# Stony Brook University

# The effects of elevated carbon dioxide concentrations on the early life history of bivalve shellfish

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Abstract of the Dissertation

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The combustion of fossil fuels has increased $CO_2$ in the world's oceans and decreased pH. Ocean acidification may alter the growth, survival, and diversity of marine organisms that synthesize shells due to decreased carbonate ion availability. I examined the response of larvae and early juveniles from three species of commercially and ecologically valuable bivalve shellfish (*Mercenaria mercenaria, Argopecten irradians,* and *Crassostrea virginica*) exposed to past, pre-industrial levels (~250 ppm), present (~390 ppm), and future (~750, 1500 ppm) levels of $CO_2$. Under higher $CO_2$ concentrations, all three species experienced decreased growth, survival, and metamorphosis with *C. virginica* being the least affected. *M. mercenaria* and *A. irradians* larvae grown under past $CO_2$ concentrations displayed significantly faster growth and metamorphosis as well as higher survival and lipid accumulation rates compared to individuals under current $CO_2$ levels. Under pre-industrial $CO_2$ levels, *M. mercenaria* and *A. irradians* larvae displayed thicker shells than individuals grown at present $CO_2$ concentrations whereas bivalves exposed to high $CO_2$ levels had shells that

were malformed and eroded. This finding suggests ocean acidification from the past two centuries may be inhibiting the survival of larval shellfish and contributing to global declines of some bivalve populations. Short term physiological effects of higher $CO_2$ on larval bivalves included decreased: growth rates, RNA:DNA ratios, calcification rates, and lipid content, all which would promote enhanced mortality in an ecosystem setting. Exposure of bivalve larvae to varying levels of $CO_2$ identified threshold duration of exposure beyond which survival was compromised. Longer term experiments (8 months) demonstrated that individuals surviving high $CO_2$ in the larval stage are capable of rapid, compensatory growth when deployed under normal $CO_2$ levels as juveniles. Experiments combining higher $CO_2$ and other environmental stressors such as increased temperature or the introduction of a harmful alga demonstrated synergistic negative effects. Collectively, this dissertation demonstrates that larval stage exposure to high $CO_2$ concentrations has profound implications for the trajectories and restoration of coastal bivalve populations.

## Dedication Page

This dissertation is the culmination of many years of hard work and determination. I have many people who have helped me in the completion of this work, and without them it never would have been accomplished. I dedicate my doctoral dissertation to my family: my mother, father, and husband. Without these three people in my life, I never would have been able to complete this feat, and I am extremely grateful for their love and support in every endeavor I pursue. At the end of each chapter, I have acknowledged everyone who provided me with logistical support for that particular set of experiments. I also need to thank my advisor and mentor, Dr. Christopher Gobler. His work ethic is truly astounding, and it is only due to his guidance that I was able to complete this work. Dr. Gobler is an inspiration through his teaching, mentoring, and ideals as a father and husband, and it has been an honor to be a graduate of his lab group.

## Table of Contents

## List of Figures

**Chapter 6**



**Chapter 7**

## List of Tables

# CHAPTER ONE:

Dissertation introduction

## Introduction

The combustion of fossil fuels and the resultant increase in atmospheric carbon dioxide ($CO_2$) during the past century (I.P.C.C. 2007) has had a multitude of effects on this planet including acidification of the world's oceans. Atmospheric $CO_2$ levels have increased from preindustrial levels of approximately 280 parts per million (ppm) to 392 ppm today (average for March, 2011 measured at the Mauna Loa Observatory by the Earth Systems Research Laboratory NOAA). The oceans have absorbed nearly one third of the anthropogenically produced $CO_2$ during the past century (Sabine et al. 2004), altering inorganic carbon chemistry and pH. Model simulations suggest that combustion of the world's fossil fuel supply in the coming centuries could result in a five-fold increase in atmospheric $CO_2$ levels to nearly 2000 ppm and a decrease in surface ocean pH by 0.77 units (Caldeira and Wickett 2003).

