, live, to the University of Massachusetts Boston where they were artificially tagged with numbers and randomly distributed between tanks using a random number table. Adult and juvenile *A. irradians,* were maintained for 25 (adult) and 21 (juvenile) days in 5 gallon plastic aquaria filled with 5 μm-filtered seawater collected from Boston Harbor at the Fox Point dock in Boston, MA. Prior to treatments all specimens were acclimated in seawater (pH ~ 7.9, pCO_2 ~ 425 ppm) for 7 days. Adults were maintained (July/August 2011) without tank replication due to significant mortality (50%) during transport from the hatchery. Juveniles were maintained (September/October 2011), in duplicate tanks, to assess tank effects within each pH treatment. Each aquarium was equipped with a salt water fan to induce water movement to promote feeding and covered with a plexiglass lid to minimize evaporative water-loss. Seventy-five percent of seawater was replaced twice a week throughout the duration of the experiment. Organisms were fed aliquots of Shellfish Diet 1800 (Reed Mariculture Inc.) twice a day to achieve $1.0x10^6$ cells per mL of seawater each day. This commercially produced feed contains four marine microalgae – *Isochrysis sp, Pavlova sp, Thalassiosira weissflogii,* and *Tetraselmis sp*.

The control pH value (NBS scale) of 7.8 was chosen based on a field survey of *A. irradian* populations found within the Nantucket Harbor (Chapter 2). Adult *A. irradians*,

were maintained for 25 days in experimental tanks equilibrated to average pCO_2 values μatm (± standard deviation, SD) of 513 (±202; Control; pH=7.86 ± 0.19), 1045 (±105; Treatment 1; pH=7.61 ± 0.16), 1880 (±223; Treatment 2; pH=7.38 ± 0.09), and 3440 (±544; Treatment 3; pH=7.19 ± 0.10). Aragonite and calcite saturations states were modeled using CO2SYS (Pierrot, 2006). The saturation state of low-Mg calcite cannot be modeled using CO2SYS, but it is expected to be between aragonite and calcite. In our experiment, Treatment 1 and Treatment 2 modeled pCO_2 concentrations are similar to those used of Ries (2009) as well as representative of values predicted for the coming millennium (Feely, 2004). We added Treatment 3 as an extreme that would likely induce an effect over the short duration of the experiments. Juvenile (average shell height from umbo to ventral edge = 18.25 mm) *A. irradians* juveniles were maintained, in duplicate, for 21 days in seawaters equilibrated to similar modeled average pCO_2 values to those in the adult exposure (Table 3.1 and in Appendix 2).

Measurement of seawater parameters and calculation of carbonate system parameters

Tanks were dosed with air-CO_2 mixtures using Allied Electronic (Fort Worth, TX) air solenoids (Part # = VX2330-02T-3CR1) and dosing maintained by pH feedback with each tank equipped with an individual Digital Aquatics (Woodinville, WA) pH probes. This experimental setup allowed dosing to be controlled by monitoring pH rather than through introduction of known concentrations of pure CO_2 and in-line monitoring of pCO_2 which is cost-prohibitive. The gas mixtures were introduced by pre-mixing CO_2 from an ultra-pure CO_2 cylinder with room air and then the mixture was bubbled through an air-stone in each tank that was secured to the base of the aquaria directly below

seawater fans to encourage mixing and rapid diffusion of the CO_2. Salinity, temperature, and conductivity were measured daily. pH, calibrated to NBS standards, was logged every 10 minutes using the DigiAquatics data management system, ReefKeeper Elite. pH was confirmed daily using an external meter to account for any drift in the pH probe influenced by algal growth or electronic noise. Alkalinity and dissolved inorganic carbon were determined, in parallel, twice a week. Alkalinity was determined following the EPA 5.10 total alkalinity method, which involved a titration with 1.6 N sulfuric acid using a Hach digital titrator. Aragonite and calcite saturation states, pCO_2, and dissolved inorganic carbon values (± standard deviation) were modeled from measured parameters using CO2SYS (Pierrot, 2006), where concentrations of total phosphate and silicate were set to 0 (Appendix 2). The juvenile experiment was performed in duplicate and data are presented as individual values (value for first tank/value for second tank).

Determination of dissolved inorganic carbon in seawater

Dissolved inorganic carbon was measured using a Tekmar Dohrmann Phoenix 8000 UV-Persulfate TOC analyzer (Model 8000). Samples were analyzed immediately after a 0.45 μm filtration at the same time as measurements of total alkalinity to reduce degassing of CO_2 enriched seawaters. For dissolved inorganic carbon (DIC), aliquots of sample were acidified with orthophosphoric acid to a pH of 3 followed by sparging with a stream of N_2(g). The method detection limit for DIC was 20.7 +/- XXXX μmol/kg of seawater.

Determination of bacterial abundance

Seawater samples were preserved by using 10 μL of glutaraldehyde per mL of seawater sample to determine bacterial abundances for the control and treatment tanks. 4',6-diamidino-2-phenylindole (DAPI) at a concentration of 50 μg/mL was used to stain bacteria for 7 minutes (Porter, 1980). Bacterial abundance values were determined only in the juvenile experiment across all treatments and control.

Net calcification and Survivorship

Net calcification rate (total calcification minus total dissolution, Δg, divided by initial weight, g_o, , all divided by days of exposure, d; $\Delta g/g_o/d$) for the various pCO_2 treatments was estimated using a buoyant weight method and qualified with shell dry weight measurements after harvesting (Ries, 2009). The buoyant weight of the *A. irradians*, in this experiment, reflects total calcification minus loss of $CaCO_3$ through dissolution. In order to obtain the buoyant weight, specimens were suspended in their dosed aquarium in a basket attached to an aluminum wire hanging on a Mettler Toledo balance. The shell dry weight measurement is the organism's shell weight after all the soft tissues were removed. Survivorship was determined based on number of live specimens at the end of the experiment ratioed to the total number at the start.

3.4 Results

Dissolved inorganic carbon (DIC), measured and modeled in the exposed seawater, were compared since DIC is the most pertinent to calcification (Ries 2009) (Figure 3-1). Measured and modeled DIC showed excellent correspondence. Measured

DIC values were only slightly lower than modeled values. However, the high R^2 values indicate that the dosing system precisely controlled pH through pCO_2 introduction.

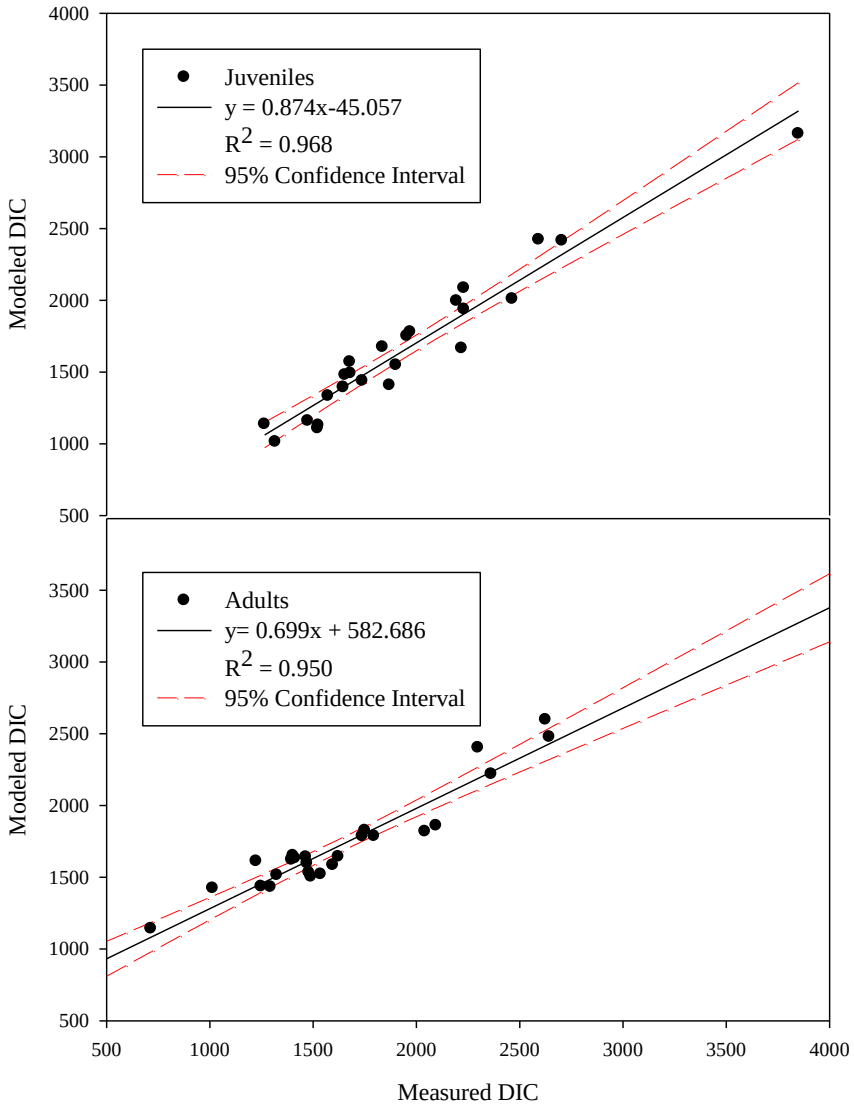

Figure 3-1: Modeled dissolved inorganic carbon (DIC) (µmol/kg seawater, SW; CO2SYS) compared to measured dissolved inorganic carbon (µmol/kg SW) for all experiments. The juvenile (A) modeled and measured DIC show strong correlation (R^2=0.968) indicating that the experimental conditions and pH-based dosing (Table 3.1; Appendix 2).

The adult (B) modeled and measured DIC similarly show a strong correlation (R^2 = 0.950) indicating precise pCO_2 control.

We also compared buoyant weight to dry shell weight to evaluate the efficacy of buoyant weight as an estimator of net calcification. The two methods correspond (Figure 3-2; R^2=0.999) and so we used buoyant weight to estimate net calcification. Shell dry weight was higher than the buoyant shell weight most likely due to the fact that shell dry weight includes the mass addition of chitin, protein, and other organic tissue, which is displaced when shells are suspended in seawater during buoyant weight measurements.