Air-sea gas exchange equilibrates surface ocean water $CO_2$ concentrations with levels present in the atmosphere on a timescale of one year (MacIntyre et al. 1995). Dissolved $CO_2$ in seawater is part of the carbonate system that includes bicarbonate ($HCO_3^-$), carbonic acid ($H_2CO_3$), and carbonate ions ($CO_3^{2-}$). The anthropogenic $CO_2$ that is entering the world's oceans is causing a continuous change in seawater carbonate chemistry. Dissolved $CO_2$ reacts with water according to the following equilibrium reactions:

1) $CO_2 + H_2O \leftrightarrow H_2CO_3$, 2) $H_2CO_3 \leftrightarrow H^+ + HCO_3^-$, and 3) $HCO_3^- \leftrightarrow H^+ + CO_3^{2-}$

Therefore, increasing levels of $CO_2$ react with water to form carbonic acid ($H_2CO_3$). Carbonic acid can then dissociate by losing hydrogen ions and $HCO_3^-$ will result. Further dissociation and release of hydrogen ions results in the release of $CO_3^{2-}$. By adding $CO_2$

to seawater, there is a release of hydrogen ions as is seen in reactions 2) and 3) above, and since $pH = -\log_{10}[H^+]$, there is an overall decrease in the pH of ocean waters, which is known as ocean acidification.

Many organisms have hard parts (tests, shells, or skeletons) that are derived from the mineral calcium carbonate ($CaCO_3$). The formation of $CaCO_3$ depends on the saturation state ($\Omega$) where $\Omega = [Ca^{2+}]\,[CO_3^{2-}] / K'_{sp}$. The apparent solubility product, $K'_{sp}$ depends on temperature, salinity, and pressure. Organisms can more readily form hard parts of $CaCO_3$ when $\Omega > 1.0$ and dissolution of $CaCO_3$ typically occurs when $\Omega < 1.0$. Because calcium levels are constant in seawater, the values of $\Omega$ generally depend on the amount of $CO_3^{2-}$ available. The increase in $pCO_2$ causes $CO_3^{2-}$ to decrease via the following reaction: $CO_2 + CO_3^{2-} + H_2O \rightarrow 2HCO_3^-$. The effects of decreasing $CO_3^{2-}$ availability on marine organisms, especially those living in coastal waters, are unclear and likely complex.

The process of fossil fuel combustion promoting increasing concentrations of anthropogenic $CO_2$ leading to declining levels of pH and $CO_3^{2-}$ in the ocean has generally been referred to as ocean acidification. While this process has been and will continue to impact coastal marine ecosystems into the future, these regions are additionally impacted by near-shore-specific processes that can further enhance dissolved $CO_2$ concentrations. These coastal oceans and estuaries are shallow ecosystems (< 200m) where terrestrial, marine, and atmospheric systems interact. Levels of $CO_2$ in marine environments will continue to rise during this century, but organisms in some coastal zones are already exposed to high levels of $CO_2$. Many estuaries are 'net heterotrophic' due to terrestrial, riverine, and wetland supplements of allochthonous carbon (Gattuso et

al. 1998; Ram et al. 2003; Koch and Gobler 2009), which can lead to waters that are supersaturated with $CO_2$. Episodic discharge of acidic river waters into the Gulf of Maine can depress aragonite Ω values with potentially negative consequences for calcifying organisms such as soft-shelled clams (*Mya arenaria*) (Salisbury et al. 2008). Additionally, upwelling can result in coastal waters with $CO_2$ levels exceeding 1,000 ppm and decreased levels of $CO_3^{2-}$ (Feely et al. 2008; Salisbury et al. 2008). Moreover, since anthropogenic nutrient loading rates in coastal zones have risen (Howarth 2008), the concomitant increase in the intensity of algal blooms (Beman et al. 2005) and subsequent heterotrophic degradation of bloom-derived organic matter may result in larger declines in pH and increases in $CO_2$. This may be especially the case in temperate coastal zones during summer when the net heterotrophic nature of these systems can be maximal (Blight et al. 1995; Ram et al. 2003; Thomas et al. 2004). Finally, sediments in estuaries are typically the most heterotrophic part of the system due to the accumulation of sinking organic matter and generally have the highest $CO_2$ concentrations. The microbial degradation of this organic matter results in the production of $CO_2$ at the sediment-water interface (Rasmussen and Jorgensen 1992) and has the same chemical effects as atmospheric fluxes of $CO_2$, reducing the marine pH and carbonate ion availability. Estuaries on Long Island, NY, and Chesapeake Bay have experienced $pCO_2$ concentrations between 500-1500 ppm, and pH levels as low as 7.6 (Talmage and Gobler 2009; Waldbusser et al. 2011) suggesting the aforementioned processes are impacting these estuaries and that coastal ocean acidification is already occurring. All of this evidence further suggests coastal zones are regions which may regularly experience high levels of $CO_2$ and may already ephemerally experience decreased $CO_3^{2-}$ availability.