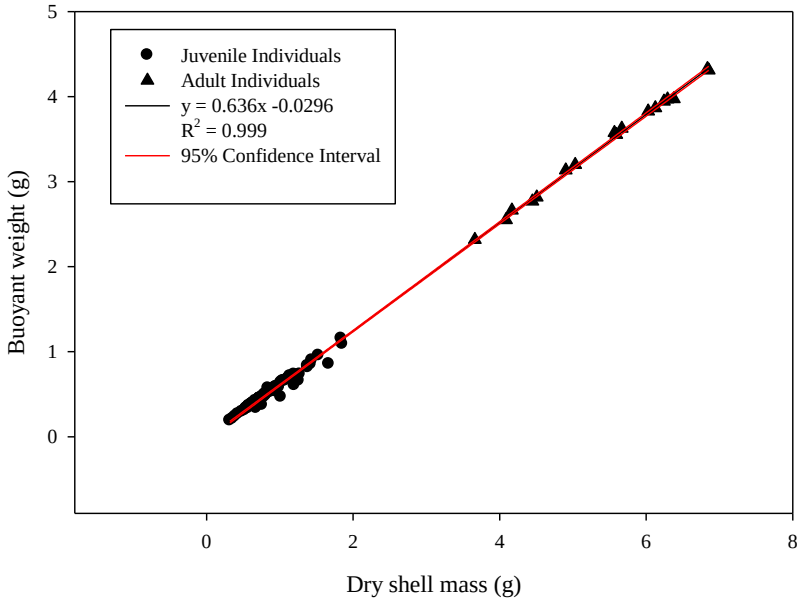

Figure 3-2: Comparison of the buoyant weight to dry shell weight showing a strong linear relation (R^2 = 0.999) (circles - juveniles, triangles - adults). Correspondence between these methods provides two independent measures of net calcification.

In the juvenile and adult experiments, a negative net calcification rate is seen in both the control and treatment exposures (Figure 3-3). The greatest difference between

age groups occurs in Treatment 3 (pH = 7.2). We also found linear relations between net calcification rate and pH in both adults (R^2 = 0.815) and juveniles (R^2 = 0.378).

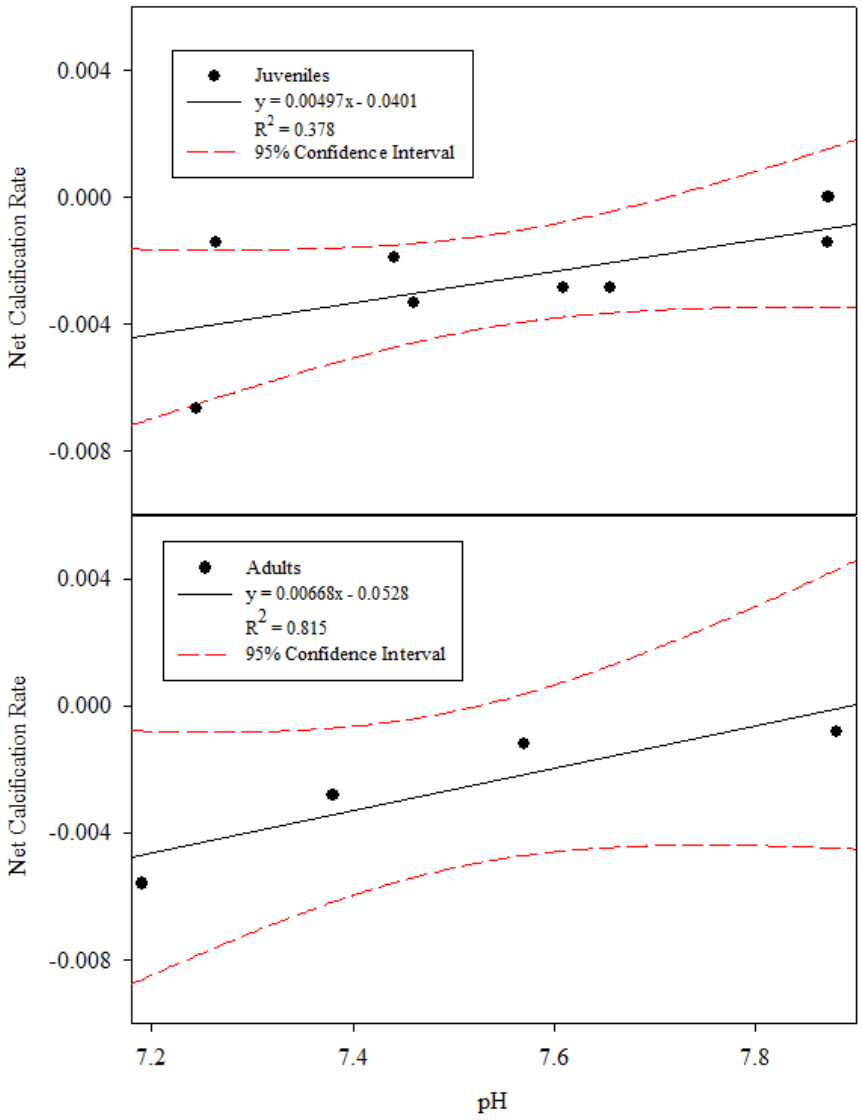

Figure 3-3: Comparison of net calcification between adults and juveniles. Net calcification is calculated as [starting buoyant weight minus end buoyant weight] / starting buoyant weight. These values were calculated as rates to account for treatment length differences between juveniles and adults assuming a constant loss. The lowest pH treatment (Treatment 3; 7.2) had the highest shell loss in both adults and juveniles.

Survivorship showed no statistically significant relation to elevated CO_2 treatment levels (Table 3-1). After approximately two weeks of exposure, adults and juveniles in Treatment 3 were observed to have hinge ligament separation (33% of the adults and 11% of the juveniles).

Table 3-1: Saturation states and percent survivorship for adult and juvenile experiment per exposure period during the four-level CO_2 experiments. The juvenile experiment was performed in duplication and is presented as individual values below (value for first tank/values for second tank). Standard deviations are provided in (Appendix 2).

	Control	Treatment 1	Treatment 2	Treatment 3
Adult $\Omega_{calcite}$	2.20	1.26	0.88	0.73
Adult $\Omega_{aragonite}$	1.42	0.81	0.57	0.47
Adult Survivorship	33%	25%	77%	17%
Juvenile $\Omega_{calcite}$	1.42/1.30	0.90/0.99	0.75/0.82	0.54/0.65
Juvenile $\Omega_{aragonite}$	0.91/0.84	0.58/0.64	0.48/0.53	0.35/0.42
Juvenile Survivorship	43%/83%	60%/34%	47%/57%	64%/79%

The average bacterial abundance count per mL of seawater ($10^8 \pm$ SD), in the juvenile Control tank was 2.6 ± 0.6. Bacterial counts declined with decreasing pH (Treatment 1: 3.3 ± 0.1; Treatment 2: 2.6 ± 0.4; Treatment 3: 1.4 ± 0.2). These data show a decline in bacterial abundance with the respect to decrease in pH which might positively impact *A. irradians* survival at lower pH values.

3.5 Discussion

Treatment 3, an extreme pH of 7.2, showed the strongest negative impact of calcification. Juveniles and adults exhibited differences in net calcification rates between

the control (pH = 7.9) and Treatment 3 (pH = 7.2). A linear regression of net calcification rate of juveniles against pH shows that pH accounts for 37.8 % of the variance in the response while the adult model explains 81.5 % of the variance in net calcification rate. The difference in the strength of the relation in juveniles versus adults may be attributable to significant dissolution seen in the adults in Treatment 3. Juveniles in Treatments 1 and 2 (pH = 7.6 and 7.4) showed similar net calcification rates and so the strength overall, of the relation in juveniles between net calcification rate and pH is less than that of adults. These results are aligned with those of Ries (2009) who found that the adult bay scallop showed lower shell accretion in response to increasing acidification. It is important to note that, in this experimentally comparative study, Ries et al. (2009) achieved a positive net calcification within all treatments while our study found evidence of shell loss, even in the Control. The differences between these two studies are best explained by differences in experimental conditions (Ries vs. this study; flow-through vs. in-tank fan, live feed vs. commercial diet, and photoperiod -10 hours/day of 213 W/m^2 irradiance vs. 12 hours/day of unmonitored irradiance). While we cannot, based on the data collected here, identify the source(s) of difference, both the Ries et al. (2009) study and our results show a negative response in calcification with respect to pH.

In adult bay scallops, our data show a stepwise progression of shell loss from Treatment 1 to Treatment 3. Adult bay scallops direct most of their energy toward the development of gonads for reproduction (Guderley, 2010). It may be that adults are unable to acclimate over the 25 day exposure and re-initiate shell growth to compensate for reduced pH conditions. In juveniles, however, the gonads are not developed and

energy is primarily directed towards somatic and shell growth (Guderley, 2010). This may explain why juveniles appear to fare better, at least in terms of net calcification rates, than the adults in Treatments 1 and 2 (pH = 7.6 and 7.4). Our data also indicate that juvenile *A. irradians* can better maintain calcification at pH values between 7.4 and 7.6 than adults. *A. irradians* are geographically limited (Brand, 2006) and their essential habitats are characterized by a narrow range of pH (~7.5 – 7.9; Chapter 2). Our results suggest that juveniles exposed to pH values at the extreme of what is predicted in future ocean acidification scenarios (Treatments 1 and 2) may have sufficient capacity to respond to decreases in pH and may exhibit phenotypic plasticity enabling them to persist in shallow marine environments that are inherently dynamic in terms of pH (Feely, 2008).

We noted that hinge ligaments, which contain aragonite (Kikuchi, 1987), separated in some of the adult and juvenile specimens in Treatment 3, where the $\Omega_{aragonite}$ was below 0.50 suggesting low-pH induced dissolution of the calcified portion of the ligament. Shell integrity, reflected by shell thickness, as well as the development and morphology of the hinge are negatively impacted at elevated CO_2 levels (Talmage, 2010). The hinge ligament provides an elastic mechanism for opening the shell that allows the scallop to effectively filter feed and transport waste away from its body (Eble, 2001), and is crucial for the scallops swimming behavior. In the wild, the inability to swim means that *A. irradians* cannot avoid predators. Moreover, the thinning of the shell can lead to opportunisitic predation (Talmage, 2010), and the weakened shell cannot provide the required physical protection for the soft tissues (Carriker, 1986). In our experiments, bay

scallops with separated hinge ligaments typically survived no more than 2 days after the hinge separated. The separated hinge ligament may lead to damage of the mantle where the extrapallial space is sealed from exchange with the surrounding seawater allowing for bacterial attack.

Previous research on *A. irradians* has indicated that ocean acidification events lead to decreases in survivorship and size at age (Talmage, 2009). We found no significant trends with respect to survivorship. Juvenile bivalves are prone to shell dissolution and shell loss (Green, 2004) and this may be exacerbated by ocean acidifying events. Bacterial abundance of water samples collected in our juvenile experiment indicated that the abundance of naturally occurring bacteria was reduced at lower pH. This decrease in bacterial abundance may explain why juveniles at the extreme pH, Treatment 3 (7.2) showed higher survivorship compared to Treatment 2. These data hint at the possibility that bacterial community composition may change as a result of ocean acidification and that lower bacterial counts may enable survival under low pH conditions.

Declines in commercial shellfish landings, nationally, can be attributed to environmental change (e.g., hypoxia) and human activities (e.g., overfishing) (Gobler, 2005; Jackson, 2001). Ocean acidification may also contribute to these declines (Talmage, 2010). With the rapid rate at which atmospheric CO_2 is increasing today compared to other ocean acidification events in the Earth's deep past (Sabine, 2004), *A. irradians*, and other calcifiers, are faced with environmental pressures that will probably require acclimation and adaptation to survive. *A. irradians* are highly vulnerable to

ocean acidification as its shell is primarily composed of low–Mg calcite, the formation of which is strongly influenced by the availability of CO_3^{2-} and, ultimately, ocean pH (Mann, 2001). These bivalves, broadcast spawners, typically show high larval and post-settlement mortalities (Guinotte, 2008). Population declines may be exacerbated by ocean acidification as the benthic juveniles experience loss of shell which may not only negatively impact shell integrity, but may lead to decreased growth and smaller size-at-age.

3.6 Conclusion

Our study revealed that CO_2-induced reductions in pH from 7.9 to 7.2 led to significant impacts on net calcification in juvenile and adult *A. irradians*. The life stage comparison indicated that juveniles are better able to maintain shell growth than adults. This is a unique finding that speaks to the need to evaluate impacts of ocean acidification across life stages. Low pH did not directly impact survivorship and this may be due to lower bacterial abundances in our low pH treatments. These data point to the complexities associated with experimental research on ocean acidification, and the need for controlled experiments that include monitoring of bacterial abundance. We found individual variation within treatments in both net calcification rate and/or hinge ligament separation, which are explained by very low saturation states for aragonite leading to dissolution of the aragonite in the hinge ligament. Future research on *A. irradians* will include studies of the biochemical responses to the stress induced by ocean acidification and the identification of biomarkers of early shell dissolution

3.7 Acknowledgments

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3.8 Ethical Standards

All dosing experiments presented here comply with the current laws of the United States of America in which these experiments were performed.

CHAPTER 4

PLASMA TAURINE/GLYCINE RATIOS AS A BIOMARKER FOR OCEAN ACIDIFICATION AND IMPACTS ON CALCIFICATION IN THE BAY SCALLOP, *ARGOPECTEN IRRADIANS* (LARMARCK 1819)

4.1 Abstract

Stress induced by energy demands for maintaining shell growth under ocean acidification conditions was hypothesized to cause an increase in plasma taurine/glycine ratios. We maintained juvenile (~15 mm from umbo to ventral edge) *Argopecten irradians* (bay scallops) under experimentally controlled pH conditions by bubbling air-CO_2 into aquaria (pH = 7.87 ± < 0.01 (control), 7.63 ± 0.03, 7.45 ± 0.01, and 7.25 ± 0.01). Alkalinity, dissolved inorganic carbon (DIC), salinity, temperature, and pH were measured throughout the experiment. Dissolved CO_2 and saturation states (calcite and aragonite) were modeled using CO2SYS. High performance liquid chromatography (HPLC) with fluorescence detection was used to measure the abundance of taurine and glycine in blood plasma (pre-and post-treatment). The ratio of taurine/glycine (T/G) was used as an indicator of environmental stress induced by high CO_2-induced reduction in

pH, because taurine and glycine are both involved in osmoregulation. Given that our results also show a correlation between increased plasma T/G with decreased net calcification, we conclude that plasma T/G can be used as a biomarker for ocean acidification induced impacts on shell calcification in *A. irradians*.

4.2 Introduction

The taurine (organic acid) and glycine (amino acid) ratio (T/G) is an indicator of environmental stress in bivalves, including stress associated with osmoregulation and metal contamination. (Jeffries 1972; Hummel 1994; Hummel 1996). Ocean acidification, an environmental stressor affecting osmoregulation, may also elicit a taurine/glycine response. An increase in plasma T/G, in *Argopecten irradians* (bay scallop), would support the hypothesis that ocean acidification affects osmoregulation in addition to its known impacts on calcification (Chapter 3).

Ocean acidification poses a threat to our oceans' ecosystems and, particularly, to their resident calcifying organisms. Model simulations predict that atmospheric CO_2 will increase from 370 ppmv (today) to 1070 ppmv (estimated as early as year 2100), which will lead to a decline in average surface ocean pH by approximately 0.50 units compared with pre-industrial values (Caldeira 2005). This decline in surface water pH changes calcium carbonate ($CaCO_3$) saturation and reduces carbonate ion concentrations. Increased absorption of atmospheric CO_2 in surface waters will shift the marine carbon dioxide-carbonate system's equilibrium towards higher bicarbonate (HCO_3^-) and CO_2 (aq) concentrations and lower carbonate (CO_3^{2-}) concentrations. The concentration of

carbonate, [CO_3^{2-}], is predicted to drop by half by 2100, despite the fact that total dissolved inorganic carbon concentrations will actually increase as more CO_2 is sequestered by the ocean (Tyrrell 2008). These changes in ocean carbonate equilibrium may directly impact the net calcification of *A. irradians* (Chapter 3).

Experimental studies show that a variety of marine calcifiers exhibit various growth, survival, and fitness responses (e.g. positive, negative, no effect) to CO_2-induced low pH (Ries 2009). In *A. irradians*, shell growth is maintained throughout the life of the organism and is influenced by both the mechanisms utilized for deposition and environmental conditions, including the availability of dissolved inorganic carbon (DIC) in surrounding water (de Paula and Silveira 2009). Therefore, a change in the pH due to increased pCO_2 of seawater will reduce the availability of dissolved inorganic carbon species such as carbonate ion (CO_3^{2-}) (Zeebe 2001). Unless the organic periostracum can protect the low-Mg calcitic shell of *A. irradians* from the surrounding seawater, dissolution of the shell will occur when the saturation state of the mineral ($\Omega = ([Ca^{2+}][CO_3^{2-}])/K_{sp}$) is less than 1.0 (Doney 2009).

Calcification in *A. irradians* is controlled by the mantle (Lowenstam 1989), which secretes the outermost protective layer, the periostracum, that isolates the biocalcifying tissues from the seawater and may also act as a substrate upon which calcification is initiated (Clark 1974). Beneath this protein, lipid, and carbohydrate rich layer is the prismatic layer (low-Mg calcite) (Checa 2000). Calcification occurs extracellularly within the extrapallial space and is controlled, in large part, by a number of amino acids

and proteins present in the extrapallial fluid (Carré 2006 and references within, Weiner 1979; Fu 2005; Dove 2010).

Recent research has revealed much about the composition (e.g., hydrophilicity) of the amino acids and proteins involved in the bivalve's calcification process, and their role in initiating, maintaining, and accelerating calcification. Calcification may be enhanced by secretion of sulfur-bearing organics (e.g. cysteine, methionine, chondroitin) (Freitas 2009). Taurine, (2-aminoethansulfonic acid), found in the extrapallial fluid , promotes $CaCO_3$ deposition (Malkaj 2006). Taurine, an osmolyte and antioxidant, is also thought to have a protective metabolic role supporting redox balance (Yancey 2002). Proteins containing glycine appear to be crucial in calcification serving as nucleation sites for $CaCO_3$ deposition or as support matrices for mineral crystallization (Fang 2011). Both taurine and glycine will likely be present in the extrapallial fluid and also within the blood of *A. irradians*, since blood is contiguous with extrapallial fluid (Nair and Robinson 1998). We hypothesize that the plasma taurine/glycine (T/G) ratio will change as a result of CO_2-induced low pH and those changes may be attributed to negative impacts on net calcification at low pH.

Considering the magnitude of anticipated ocean pH decline over the next century and the economic importance of the bay scallop, evaluation of the plasma T/G under various pH conditions and associating changes to impacts on net calcification is crucial for early identification of pH-induced stress. The development of a biomarker, such as

plasma T/G, is also critical to understanding impacts across bivalve species and to identifying at risk or negatively impacted populations.

4.3 Methods

We evaluated plasma T/G as an ocean acidification stress indicator in the bay scallop, *Argopecten irradians*, and determined relations among pH, net calcification, and plasma T/G. Experiments were performed, in duplicate, on juveniles (~15 mm umbo to edge) over 21 days at ambient temperature. Based on a field survey of the Nantucket Harbor *A. irradians* population (Chapter 2), we maintained organisms under controlled conditions with three CO_2-induced low pH experimental treatments resulting in pH values of 7.87 ± < 0.01 (control), 7.63 ± 0.03, 7.45 ± 0.01, and 7.25 ± 0.01. The treatment pH values are correlative to pH reductions caused by an increase of atmospheric CO_2 levels of 3, 8, and 15 times, respectively, pre-industrial values (~280 ppmv).