Increases in the ocean's $pCO_2$ levels and the subsequent reduced availability of $CO_3^{2-}$ in seawater can have detrimental effects on organisms that utilize the $CO_3^{2-}$ for the formation of their hard, structural parts. Calcifying organisms' biomineralization processes involve both passive and active movement of necessary ions isolated from ambient seawater in and out of a calcification compartment (Weiner and Dove 2003). The majority of marine $CaCO_3$ is produced by organisms which secrete two different forms of $CaCO_3$: calcite (rhombohedral crystals) or aragonite (orthorhombric crystals). The major marine calcite producers include coccolithophorids, foraminifera, crustose corraline algae, and some corals while pteropods and many reef building corals are primarily composed of aragonite. Many species of shellfish are predominantly comprised of a combination of calcite and aragonite (thermodynamically stable structures), although some of their earliest developmental stages are mostly amorphous calcium carbonate and later aragonite (Weiss et al. 2002). Amorphous calcium carbonate is the least stable form of the six known phases of $CaCO_3$ (aragonite, calcite, vaterite ($\mu$-$CaCO_3$), calcium carbonate monohydrate, calcium carbonate hexahydrate, and amorphous calcium carbonate) (Mann 2001). Vaterite has the highest solubility of all phases, and once exposed to water it converts to calcite or aragonite (Mann 2001). Magnesium ions are also readily accommodated in the calcite lattice and many biological calcites also contain $CO_3^{2-}$ as magnesite, ($MgCO_3$) or dolomite ($CaMg(CO_3)_2$) (Mann 2001). Amorphous calcium carbonate often comprises the earliest stages of development in many organisms since it is known to easily dissolve and be replaced on rapidly growing crustaceans, mollusks, and echinoderms (Raz et al. 2002; Weiss et al. 2002; Politi et al. 2004). Since aragonite is 50% more soluble than calcite (Mucci 1983), small decreases in $\Omega$ values for

aragonite associated with decreases in pH can lead to biomineralization problems for some animals including corals and pteropods (Orr et al. 2005; Albright et al. 2008; Doney et al. 2009).

There is growing recognition that increases in $CO_2$ and resulting decreases in pH and $CO_3^{2-}$ concentrations can have profound implications for a diverse set of $CaCO_3$ synthesizing organisms. For example, increasing $CO_2$ and decreasing $CaCO_3$ saturation can promote shell dissolution in pteropods (Orr et al. 2005). In experiments with two of the major calcifying coccolithophorid species, *Emiliania huxleyi* and *Gephyrocapsa oceanica*, Riebesell et al. (2000) increased $CO_2$ concentrations to a future level predicted for this century, from 280 to 365 and 750 ppm. This increase caused calcite production to be significantly reduced, malformation of the coccoliths to occur, and calcification rates of the coccolithophores to decrease from present levels by 16% and 45%, respectively (Riebesell et al. 2000). In contrast, another study has demonstrated that calcification and net primary production of *Emiliania huxleyi* increased under the same $CO_2$ partial pressures used by Riebesell et al. 2000 (750 ppm); (Iglesias-Rodriguez et al. 2008b). These conflicting results were the result of different experimental methodologies between the two studies. Culturing techniques may not have allowed for true acclimation of cells to experimental conditions in the later study which would have not represented the formation of the coccolithophores in a proper way (Riebesell et al. 2008). In addition, the results of (Riebesell et al. 2000) may have been confounded by using acid/base manipulation of seawater to manipulate $CO_2$ as this may not accurately represent the future chemistry of the high $CO_2$ oceans (Iglesias-Rodriguez et al. 2008a; EPOCA 2009). In mesocosm experiments bubbled with different $CO_2$ levels, it was noted that the

median coccolith size of *Emiliania huxleyi* did not vary significantly with increases in $CO_2$ to 700 ppm (Engel et al. 2008). Given that biological responses to ocean acidification may vary even within a species, the need for research focusing on the effects of this phenomenon on marine species seems clear.

In addition to its effect on calcifying phytoplankton, decreases in $CO_3^{2-}$ concentration can also reduce the ability of reef-building corals to produce $CaCO_3$ skeletons, affecting the ability of the reef system to overcome reef erosion and maintain the coral growth rates observed today (Kleypas et al. 2006). With an estimated increase in oceanic $CO_2$ levels to 560 ppm, well within what is expected this century, aragonite saturation states around the globe are projected to decrease to levels forcing the calcification rates of many coral reef communities to decrease (Kleypas et al. 2001). By 2100, the aragonite saturation state ($\Omega$) of tropical surface oceans are predicted to decrease approximately 30%, potentially leading to declines in reef calcification rates by 14-30% from preindustrial $CO_2$ levels (Kleypas et al. 1999). Since calcifying species play a central role in the growth and stabilization of carbonate reefs, future changes associated with acidification could greatly impact coral reefs throughout the world, even pushing some to extinction (Doney 2006).