Experimental treatment

Juvenile *A. irradians* were maintained for 21 days in 5 gallon plastic aquaria filled with 5 μm-filtered seawater collected from Boston Harbor at the Fox Point dock in Boston, MA. Each aquarium was equipped with a saltwater fan to induce water movement and promote feeding, and covered with a plexiglass lid to minimize evaporative water-loss. Tanks were randomly assigned to the right and left side of the room. Seventy-five percent of seawater was replaced twice a week throughout the duration of the experiment. Organisms were fed aliquots of Shellfish Diet 1800 (Reed

Mariculture Inc.) twice a day to achieve $1.0x10^6$ cells per mL of seawater each day. This commercially produced feed contains four marine microalgae – *Isochrysis sp, Pavlova sp, Thalassiosira weissflogii,* and *Tetraselmis sp*.

Experimental air-CO_2 mixtures were combined prior to bubbling into aquaria. CO_2 (g) was mixed with air from aquaria air pumps, where diffusion was encouraged by securing air-stones to the base of the aquaria directly below saltwater fans. To control the pH of each tank, Allied Electronic (Fort Worth, TX) gas solenoids (Part # VX2330-02T-3CR1) were triggered using a feedback system attached to a Digital Aquatics (Woodinville, WA) pH probe causing CO_2 to be injected into the air stream to the aquarium using a pH = 0.01 rise as trigger to dose. The pH of each tank was logged using a ReefKeeper Control (Digital Aquatics) set consisting of: 8 RKM – SL1 units connected to a ReefKeeper Elite Head Unit and logged data collected from a Net box. pH, calibrated to NBS standards, was logged at 10 minute intervals using this DigiAquatics data management system over the entire duration of the experiment. Using an Orion 5-Star Plus benchtop meter (Thermo Scientific), pH, calibrated to NBS standards, was confirmed externally to account for any drifts in the Digital Aquatics pH probe influenced by algal growth or electronic noise.

Salinity, temperature, dissolved oxygen, and conductivity were measured daily using a YSI Professional Plus hand meter. Alkalinity (ALK) and dissolved inorganic carbon (DIC) were analytically determined in parallel twice a week at each water change. ALK was determined by a titration with 1.6 N sulfuric acid using a digital titrator (EPA 5.10).

DIC was determined using a Tekmar Dohrmann Phoenix 8000 UV-Persulfate analyzer, where laboratory standards were prepared using sodium carbonate and potassium hydrogen pthalate. Aragonite/calcite saturation states (Ω), $pCO_{2(aq)}$, and DIC were modeled using CO2SYS (Pierrot 2006) (Table 3.1; Appendix 2).

We used the buoyant weight method to calculate net calcification (Chapter 3). Net calcification was calculated as starting weight minus end weight divided by starting weight.

Collection of plasma

Whole blood samples were collected using a 10 cc glass syringe equipped with a 21 gauge needle. Samples were taken by sliding the needle between the mantle and shell. Cell free plasma (< 100 μL) was obtained by centrifuging whole blood samples at 15,000 g for 1 minute. The supernatant was collected in cryo-vials and stored at -80°C until analyzed. Samples were collected from randomly selected specimens (n=18) prior to treatment to establish a baseline (time-zero) value for T/G.

Taurine and glycine analysis

Taurine and glycine were analyzed by high performance liquid chromatography (HPLC; Varian ProStar gradient pump, Model 240, CA, USA), using a Gemini C18 column for separation (100 x 4.6 mm I.D., 3 μm particle size; Phenomenex, CA, USA) protected by a Gemini C18 guard column (4 x 3.0 mm I.D.; Phenomenex, CA, USA), connected to a Dionex fluorescence detector (Model RF2000) at 340 nm for excitation and 455 nm for emission. The C18 column was maintained at 30°C throughout each

analytical run (Varian ProStar autosampler, Model 410, CA, USA). Mobile phases were prepared as described in Piepponen (2001). The first mobile phase, A, was composed of 0.05 M disodium hydrogenphosphate, 0.5% (v/v) acetonitrile, 1% (v/v) tetrahydrofuran at a pH of 6.1 (adjusted with 85% phosphoric acid). The second mobile phase, B, was an acetonitrile-tetrahydrofuran-water mixture (70:1:29, v/v). A 100% Mobile phase A was pumped at a flow-rate of 1.5 mL/min and the column allowed to equilibrate for 2 minutes. Once the sample was injected and held for one minute, the pump was programmed to ramp to 100% mobile phase B (linear gradient) over 21 minutes. At 23 minutes, the system was programmed to ramp back to mobile phase A over one minute and held for column equilibration.

An 8- μL volume sample of plasma was diluted with 200 μL of methanol-water (50:50, v/v), and alanine was added as an internal standard. Sample derivatization was automated using the Varian ProStar Model 410 autosampler by adding 200 μL of the derivatizing agent (phthaldialdehyde) to the sample of plasma. After a 2 minute reaction time, a 3 μL injection was analyzed for alanine, taurine and glycine. Peak area data were processed using Varian Galaxie software.

Data Analysis

Significant differences in T/G between and within treatments were tested using two-way ANOVA with Tukey's HSD *post hoc* test ($\alpha \leq 0.05$). Relations between T/G and net calcification were examined using Pearson correlation. A general linear model was used to evaluate the relation between pH and T/G. This model also tested for tank

effects (right vs. left side of room). Linear regression was used to evaluate the relation between net calcification and T/G in blood plasma.

4.4 Results

Dissolved inorganic carbon (DIC), both measured and modeled, was compared since DIC is the measured parameter that is most pertinent for calcification (Ries 2009) (Juvenile experiment Chapter 3, Figure 3-1). Measured and modeled DIC showed excellent correspondence (87%) indicating the dosing system precisely controlled pH through pCO_2 introduction.

Growth, survival, and injury

The average net calcification (± SE) was highest in the control (pH = 7.86 ± < 0.01) and lowest at pH 7.19 ± 0.10 (Figure 4-1). All treatments, including the control, experienced a net loss of shell as portrayed by negative mean values in net calcification. A linear regression was performed to explore the relation between net calcification and pH ($R^2 = 0.378$), with the greatest difference occurring in Treatment 3 (pH = 7.2) (Chapter 3, Figure 3-3). We also found linear relations between net calcification and pH in both adults ($R^2 = 0.815$) and juveniles ($R^2 = 0.378$).

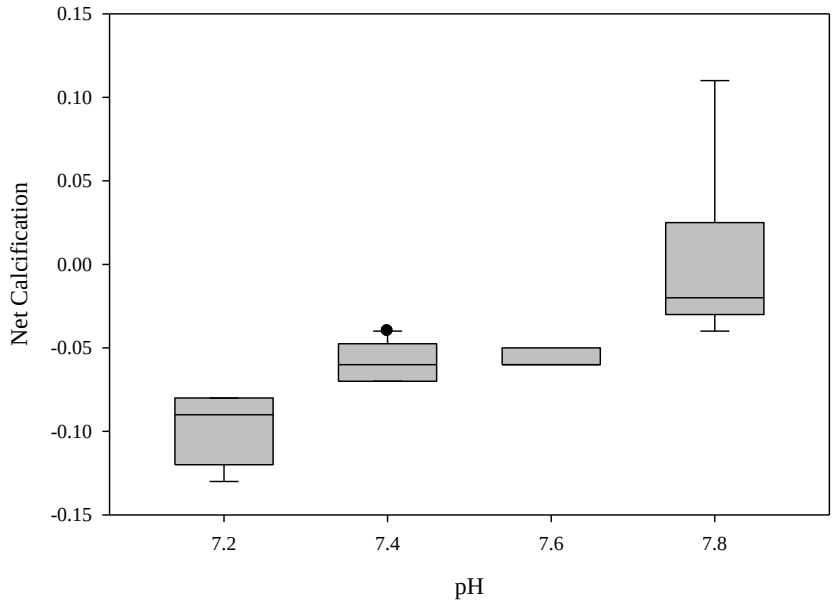

Figure 4-1: Change in net calcification (±S.E) with respect to pH treatment indicates a loss of shell with respect to a decrease in pH.

Taurine/Glycine

T/G ratios were log normalized to achieve equality of variance and, pH was used as a continuous variable in a general linear model (GLM) to test for a relationship between these two variables. There was no evidence of a y-intercept or interaction effect between the pH treatment and the location in the room ($F_{1,5} = 2.35$, $p = 0.186$, Figure 4-2). The baseline average T/G for *A. irradian* was 0.30 ± 0.09. GLM revealed a moderate pH effect ($F_{1,5} = 4.68$, $p = 0.083$) and a weak effect of tank position (left vs. right) ($F_{1,5} = 2.35$, $p = 0.186$). Using a nested ANOVA, the tank-to-tank variance within treatment was found to be insignificant ($F = 1.09$, $p = 0.375$). A linear regression on the T/G across pH treatments indicates a strong, linear relationship ($R^2 = 0.719$)

between plasma T/G and pH (Figure 4-2). Typically, the increase in T/G was caused by an increase in taurine concentrations with little variation in glycine.

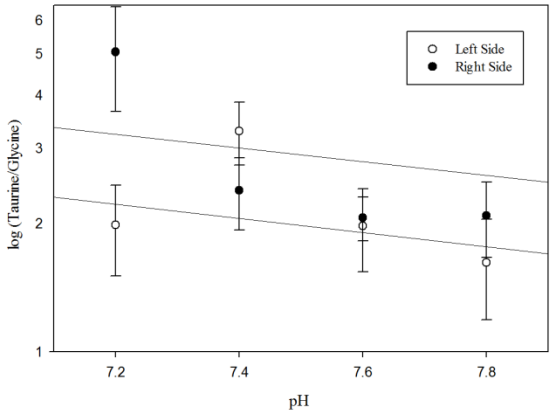

Figure 4-2: Mean taurine/glycine ratio on a log scale with respect to pH. Left = left side of room, Right = right side of room. Using a nested general linear model a modest pH and weak room side effect were found ($F_{1,5} = 4.68$, $p = 0.083$; $F_{1,5} = 2.35$, $p = 0.186$).