While some of the effects of increasing $CO_2$ levels on many calcifying marine organisms are being investigated, research on ocean acidification is in its infancy. In the past three years, new research has demonstrated many types of marine calcareous organisms can be affected by modest changes in $CO_2$. In a seven-week experiment in Hawaii, crustose coralline algae showed a significant decrease in recruitment and growth with increases of $CO_2$ expected this century (Kuffner et al. 2008). Planktonic

foraminifera, echinoderms, and tropical corals have demonstrated reduced calcification and negative responses to increased $CO_2$, and some species of prokaryotes and seagrasses have demonstrated increased photosynthesis under increased $CO_2$ concentrations demonstrating the mixed response between calcifying and photosynthesizing marine organisms (Doney et al. 2009). Species specific results such as these demonstrate the importance of researching responses to increased levels of $CO_2$ through diverse sets of calcifying organisms.

Another group of marine organisms that are affected by increasing $CO_2$ levels are shellfish. Filter feeding bivalves are considered ecosystem engineers in coastal waters due to the filtration services they provide (Colson and Sturmer 2000; Gutierrez et al. 2003). This filtration has the potential to control eutrophication and harmful algal blooms (Officer et al. 1982; Cerrato et al. 2004), increase pelagic light penetration (Newell and Koch 2004), with the latter two processes providing benefit to submerged aquatic vegetation (Carroll et al. 2008; Wall et al. 2008). As such, these organisms can have major effects on ecosystem structure and function (Raillard and Menesguen 1994; Grant 1996; Arnold et al. 2002). Beyond benefits to marine ecosystems, many shellfish are also commercially valuable as a food source. For example, in 2007, United States landings of clams, mussels, oysters, and scallops exceeded $700 million (NMFS 2008) with their ecosystem services as filter feeders far exceeding that value (Costanza et al. 1997).

In recent decades, wild populations of shellfish such as the hard clam or northern quahog, *Mercenaria mercenaria* (*Linnaeus*, 1758), the Eastern oyster, *Crassostrea virginica* (*Gmelin, 1791*), and the bay scallop, *Argopecten irradians* (*Lamarck, 1819*),

have been under increasing pressure from overfishing, loss of habitat, hypoxia, and harmful algal blooms, and their populations have experienced precipitous declines (Jackson et al. 2001; Myers et al. 2007). New York estuaries offer prime examples of such declines, as landings from both the hard clam fishery in Great South Bay and the bay scallop fishery in the Peconic Estuary have declined by more than 99% since the early 1980s (NYSDEC 1950-2009). Factors which have been cited as contributing to these precipitous declines include overharvesting (Kraeuter et al. 2008), reduced reproductive success, predation (Kraeuter 2001), harmful algal blooms (Greenfield et al. 2004; Bricelj and MacQuarrie 2007), and a changing food supply (Greenfield et al. 2005; Lonsdale et al. 2007). No study to date has considered the manner in which $CO_3^{2-}$ availability may impact these species.

Shellfish produce calcareous shells, and the production of these shells depends on the same calcification processes and availability of $CO_3^{2-}$ discussed above. However, studies investigating the effects of increasing $CO_2$ levels on bivalve growth and shell production have been rare. Studies of $CaCO_3$ secreting shellfish have found that sediments undersaturated with respect to aragonite ($\Omega = \sim 0.3$) can cause enhanced mortality of juvenile clams (*Mercenaria mercenaria* at 0.2 mm, 0.3 mm, 1 mm, and 2 mm size classes) (Green et al. 2004). Coastal ocean acidification is already evident in many estuary settings due to eutrophication, anthropogenic inputs, upwelling, and freshwater inputs (Feely et al. 2008; Salisbury et al. 2008; Waldbusser et al. 2011). Elevated $CO_2$ concentrations (~740 ppm) have been shown to cause decreased calcification rates in mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) (a decrease of 25% and 10%, respectively, over present levels; (Gazeau et al. 2007). Berge et al.

(2006) described decreased growth and metabolic rates in the blue mussel (*Mytilus edulis*) at pH levels of 7.4. Similarly, in the marine mussel, *Mytilus galloprovincialis,* a reduction in sea-water pH to 7.3 resulted in decreased rates of oxygen consumption, increased nitrogen excretion indicating the net degradation of proteins, and an overall reduction in growth (Michaelidis et al. 2005).