A Pearson's correlation confirmed that plasma T/G correlated to pH ($p < 0.001$), and net calcification ($p = 0.038$). A linear regression of T/G to net calcification indicated an increase in plasma T/G with greater loss of shell material ($R^2 = 0.715$) (Figure 4-3). Linear regression indicated a correlation between plasma T/G and shell loss across pH levels: 7.2, 7.4, 7.6, and 7.8.

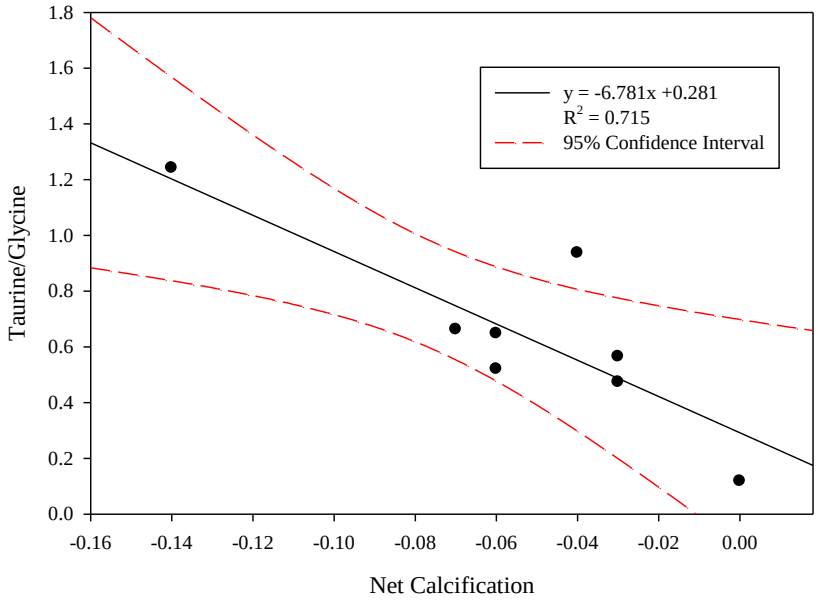

Figure 4-3: Taurine:glycine ratio mean with respect to net calcification. A linear regression indicated a correlation between taurine:glycine values in plasma to shell loss experienced in juveniles exposed to various pH levels (pH = 7.2, 7.4, 7.6, and 7.8).

4.5 Discussion

The juveniles maintained at reduced pH exhibited a decrease in net calcification and an increase in plasma T/G. Control plasma T/G (1.85 ± 1.32) was elevated above baseline (0.30 ± 0.09) indicating some experimental stress. Cummings (2011) found that, in Antarctic clam (*Laternula elliptic*), stress caused by lowered pH impacted the expression of CHS, a gene that codes for a key enzyme in bivalve shell synthesis, suggesting that shell formation is negatively impacted. Others have shown that protein synthesis in marine bivalves is deleteriously impacted by increases in freshwater input (Clark 2009), decreased osmotic stress (Chang 2004), and lowered pH (Cummings 2011).

Although T/G is not a direct measure of net calcification, our data show that they are strongly related.

Previously published research on juvenile *A. irradians* indicates that there are no statistically significant trends with regards to survivorship and elevated CO_2 (Chapter 3). However, 11% of the organisms maintained at the lowest pH (pH = 7.2) in one of our replicate tanks suffered hinge separation. The hinge ligament is composed of proteins and aragonite (Kikuchi, 1987). It is uncertain whether hinge separation was caused by dissolution of the aragonite (since $\Omega_{aragonite} < 1$) or damage to the organic materials (collagen, elastin, resilin, and silk fibroin). Since the hinge separation occurred in the tank with the lowest aragonite saturation ($\Omega_{aragonite} = 0.35$), it is reasonable to assume that hinge separation was caused by aragonite dissolution. Individuals with hinge separation died within 2 days of separation, and so plasma T/G could not be measured for comparative purposes.

Our results show that T/G is inversely related to net calcification. We interpret these results as supportive evidence that plasma T/G can be used as a biomarker of ocean acidification-induced stress. Proteins containing glycine appear to be crucial in calcification, serving as nucleation sites for carbonate mineral deposition, or as support matrices for mineral crystallization (Fang 2011). Higher plasma T/G in specimens maintained at lower pH may be due to increased consumption of glycine reducing the glycine concentration in the plasma.

Higher plasma T/G may also be related to increased taurine concentrations within the extrapallial fluid since taurine may be used to accelerate the shell accretion. Increased taurine in the extrapallial fluid, as reflected in plasma concentrations, suggest that taurine is being used to compensate for the loss of shell caused by the pH reduction. This contention is supported by the work of Malkaj (2006) who showed that the fluid saturated with taurine accelerated calcification. Yancey (2002) linked the concentrations of taurine in intracellular fluids of deep-sea shrimp and skates to depth/pressure, and proposed that a derivative of taurine, trimethylamine *N*-oxide, was utilized as a universal protein stabilizer to counteract the effects pressure may have on inhibiting protein folding (Yancey 2002). Taurine enhances other cellular antioxidant functions as well (Yancey, 2005). The derivatives of taurine, hypotaurine and thiotaurine, found in tissues are linked to sulfide exposure (Brand 2007). Taurine is abundant in algae (Malkaj 2006) and could also be sourcing from the diet used in our experiments. However, to attribute changes in plasma T/G to taurine from algae, we should see no difference in taurine concentrations in each of the Treatments, since all organisms were fed the same composition and amount. If lower pH caused inefficient feeding, then plasma T/G should decline, but our data show an increase in plasma T/G with lower pH. We contend that the increase plasma T/G indicates that taurine is being concentrated in the extrapallial fluid to accelerate calcification and/or to increase the sites available for calcite deposition.

Since free amino acids, such as glycine and the organic acid taurine, are involved in osmoregulation, the relations between the plasma T/G, net calcification, and reduced

pH suggest that ocean acidification may lead to osmotic stress. In the purple Washington clam, *Saxidomus purpuratus*, taurine was specifically linked to osmoregulation of body fluids induced by salinity changes (Wada 1976), and here we link it to pH. Under ocean acidification conditions, ion-transport systems in the gill epithelia may compensate for changes in pH enabling some bivalves to actively accumulate bicarbonate ion to stabilize the extracellular pH (Melzner 2009). This may, in combination with increased taurine concentrations and glycine consumption, enable some bivalves to maintain shell growth at low pH. Mechanisms for ion exchange are, however, still not fully understood.

4.6 Conclusion

While different species exhibit a variable tolerance to the future conditions of ocean acidification (e.g., "business as usual" scenario), we identified a biomarker of stress that links stress induced by low pH to net calcification in *A. irradians*. It is reasonable to assume that this biomarker will be useful in studies of other pectinoids. The ocean is becoming more acidic and the resulting lower pH, lower carbonate ion concentration, and higher dissolved CO_2 may impact plasma T/G due to the role of these two organic molecules in calcification and osmoregulation. The overall elevation of plasma T/G at low pH, when compared to baseline plasma T/G suggests that even under control conditions the organisms were stressed. However, the correlation between net calcification and plasma T/G is informative for future research that will evaluate ion exchange and maintenance of shell growth under low pH.

If the increase of pCO_2 in the ocean quickly surpasses the ability of *A. irradians* to maintain shell deposition, it is possible that the hinge separation seen in our experiments could occur in the wild. If this were to occur, conservation efforts will have to adapt to quickly mitigate the ecological effects of ocean acidification since the inability to maintain shell integrity could lead to greater mortality. It is not possible to predict how *A. irradians* will respond to predicted declines in ocean pH, but monitoring of plasma T/G may enable early detection of negative impacts on net calcification.

4.7 Acknowledgments

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CHAPTER 5

SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Overview

For the management of important shellfish resources such as the bay scallop (*Argopecten irradians)* identification of essential habitat is crucial. More recently U.S. Department of Agriculture, U.S. Fish and Wildlife Service, and the National Marine Fisheries Service are using elemental data from shells and tissues to establish food provenance and management of commercial populations to meet growing demand for seafood resources. Economically, especially for small fisheries such as bay scallop, identification of environmental risk factors is critical to sustaining yields and maintaining the fishing culture. This is particularly true for Nantucket, MA and its bay scallop fishery. Annual bay scallop landings in MA alone are estimated at a value of $1.1M all of which are caught within 4.8 km of the coast. Nantucket landings account for 20% of the MA landings. Given the economic importance of the bay scallop fishery to MA and to Nantucket there is pressing need to understand the potential impacts of climate change on the population.

The research presented here examined current populations of the bay scallop in Nantucket Harbor where much of the economy and culture is influenced by recreational

and commercial harvest of the bivalve. A snapshot sampling occurred in 2009 to assess if geochemical signatures in the shells could identify areas of the harbor that could host more individuals. Once pH was identified as a an important variable for the bay scallop, laboratory experiments with bay scallops in pCO_2 enriched waters were used to determine how the bay scallop would respond under future conditions of projected ocean acidification. As bay scallop habitat is coastal estuarine and so subjected to variations in pH due to tide and freshwater discharge, it is important to note that they are already exposed to slightly lower pH values (pH = 7.8) than open ocean (pH = 8.1). Tidal influences, human activities, and rain events are intrinsic and extrinsic variables that influence bay scallop habitat quality and, specifically, pH. The research presented here examines the response of the bay scallop to acute doses of acidified waters.