The earliest developmental stages of shellfish, larvae, are critical to the population dynamics of the adult populations, as any decline in larval populations can have profound implications for future shellfisheries (Caley et al. 1996; Gosselin and Qian 1997; Carriker 2001; Cragg 2006). These earliest developmental stages of calcifying shellfish may also be highly sensitive to increased $CO_2$ concentrations. Green et al. (2009) demonstrated that increasing surface sediment aragonite saturation state ($\Omega$) from 0.25 to 0.53 can increase the settlement of *Mya arenaria* by three-fold, suggesting that recently settled larvae may be sensitive to $CO_3^{2-}$ availability (Green et al. 2009). Experimentally enhanced $CO_2$ has been shown to decrease the development rate of Pacific oyster larvae *Crassostrea gigas* (Kurihara et al. 2007) and the mussel, *Mytilus galloprovincialis* (Kurihara et al. 2008). When $CO_2$ levels were increased from ambient air to 2,000 ppm, sea urchin larvae (*Hemicentrotus pulcherrimus* and *Echinometra mathaei*) and coral larvae (*Acropora tenuis*) all showed degradation in morphology that could later lead to negative consequences in skeleton and shell synthesis for juvenile and adult populations (Kurihara 2008). Given the impacts of elevated $CO_2$ on the larvae of these invertebrates, shellfish larvae may also be threatened by these conditions.

In addition to rising levels of $CO_2$, there are other environmental pressures on larval shellfish in coastal ecosystems. For example, the sharp rise in anthropogenic

nutrient loading rates during the past century (Nixon 1995; Howarth 2008) is one of the factors responsible for the increasing frequency of harmful algal blooms (HABs) (Anderson et al. 2008; Heisler et al. 2008). HABs afflict most temperate and tropical coastal nations and the frequency of HAB events and their negative impacts on fisheries have increased markedly in recent decades (Anderson et al. 2002; Hoagland et al. 2002; Heisler et al. 2008; Jin et al. 2008). HABs can be toxic to shellfish (Shumway 1990; Bricelj and Shumway 1998), and more recently, the lethal effects of HABs on shellfish larvae have been documented (Matsuyama et al. 2001; Yan et al. 2001; Yan et al. 2003; Leverone et al. 2006; Padilla et al. 2006; Stoecker et al. 2008). In the United States, blooms of the dinoflagellate *Cochlodinium polykrikoides* have emerged to become annual HAB events on both the Atlantic and Pacific coasts (Curtiss et al. 2008; Gobler et al. 2008), and this species is known to bloom during the months in which shellfish spawn, causing enhanced mortality in larval shellfish (Tang and Gobler 2009). While both HABs and rising levels of $CO_2$ are factors which will negatively impact larval shellfish in coming decades, the combined effects of these factors on larval growth and survival are unknown.

An additional environmental stressor likely to affect the survival of larval shellfish in coastal ecosystems is temperature. The earth's surface temperature increased by approximately $1^{o}$C during the $20^{th}$ century (I.P.C.C. 2007). In the current century, global temperatures are expected to increase an additional 2 to $5^{\circ}$C (Houghton et al. 2001) with coastal ocean temperatures expected to display a similar trend (Nixon et al. 2004; I.P.C.C. 2007). Temperature is a key factor which influences the development of larval shellfish. *Mercenaria mercenaria* development and growth has been demonstrated to

slow above 25ºC and to cease at 31ºC (Fritz 2001). For the Eastern oyster (*Crassostrea virginica*), most fertilized eggs developed normally at 30ºC, but experience decreased survival at higher temperatures (Loosanoff 1965). For the bay scallop, *Argopecten irradians*, larvae exposed to water temperatures outside of the optimal range (23-28ºC) can experience increased mortality rates (Cragg 2006). Together, the effects of increasing temperature and increasing $CO_2$ could have large effects on future shellfish populations.

My research will investigate the effects of increasing $CO_2$ concentrations on the growth and survival of the larvae of three species of $CaCO_3$-synthesizing bivalves native to the east coast of North America: the hard clam or northern quahog, *Mercenaria mercenaria* (*Linnaeus*, 1758), the Eastern oyster, *Crassostrea virginica* (*Gmelin, 1791*), and the bay scallop, *Argopecten irradians* (*Lamarck, 1819*). These shellfish are important economic resources and ecosystem engineers in shallow coastal waters (Newell 2004). Larvae represent an important life stage for shellfish populations as reductions in the growth and survival of larvae have the potential to translate into declines in adult populations (Caley et al. 1996; Gosselin and Qian 1997; Schneider et al. 2003; Arnold 2008).