5.2 Summary and Conclusions of Research Findings

Calcareous shells record the ambient conditions of the water in which the organism lived at the time of shell deposition and the processes of shell mineralization are also influenced by these conditions. With essential habitat identification in mind, the relation between elemental chemistry of shells (elemental/Ca signatures, carbon, nitrogen), water quality (pH, salinity, depth, dissolved oxygen, etc.), and bay scallop abundances was examined. Here the abundance of the bay scallop is assumed to be a measure of optimal conditions for persistence over the life span of the organism since, in the adult stage; the bay scallop can elicit an escape response from non-optimal conditions. Relations between the shell chemistry (La/Ca, Pb/Ca, and Zn/Ca) and both

scallop abundance and physical water chemistry were found. Based on these relationships, a linear discriminate model was built that differentiated between abundance zones with 73.6% accuracy. The model revealed that pH and salinity, which strongly influence the free metal activity of the elements incorporated into the shell, were important habitat quality indicators. This research also revealed potential sites within the Nantucket Harbor that could support bay scallops and increase harvestable annual populations.

pH, dissolved oxygen, temperature, salinity, and depth were identified as components in defining the essential habitat of the bay scallop. However, pH is also a controlling factor for shell growth in marine calcifiers, therefore the research followed the line of reasoning that ocean acidification may impact net calcification of bay scallop. Experimental pH treatment tanks were designed and deployed in which juvenile and adult bay scallops were maintained under different pCO_2-induced low pH conditions to test the effects of ocean acidification on net calcification. Juveniles exhibited statistically different calcification rates between the control and the most acidified tank as did adults. Adults exhibited significantly lower negative net calcification rates than juveniles. The most profound effects were seen in the lowest pH treatment (pH 7.2) where the hinge separated in a number of specimens ultimately leading to death of the individuals. The differences in net calcification rate/shell loss were attributed to stress induced changes in energetics, where juveniles retain plasticity with regard to pH decline.

Plasma taurine/glycine ratio, a biomarker for environmental stress induced by pH decline, was found to be associated with net calcification. A decrease in net calcification

and an increase in the taurine/glycine ratio were found in scallops maintained at low pH. A Pearson's correlation indicated that the taurine/glycine ratio was correlated to both net calcification ($p = 0.038$) and pH ($p < 0.001$), while a linear regression indicated a strong relation between T/G in plasma and net calcification ($R^2 = 0.715$) (Chapter 4). Although taurine/glycine ratios are not a direct measure of calcification, our results indicate that the taurine/glycine is a reasonable indirect proxy for ocean acidification-induced net calcification decline.

5.3 Recommendations for Future Studies

Bay scallops, since they are geographically restricted and their shell is composed of low-Mg calcite, may serve as a unique "miner's canary" with respect of changes in ocean pH. There are gaps in our understanding of the calcification process, such as the components involved in the organic matrix, and overall ecosystem responses to future ocean conditions. Factors such as temperature, salinity, dissolved oxygen, and pH all define the quality of habitats for a bay scallop, and ultimately the sustainability of the population. These factors by themselves (e.g., only pH) and in combination affect the survivorship, net calcification rate, growth, reproduction, recruitment, and ultimately the economic viability of the fishery. However, some parameters or combinations of parameters can be used to evaluate the quality of a given habitat.

Ocean acidification impacts the net calcification of the bay scallop. Other important factors in assessing the impact of future climate change are temperature and salinity. As seen from identification of essential habitats within Nantucket Harbor, there

appears to be an optimal salinity for the organism where large populations of scallops resided. Future studies should be focused on determining the combined effects of these variables (pH and salinity) and model impacts based on future changes in both. It is also relevant to determine to what extent calcification processes are related across calcifying organisms (genus vs. species, etc). For example, are the biochemical indicators (taurine and glycine) only viable in the bay scallop or can they extend to other bivalves (e.g. mussel) or even into other calcifiers (e.g. shrimp)? Additionally, in an effort to understand and elucidate mechanisms involved in the calcification process, multiple indicators should be developed that complement existing stress biomarker research, such as suites of amino acids, heat shock protein, glucose, and genetic variances.

Salinity and pH

The response of survivorship and net calcification need to be considered in response to a combined water freshening and acidification of the ocean. A combination of pH and salinity levels should be investigated. The general procedure for the treatment would involve setting up flow-through treatment tanks that paralleled the 4 pH treatment level (7.8, 7.6, 7.4, and 7.2) with different salinity levels (e.g. 30 and 15 $^{o}/_{oo}$) in a nested block design taking into consideration tank effects. Every level of pH and salinity should be measured in duplicate (total of 16 tanks). Measurements of pH and total alkalinity should be made daily for modeling in CO2SYS, where the other carbonate system components can be modeled. To control for salinity, conductivity and total suspended solid measurements should also be collected. Seawater should be stored over short periods of time (< 3 days) once equilibrated to the pH and salinity conditions and bubbled

continuously to avoid anoxia. Careful consideration should be given to measuring the algal response to these conditions as extreme pH and salinity conditions may cause adverse effects on feeding. Organisms should be collected and held for 7 days for acclimation to laboratory conditions prior to initiation of experiments. Based on the results presented in this dissertation, experimental dosing should last 21 days so that they can be compared to the results present here.

Taurine/glycine in other calcifying species

Comparison of plasma taurine/glycine across calcifying species has not been investigated. Based on published ocean acidification research, organisms exhibiting different net calcification responses are known and should be selected for such as study (e.g., negative response, positive response, neutral, and threshold response). For example, the bay scallop, shrimp, hard clam, and blue mussel all have varied net calcification responses when reared at low pH (Ries et al. 2009). Using a single factor model as implemented in this dissertation for pH, the organisms could all be exposed for the 21 day period. Throughout the duration organisms can be sacrificed at time zero, day 10, and day 21 to measure plasma extracted from blood samples. Plasma should be analyzed for taurine/glycine ratios using high performance liquid chromatography with a fluorescence detector. The resulting data should be compared to net calcification rates and pH treatments to determine if taurine and glycine changes such as seen here occur across calcifiers.

Multiple stress indicators

In order to determine how well plasma T/G predicts impacts on net calcification, it is important to rely on multiple measures of stress. For example, raising organisms outside of their natural habitat may artificially increase stresses due to space restrictions and poor replication of their native habitat. Although there may be natural variations in the availability of food in the wild, it is essential to isolate the stress effect caused by poor diet. In the case of underfeeding caused by algal death from acidification, glycogen can be monitored in tissues as a biomarker of feeding. Also, free amino acids are often used to assess osmoregulation and bioenergetics, such that amino acid profiles may identify species-specific physiologic responses to low pH.

This study serves to enhance our understanding of the characteristics of the bay scallop habitat and the resulting impacts of ocean acidification on calcification. Understanding the link between environmental characteristics and the effects of climate change on calcification should be used to verify geochemical signatures in long term food safety certification techniques. In this study both field and experimental laboratory approaches were used to determine the vulnerability of the bay scallop to ocean acidification in both the juvenile and adult life stages. It is relevant to state that although predictions of future pH decline due to ocean acidification have been developed, the ultimate conditions will only be realized with time. Although this study does not predict the future survival or market impacts for the Nantucket bay scallop under future climate change scenarios, it does inform our understanding of the vulnerability of the bay scallop

to these changes and provides context for mitigation of impacts through protection of essential habitat.

APPENDIX 1

REFERENCES FOR PHYLOGENETIC DATA ON SHELL STRUCTURE AND MINERALOGY FOR TABLE 1-1

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APPENDIX 2

CO2SYS MODEL DATA

	Salinity	Temp. (ºC)	pCO_2 SW (µatm)	pH	A_T (µmol · kg^{-1} SW)	DIC (µmol · kg^{-1} SW)	$\Omega_{calcite}$	$\Omega_{aragonite}$	Surv. (%)
Adult	27.8 ± 0.5	24.1 ± 0.5	468 ± 191	7.88± 0.12	1510± 170	1383 ± 181	2.20± 0.462	1.42 ± 0.298	33
	27.8 ± 0.5	23.9 ± 0.6	1029 ± 130	7.57± 0.04	1616± 86	1566 ± 83	1.26± 0.116	0.81 ± 0.075	25
	27.7 ± 0.5	24.1 ± 0.5	1823 ± 220	7.38± 0.02	1683± 119	1679 ± 117	0.88 ± 0.095	0.57 ± 0.064	77
	27.7 ± 0.3	24.0 ± 0.4	3437 ± 518	7.19± 0.01	2151 ± 290	2208 ± 302	0.73± 0.102	0.47 ± 0.066	17
Juvenile	28.5 ± 0.2	23.2 ± 0.5	535 ± 188	7.87 ± 0.16	1275 ± 260	1195 ± 218	1.42 ± 0.769	0.91 ± 0.494	43
	28.6 ± 0.2	23.4 ± 0.6	1180 ± 280	7.61 ± 0.11	1459 ± 140	1436 ± 121	0.90 ± 0.266	0.58 ± 0.171	60
	28.3 ± 0.3	23.3 ± 0.7	2236 ±373	7.44 ± 0.06	1791 ± 164	1813 ± 168	0.75 ± 0.115	0.48 ± 0.074	47
	28.4 ± 0.2	23.3 ± 0.7	3546 ± 686	7.26 ± 0.07	1891 ± 426	1966 ± 434	0.54 ± 0.170	0.35 ± 0.108	64
Juvenile	28.6 ± 0.2	22.8 ± 0.3	537 ± 207	7.87 ± 0.18	1206 ± 58	1131 ± 26	1.30 ± 0.486	0.84 ± 0.314	83
	28.7 ± 0.1	22.9 ± 0.3	1110 ± 248	7.66 ± 0.08	1485 ± 40	1454 ± 53	0.99 ± 0.159	0.64 ± 0.102	34
	28.3 ± 0.2	23.1 ± 0.3	2253 ± 233	7.46 ± 0.02	1903 ± 214	1922 ± 218	0.82 ± 0.105	0.53 ± 0.067	57
	28.6 ± 0.1	23.1 ± 0.3	4162 ± 837	7.24 ± 0.06	2343 ± 693	2444 ± 705	0.65 ± 0.280	0.42 ± 0.181	79

Table 4-1: pH, carbonate chemistry, alkalinity, salinity, temperature, and percent survivorship (surv.) of juvenile and adult *Argopecten irradians* per exposure period (± standard deviation) during the four-level CO2 experiments. Total alkalinity (AT), pH (NBS), salinity, and temperature were used to derive modeled pCO2, DIC, and saturation states (Ω) for calcite and aragonite.