Establishing the effects of elevated $CO_2$ levels on the growth and development of the early life stages of bivalves is crucial to understanding how past and future environmental changes have affected and will affect their success in coastal ecosystems. To date, there are a limited number of investigations on the effects of increased $CO_2$ levels and decreased pH on bivalves, especially larval bivalves. This dissertation

represents a comprehensive investigation of the effects of increasing $CO_2$ levels on the early development of bivalves. The objectives of my dissertation were as follows:

**Objectives:**

1. To assess the effects of current, and elevated $CO_2$ levels on the survival and metamorphosis of larval bivalves native to Long Island.

2. To assess the effects of past, current, and elevated $CO_2$ concentrations on bivalve larvae: survival, development, accumulation of lipids, and three-dimensional growth.

3. To evaluate short term (RNA synthesis and calcification rates) vs. long term (growth and survival) and varying exposure effects of past, current, and elevated $CO_2$ concentrations on bivalves.

4. To assess the interactive effects of increasing levels of $CO_2$ and other common environmental stressors in coastal zones, including elevated temperature and poor food, on the growth and survival of larval bivalves.

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## CHAPTER TWO:

# The effects of elevated carbon dioxide concentrations on the metamorphosis, size, and survival of larval hard clams (*Mercenaria mercenaria*), bay scallops (*Argopecten irradians*), and Eastern oysters (*Crassostrea virginica*)

**(Published in Limnology and Oceanography, November 2009)**

**Abstract**

These experiments examined the metamorphosis, growth, and survivorship of larvae from three species of commercially and ecologically valuable shellfish (*Mercenaria mercenaria, Argopecten irradians,* and *Crassostrea virginica*) at the levels of $CO_2$ projected to occur during the 21st century and beyond. Under $CO_2$ concentrations estimated to occur later this century (~650 ppm), *Mercenaria mercenaria* and *Argopecten irradians* larvae exhibited dramatic declines (> 50%) in survivorship as well as significantly delayed metamorphosis and significantly smaller sizes. Although *Crassostrea virginica* larvae also experienced lowered growth and delayed metamorphosis at ~ 650 ppm $CO_2$, their survival was only diminished at ~1500 ppm $CO_2$. The extreme sensitivity of larval stages of shellfish to enhanced levels of $CO_2$ suggest that current and future increases in pelagic $CO_2$ concentrations may deplete or alter the composition of shellfish populations in coastal ecosystems.

## Introduction

The combustion of fossil fuels and the resultant increase in atmospheric $CO_2$ during the past century has had a multitude of effects on this planet including acidification of the world's oceans. The oceans have absorbed nearly half of the anthropogenically produced $CO_2$ during the past century (Sabine et al. 2004), altering its inorganic carbon chemistry and pH. Model simulations suggest that combustion of the world's fossil fuel supply in the coming centuries could result in a five-fold increase in atmospheric $CO_2$ levels to nearly 2,000 ppm and a decrease in surface ocean pH by 0.77 units (Caldeira and Wickett 2003). This decline in the pH of surface waters will concurrently reduce carbonate ion ($CO_3^{2-}$) concentrations and the degree of calcium carbonate ($CaCO_3$) saturation ($\Omega$) in surface waters with potentially negative consequences for $CaCO_3$ synthesizing marine organisms.

Although levels of $CO_2$ in marine environments will continue to rise during this century, organisms in some coastal zones are already exposed to high levels of $CO_2$. Many estuaries are 'net heterotrophic' due to terrestrial, riverine, and wetland supplements of allochthonous carbon (Gattuso et al. 1998; Ram et al. 2003; Koch and Gobler 2009), which can lead to waters that are supersaturated with $CO_2$. Moreover, in temperate coastal zones, many bivalve mollusks such as Eastern oysters (*Crassostrea virginica* (Gmelin, 1791)), bay scallops (*Argopecten irradians,* (Lamarck, 1819)), and hard clams or northern quahogs (*Mercenaria mercenaria,* (Linnaeus, 1758)) spawn during summer (Kennedy and Krantz 1982; Bricelj et al. 1987; Kraeuter and Castagna 2001) when the net heterotrophic nature of these systems is maximal (Blight et al. 1995; Ram et al. 2003; Thomas et al. 2004). Additionally, coastal upwelling and riverine discharge can result in coastal waters with $CO_2$ levels exceeding 1000 ppm and sub-saturating levels of carbonate ion (Feely et al. 2008; Salisbury et al. 2008). Hence, it is likely that calcium

carbonate synthesizing organisms in coastal zones are often challenged with high levels of $CO_2$ and low levels of pH and carbonate ion.

Calcite and aragonite are the primary biogenic forms of calcium carbonate in ocean animals. Studies to date have documented shell dissolution in pteropods (aragonite) as well as in calcifying coccolithophores (calcite) with increasing $CO_2$ and decreases in $CaCO_3$ saturation (Riebesell et al. 2000; Orr et al. 2005). Decreases in $CO_3^{2-}$ concentrations can reduce the ability of reef-building corals to produce $CaCO_3$ skeletons (Kleypas et al. 2006). Sediments undersaturated with respect to aragonite can cause enhanced mortality of juvenile clams (*Mercenaria mercenaria*, Green et al. 2004), and settlement of *Mya arenaria* have been shown to increase when sediment saturation states were increased by buffering from crushed *M. arenaria* shells suggesting that juvenile settlement is influenced by $CO_2$ levels (Green et al. 2004; Green et al. 2009). Moreover, elevated $CO_2$ can cause decreased calcification rates in mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*; (Gazeau et al. 2007)), and decreased growth and metabolic rates in mussels (Berge et al. 2006). The earliest developmental stages of calcifying marine organisms also may be highly sensitive to increased $CO_2$. Experimentally enhanced $CO_2$ has been shown to decrease the development rate of Pacific oyster larvae (*Crassostrea gigas*; (Kurihara et al. 2007)), and have negative effects on the early development of sea urchins (*Hemicentrotus pulcherrirnus, Echinometra mathaei*; (Kurihara 2008)). *Crassostrea virginica* larvae have displayed smaller shell area when exposed to $CO_2$ levels predicted for the end of the 21$^{st}$ century (Miller et al. 2009). To date, no study has examined the effects of the $CO_2$ levels predicted to occur this century on the metamorphosis, growth, and survival of larval shellfish.

This study investigated the effects of increasing $CO_2$ levels on the growth and survival of the larvae of three species of $CaCO_3$ synthesizing, bivalves: the hard clam, *Mercenaria*

*mercenaria,* the Eastern oyster, *Crassostrea virginica*, and the bay scallop, *Argopecten irradians*. These shellfish are important economic resources and ecosystem engineers in shallow coastal waters (Newell 2004). Larvae represent an important life stage for shellfish populations as reductions in the growth and survival of larvae have the potential to translate into declines in adult populations (Gosselin and Qian 1997; Schneider et al. 2003; Arnold 2008). I conducted a series of experiments to determine how $CO_2$ levels estimated to occur this century (~400-750 ppm) and through the year 2250 (~1500 ppm); (Caldeira and Wickett 2003; Zeebe et al. 2008) would affect larval survival, growth, and metamorphosis into juvenile stages.

**Methods**

*Carbon dioxide treatments and measurements* - A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used to deliver $CO_2$ gas to seawater treatments at multiple rates. The gas proportionator mixed appropriate flow rates of 5% carbon dioxide gas and pressurized air (~390 ppm $CO_2$) to yield the concentrations of carbon dioxide desired for experiments at a net flow rate which resulted in a total volume of gas (350 ± 5 mL $min^{-1}$) which turned over the volume of experimental beakers ~ 100 times daily. For experiments, the $CO_2$ gas mixtures from the proportionator system were continuously delivered to the bottom of four replicated, polypropylene 1-liter beakers containing 0.2 $\mu$m filtered seawater from eastern Shinnecock Bay, New York, United States. Control containers were continuously bubbled with air at the same rate as the treatment containers with elevated levels of $CO_2$. With continuous bubbling, all treatment beakers were saturated with respect to oxygen (~8 mg $L^{-1}$). To quantify precise $CO_2$ levels attained in experimental beakers, seawater in beakers was bubbled for 24 hours and then analyzed before (no larvae or phytoplankton in seawater) and immediately after