APPENDIX 3

PREPARING THIN SECTIONS FOR LA-ICP-MS

Shells collected from the Nantucket were assigned identification numbers by indicating site and a number to indicate number of organisms collected. For example, a shell from site 19 where 30 individuals were collected could be labeled 19 – 01 or 19 – 30, or any number between 1 and 30. Excel was used to randomly select (=RANDBETWEEN(1,30)) which individual shells would be prepared for LA-ICP-MS. The randomly selected individual and site association were recorded in a notebook. The right valve was used for all analysis. The important distinction is that for this type of analysis the same side should be used between individuals to account for physiological differences in depositional rates between the left and right valve. The description for sample preparation will allow for analysis of edge and umbo chemistry individually, but also for investigation along the life of the shell (umbo to edge).

For adult shells, the rock saw (Rascal, Model T) in S-3-35 was used. WEAR SAFETY GOGGLES! A cut to the slight left or right of the nub was made from the umbo to edge. For these samples, you are hand guiding the shell across the blade so it is necessary only for large samples and 2 people should be present for safety. A second cut

must be made to one of the sides so that the shell is short enough to fit on the microslides. Shells should be placed in a bag and carried to the IsoMet low speed saw (Buehler), currently in S-1-47.

To adhere the shell to the microslide, Crystalbond was used to attach the quarter of the shell. A dremel was used to etch the ID onto the slide indicating a "U" for umbo or "E" for edge. The respective umbo or edge was mounted closest to the identification mark. Two slides were used from the adult shells (Eg. 19-01-U and 19-01-E). The slide was mounted onto the IsoMet low speed saw so that the blade will prepare a thin section. It is important to use water in the pan to keep the blade wet, and weights can be added to perform a quicker cut. All cuts were started on a speed of 2 until the blade had successfully entered the front and back of the cut, after initial cut is made the speed can be adjusted up to 5. Thin sections were stored in a petrographic slide case.

For juveniles shells, the entire valve can be attached to the petrographic slide using Crystalbond (i.e. it is unnecessary to make initial cut with the rock saw). Mount two diamond blades on the isomet saw using a separator between the blades. Orientate the shell so that the thin section to be obtained is between these two blades. Since the juveniles are typically not as thick as the adults and two blades are being implemented, it is important to only use low speeds. Start the blade and slowly lower the sample onto the blade (Speed 2). Weights should be avoided for these samples. To complete the cut, you can raise the speed on the Isomet saw to 3. Once the glass is starting to etch, the cut is complete. Reheat the sample on a hot plate to extract the thin section and mount (cut

must be up) to an etched petrographic slide indicating which side is umbo and which is edge.

Adult and juvenile shells must both be hand polished to a thickness that light can pass through for microscopic images. Having a smooth surface also helps for laser ablation efficiencies. For hand polishing, make sure that you have multiple grades of sandpaper. Start the polish with the roughest paper by adding water to the surface and make figure 8 patterns. The hand pattern should always be a figure 8, but it is important to reverse the direction to prevent striations in the pattern. End on the smoothest paper to finely smooth the surface. Inspect the shell under a microscope to see if sufficient polishing has occurred.

At this point, the samples are ready for LA-ICP-MS (laser ablation inductively coupled plasma mass spectrometry). This standard operating procedure will not include tuning of the ICP-MS or the hardware for connecting to a laser. However, a guideline for someone who is performing the exact analysis presented in Chapter 2 is given. It is assumed that a trained laboratory technician has already warmed the laser head (30 minutes) and opened the software for the user along with performing the daily performance checks on the ICP-MS.

1. On the ICP-MS software (Elan) click the sample tab and provide the name of your sample under Sample ID. Choose the appropriate method for analysis (brybry/scallop_shell_040611.mth) by double clicking on the method cell and searching for the file. This method includes an automated trigger for

signaling the ICP-MS to start collection. All sample lists should begin with triplicates of each standard to be used (Eg, MACS-1, and MACS-3)

2. Load the sample into the laser cell. To do this, open the laser front and GENTLY pull out the sample cell. Twist the black cap to the left and remove previous sample (if one is there). Load sample mounted on petrographic slide so that the sample number is always at the top or bottom (this will speed finding the end to be ablated between samples). Twist the cap clockwise to tighten and GENTLY push sample cell back inside and close the laser front door.
3. Allow the system to purge the cell for about 30 seconds. Lower the helium flow control on DigiLaz software to 300 mL/min and press load. Raise the helium flow control on DigiLaz software to 750 mL/min and press load. If the helium does not report near 750 mL/min, contact the laboratory technician who set the system up for you. Using this purge and helium flow control, you should be able to avoid blowing the plasma on the ICP-MS out.
4. Using DigiLaz software, connect dots to form the line of ablation. The method presented here recommends ablating the prismatic layer (most consistent form of carbonate layer). To do this, the line should start on the shell around the nacre side and should ablate to the periostracum (area normally has color). If you are having problems finding the spot for ablation, on DigiLaz software go to Tools > Sample Mapping and choose a 4 x 4 map.

You can simply left click on the image where your sample is located to navigate the image.

5. Once the line has been drawn, the DigiLaz software will give an estimation of time required for the analysis. On the Elan (ICP-MS) software, ensure that the method time is at least 1 minute longer than the scan time. On Elan, click File > Save. Then click back to the Sample tab, highlight the sample to be analyzed, and hit Analyze sample.
6. On DigiLaz software, click Go.
7. On the Elan software, you can watch data acquisition, by choosing clicking the Real Time tab.
8. Repeat for all the samples. Run a set of external standards periodically (1 set per 15 samples). Monitor Calcium 43 and record everytime you analyze MACS-1. A 20 – 30% loss in intensity means that analysis should cease and the ICP-MS and laser should be cleaned.

DigiLaz Method:

On DigiLaz software, choose the blue notebook to begin a method and choose Single Line Scan. Parameters outlined in DigiLaz software.

LA-ICP-MS operating conditions	
Power	70%
Pulse Repetition Rate	10Hz
Spot Size	50 μm
Scan rate	8.25 μm/sec

All data from this was analyzed using GeoPro. The steps provided here are basic guidelines to reproduce with some guidance from an experienced user.

Data Integration

1. Open integ722 Excel file, enable this content on options.
2. Choose load raw data (select your file). A spreadsheet will appear with the file name you chose, click Chart 1.
3. Select log scale on the upper left of chart.
4. Move the lines on the software to identify areas to be integrated. Bracket the gas blank (left two lines that are dotted). Bracket sample signal plateau (two solid lines). Bracket the matrix (dot dash lines on right).
5. Tab back to the spreadsheet with sample name, Save Integrals, Yes to save changes, Click Done.
6. Return to integ722. Repeat this process with all the samples collected.
7. Click done on integ722.

Quantitative Analysis

8. Open the quant789 Excel file, enable this content under options.
9. Load samples by choosing the first standard in the file. Click OK.
10. Pull down the standard options on the Master sheet to label all the standards in the run.
11. Put in internal standard concentration as reported.

12. Check the use of internal standards (MACS-1: Ca 43 – 38% and MACS-3: Ca 43 – 37.69%).
13. Choose Get integrals.
14. Check calibrations and adjust sensitivity by the raw data.
15. On Master, Click Calculate.
16. On Master sheet, Click Report (values in ppm).
17. Hit Stats tab for data.
18. Data from this point should be converted to molar ratios.
19. Relative error can be calculated from ppm data: $\frac{|measurement - true\ value|}{true\ value}$

APPENDIX 4

TIC/TOC REAGENTS AND STANDARDS

To prepare 10% Persulfate/5% Phosphoric Acid Reagent: **WEEKLY**

1. Measure **100** g of 98% sodium persulfate into a rinsed 1L glass bottle.
2. Add **852** mL (by mass) of mQ water and mix well using a stir bar and stir plate.
3. Remove stir bar.
4. Add **36** mL of 85% phosphoric acid (measured with a graduated cylinder).
5. Cap bottle, invert 3 times.
6. Store in the dark by covering with aluminum foil.
7. Wait 12 hours before use.

Expires within one week. The seawater method for 0.1 to 20 ppm carbon consumes about 10mL of this reagent. You can adjust these values accordingly. Write the date, time, and who made the reagent.

To prepare 21% Acid Reagent: **MONTHLY**

1. Measure **752** mL (by mass) of mQ water into a rinsed 1L glass bottle.
2. Add **148** mL of 85% phosphoric acid to the solution.
3. Store in the dark by covering with aluminum foil.

Expires within one month. Reagent does not have to sit for 12 hours before use.

To prepare 1000ppm sodium carbonate stock:

1. Use a 50 mL volumetric flask and add mQ water about ½ full.
2. Weigh 0.441 g of sodium carbonate on to a weigh dish and transfer to the glass flask.
3. Rinse down all the solid and dilute to the mark.
4. Transfer to a storage container and use for making calibration solutions.

Expires within one month.

Prepare calibration standards and quality control solutions each run.

Identification	Concentration (ppm)	mQ Water (mL)	Actual Water (mL)	1000ppm Sodium Carbonate (uL)	KHP (uL)
Blank	0	50.000		0	0
Standard 1	0.1	49.990		5	5
Standard 2	1	49.900		50	50
Standard 3	5	49.500		250	250
Standard 4	10	49.000		500	500
Standard 5	20	48.000		1000	1000
Check Standard	7.5	49.250		375	375
MDL 1	0.9	49.910		45	45
MDL 2	0.9	49.910		45	45
MDL 3	0.9	49.910		45	45
MDL 4	0.9	49.910		45	45
MDL 5	0.9	49.910		45	45
MDL 6	0.9	49.910		45	45
MDL 7	0.9	49.910		45	45

An instrumental SOP is already developed and in use for the Tekmar Dohrmann Phoenix 8000 UV-Persulfate TOC Analyzer, and therefore, will not be detailed here. However, with reference to CO_2-enriched samples, the best, acceptable analytical precision as

determined by CO2SYS calculated versus modeled graphs, prepare the samples as follows:

Seawater sample preparation:

Prepare a 100 mL volumetric flask by filling it about ½ way with mQ water and record this mass. This dilution factor will ensure that the high CO_2 samples will fall within the standard range provided here (1 – 20 ppm). Because of this factor, the instrument reported value must be corrected by using the equation (100 mL / (100 – weight of distilled water)) where the density is assumed to be 1 g/mL. Using a plastic syringe, remove the filter and pull up seawater directly from the tank (no airspace). Attach the 0.45 μm filter, and deliver the seawater into a 100 mL volumetric flask. About halfway through filling the volumetric flask add two drops of 85% phosphoric acid and dilute to the 100 mL mark. This will allow for sufficient sample to be analyzed in triplicate. Do not allow samples to sit. Run the sample immediately after collecting. The water for the TIC/TOC and alkalinity (EPA method 5.10 Total Alkalinity) should be analyzed in parallel so that the water is not dosed in between collections.

To convert and correct the DIC value reported use the following Equation 5:

$$DIC\ \left(\frac{umol}{kg}\right) = \frac{[IC\ reported * C.F. * Dilution\ Factor] - blank}{1000} * \frac{10e-6}{12.011} \qquad \text{Equation 5}$$

APPENDIX 5

TAURINE AND GLYCINE MEASUREMENTS BY HPLC-F

The fluorescence detector should be set at 340 nm for excitation and 455 nm for emission and allowed to warm for about a half hour. Before starting, mobile phases must be prepared as follows.

To make mobile phases:

Mobile Phase A:

1. Weight 7.1 g of sodium phosphate dibasic into a glass beaker.
2. Dilute to 1 L with 18.2 Meg-Ohm water in a volumetric 1 L glass flask.
3. Calibrate a pH probe using a 4, 7, and 10 NBS standard.
4. Transfer 1 L solution to a glass beaker and add a stir bar, place on stir plate.
5. Adjust pH to 6.1 with 85% phosphoric acid and record final pH.
6. Transfer 985 mL of the 6.1 sodium phosphate dibasic solution to a graduated cylinder and deliver this to the HPLC plastic bottle that will hold Mobile Phase A.
7. Measure 5 mL of acetonitrile into a graduated cylinder and transfer to Mobile Phase A bottle.
8. Measure 10 mL of tetrahydrofuran into a graduate cylinder and transfer to Mobile
9. Phase A bottle.
10. Degas solution for 10 minutes with helium flow in a vented hood.

Mobile Phase B:

1. Transfer pure methanol into this bottle as a wash for end of the run.
2. Degas the solution for 10 minutes with helium flow in a vented hood.

Mobile Phase C:

1. Measure 350 mLof acetonitrile in a graduated cylinder.
2. Measure 5 mL of tetrahydrofuran in a graduated cylinder.
3. Measure 145 mL of 18.2 Meg-Ohm water into a graduated cylinder.
4. Combine all 3 into the Mobile Phase C plastic bottle.
5. Degas the solution for 10 minutes with helium flow in a vented hood.

Prepare standards of all analytes of interest to confirm retention times on the column (taurine, glycine, and alanine). Use a glass syringe to deliver accurate amounts. To clean syringe between deliveries, rinse the syringe with HAM (hexane/acetone/methanol) three times each.

Prepare plasma samples by putting 200 μL of methanol-water (50:50) into an autosampler amber vial. Add 8 μL of cell free plasma and an internal standard (alanine for bay scallops) to the vial. Cap and place on autosampler.

You will also need to fill a reagent vial with complete phthaldialdehyde reagent and place this on the autosampler. The taurineglycine method with automate this reaction time and will provide a stable heat for the column oven.

Before starting the instrument, purge the lines of air. Open the Galaxie software and choose the configuration for fluorescence (further guidance for this can be found in the User Manuals which are located as PDF's on the computer). Choose to wash the autosampler needle with methanol. Turn on the pumps allowing Mobile Phase A to circulate system for about 10 minutes. Record the PSI on the column (e.g. 1954.0). Zero the fluorescence detector (read should be between -20 and 20), and make sure flow is exiting into waste container.

APPENDIX 6

SPSS CODE FOR NESTED TWO-WAY GENERAL LINEAR MODEL

SPSS code used to look at normality and distribution of trends through box plots.

```
EXAMINE VARIABLES=TGRatio BY pH
 /PLOT BOXPLOT
 /COMPARE VARIABLES
 /STATISTICS DESCRIPTIVES
 /CINTERVAL 95
 /MISSING LISTWISE
 /NOTOTAL.
```

SPSS code that indicated a parallel trend between tank sides. Note that the Dunnett post-hoc test was also implemented since the experimental design for ocean acidification included a control and treatments. In order to use this test in SPSS, the user must group the control data either at the top or the bottom of the spread sheet.

```
UNIANOVA lnTGRatio BY pH Side
 /METHOD=SSTYPE(3)
 /INTERCEPT=INCLUDE
 /POSTHOC=pH(TUKEY DUNNETT)
 /PLOT=PROFILE(pH*Side)
 /EMMEANS=TABLES(pH)
 /EMMEANS=TABLES(Side)
 /PRINT=HOMOGENEITY
```

```
/CRITERIA=ALPHA(.05)
/DESIGN=pH Side.
```

SPSS code used to reject null for pH and assess pseudoreplication. Here lnTGratio is my response variable, while pH is my treatment level and side is a controlled factor (tank placement in a room).

```
UNIANOVA lnTGRatio BY Side WITH pH
/METHOD=SSTYPE(3)
/INTERCEPT=INCLUDE
/EMMEANS=TABLES(Side) WITH(pH=MEAN)
/PRINT=HOMOGENEITY
/CRITERIA=ALPHA(.05)
/DESIGN=pH Side.
```

Comment Reject null for pH, but not potential pseudoreplication.

SPSS code here

```
UNIANOVA lnTGRatio BY Side  Tank WITH pH
/METHOD=SSTYPE(1)
/INTERCEPT=INCLUDE
/PRINT=HOMOGENEITY
/CRITERIA=ALPHA(.05)
/DESIGN=pH Side Tank(pH).
```

APPENDIX 7

NANTUCKET FIELD DATA

Site Number	Zone	Date	Depth (m)	Latitude (N)	Longitude (W)	D.O. (mg/L)	Temp (°C)	pH	Salinity (‰)
1	3	8/11/2009	1.1	41.314722	70.033056	7.84	26.5	7.54	25.8
2	1	8/5/2009	1.3	41.301667	70.032222	9.14	24.7	7.92	26.0
3	3	8/11/2009	1.6	41.297778	70.041944	9.90	24.7	7.89	25.7
4	3	7/28/2009	1.9	41.296667	70.054444	6.65	23.9	8.08	25.7
5	3	8/12/2009	1.1	41.297222	70.062500	8.87	23.4	7.98	25.8
6	3	7/29/2009	0.9	41.280000	70.085000	8.81	23.7	7.78	25.7
7	3	8/12/2009	0.9	41.284722	70.078611	9.43	23.6	7.93	25.7
8	3	7/21/2009	2.1	41.290000	70.074167	7.88	22.9	7.77	32.4
9	2	7/29/2009	0.9	41.293889	70.068333	7.89	23.9	7.91	25.8
10	3	8/4/2009	1.4	41.285833	70.083056	7.80	24.5	7.63	25.9
11	1	7/28/2009	2.1	41.324167	70.037778	8.52	24.4	7.82	26.0
12	3	8/11/2009	1.4	41.318056	70.045278	6.89	25.1	7.59	26.0
13	3	7/22/2009	1.4	41.314722	70.047778	9.08	23.3	7.63	28.5
14	3	8/4/2009	1.2	41.311111	70.053611	7.29	26.0	7.76	26.1
15	2	8/5/2009	1.2	41.308056	70.058889	6.32	24.7	7.86	26.0
16	2	7/22/2009	1.5	41.305	70.063611	9.09	23.3	7.86	28.4

Table 5-1: Physical water quality parameters of *Argopecten irradians* collection sites. Date of collection and abundance zone (1: no scallops, 2: 1 – 70 individuals, 3: > 70 individuals).

Site Number	Zone	Date	Depth (m)	Latitude (N)	Longitude (W)	D.O. (mg/L)	Temp (°C)	pH	Salinity (‰)
17	2	8/12/2009	1.2	41.300833	70.071944	7.66	23.6	7.84	26.0
18	2	7/21/2009	1.4	41.298056	70.078611	6.67	22.4	7.74	32.7
19	1	7/21/2009	1.2	41.292222	70.086111	7.85	22.5	7.71	32.4
20	3	8/12/2009	0.9	41.294167	70.090278	8.05	23.0	7.79	25.8
21	2	8/5/2009	1.9	41.294444	70.100833	2.67	24.0	7.76	25.7
22	2	7/29/2009	2.4	41.291944	70.094167	8.95	24.0	7.94	25.8
23	1	8/4/2009	1.2	41.332222	70.031667	6.84	26.1	7.55	26.1
24	1	8/11/2009	2.3	41.344167	70.016111	6.36	25.3	7.50	26.0
25	1	7/22/2009	2.1	41.325833	70.003611	9.08	23.7	8.01	28.4
26	2	8/5/2009	3.9	41.298333	70.073333	9.43	24.8	7.87	25.9
27	2	7/29/2009	2.5	41.303611	70.057500	7.77	23.0	7.68	25.8
28	3	7/28/2009	2.1	41.310556	70.048611	6.99	24.4	7.63	25.9
29	1	7/21/2009	7.3	41.306389	70.040556	6.91	25.6	6.70	30.5
30	1	7/22/2009	2.7	41.320000	70.038889	8.44	23.2	7.85	28.6
31	1	7/28/2009	5.6	41.334167	70.014722	6.97	24.1	7.76	25.9
32	1	8/4/2009	1.5	41.318056	70.021944	7.93	25.1	7.47	26.0

Table 5-1 (continued): Physical water quality parameters of *Argopecten irradians* collection sites. Date of collection and abundance zone (1: no scallops, 2: 1 – 70 individuals, 3: > 70 individuals)

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