(larvae removed, phytoplankton present) each experiment using an EGM-4 Environmental Gas Analyzer® (PP Systems) system that quantifies total dissolved inorganic carbon levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). Levels of $CO_2$ were subsequently calculated based on measured levels of total inorganic carbon, pH (mol kg seawater$^{-1}$; NBS scale), temperature (~24°C), salinity (~28), and first and second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/). All beakers were also monitored daily for pH level, using an Oakton® or Thermo Scientific Orion Star Series™ Benchtop pH meter (± 0.01 and 0.001, respectively), to provide further assurance that the constant $CO_2$ levels were administered throughout the experiment. Spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson *et al.* (2007) and corrected for scale (Dickson 1993) were never significantly different from those obtained with the high sensitivity microprocessor. For comparison of the levels of $CO_2$ administered during experiments to those found today in coastal zones, bi-weekly measurements of total inorganic carbon, pH, temperature, and salinity were made in eastern Shinnecock Bay (40.87°N, 72.45°W) during the typical shellfish spawning period (June through September; (Kennedy and Krantz 1982; Bricelj et al. 1987; Kraeuter and Castagna 2001). Water samples were collected at 1200 (±1 h) on each sampling day from 0.5 meters below the water column using a Van Dorn bottle. Water was immediately transferred without bubbling to 300 mL, borosilicate bottles, and measured for total inorganic carbon as described above. Levels of $CO_2$ were estimated as described above using CO2SYS.

*Experimental design* - Experiments were conducted using three species of bivalves: *Mercenaria mercenaria, Crassostrea virginica*, and *Argopecten irradians*. For each experiment, three levels of carbon dioxide were administered: a high level (~1500 ppm $CO_2$), predicted for

the year 2250, a moderate level (~650 ppm $CO_2$), predicted for the year 2100 (Caldeira and Wickett 2003; Zeebe et al. 2008), and ambient air (~390 ppm $CO_2$). An additional experiment was conducted in which the range of $CO_2$ levels predicted during this century (~400, 500, 600, and 700 ppm; (Joos et al. 1999)) were administered to *Mercenaria mercenaria* larvae. *Argopecten irradians*, *Crassostrea virginica,* and *Mercenaria mercenaria* larvae were obtained from the East Hampton Shellfish Hatchery, East Hampton, New York, United States, 24 h after fertilization.

As a food source, a culture of *Isochrysis galbana* (Tahitian strain, T-Iso) was maintained in exponential phase growth using standard culture conditions and added at a density of $2 \times 10^7$ cells daily to each experimental beaker ($2 \times 10^4$ $mL^{-1}$). This algal species administered at this density and at this rate is known to produce high growth rates and survivorship of shellfish larvae through metamorphosis (Carriker 2001; Cragg 2006; Padilla et al. 2006). To promote the high survivorship, all containers that were in contact with larvae were never exposed to chemicals or detergents (Padilla et al. 2006). To discourage the growth of bacteria during experiments, an antibiotic solution (Sigma-Aldrich No.4083, 5000 units of Penicillin, 5 mg of Streptomycin, and 10 mg of Neomycin per milliliter of solution) was added to each beaker at 1% its original concentration at the beginning of each experiment and at the time of each water change (approximately 2 times weekly). This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae (Padilla et al. 2006). For each experiment, approximately 100 larvae were distributed to each experimental beaker. Each treatment began with approximately 900 mL to allow beaker volume for the algal culture to be added daily as a food source. Twice weekly during experiments, larvae were gently poured onto a 64 $\mu$m mesh, and the condition (live or dead) and developmental stage of each larvae

(veligers, pediveligers, and metamorphosed) was determined visually under a dissecting microscope; every individual larvae was counted at every water change. Larvae from each beaker ($n$ =4, per treatment) were removed, counted, observed, and transferred into a new beaker with new filtered seawater, food, and antibiotics within a 15 minute period. All beakers were submerged in a water bath maintained at 24˚C and recorded every 6 minutes throughout experiments using in situ loggers (Onset©). This temperature generally yields high growth rates for *Argopecten irradians*, *Crassostrea virginica,* and *Mercenaria mercenaria* larvae (Carriker 2001; Matthiessen 2001; Cragg 2006). Percent survivorship of all larvae was determined by movement or lack of movement of individuals at each of the bi-weekly water changes when the numbers of larvae in each stage of veligers, pediveligers, and metamorphosed juveniles were quantified. Experiments were terminated when at least 50% of the remaining larvae had metamorphosed. At this time, digital photos of 15 randomly selected metamorphosed larvae were obtained from under a dissecting microscope and lengths were determined using Image J® software. Statistical differences among replicate beakers were examined using $Q$-tests ($p$<0.1) although none were present in any experiment for all parameters quantified. To statistically assess the effect of $CO_2$ treatments on larval survival, a goodness of fit test or $G$-Test was calculated for each experimental treatment since this is a robust for analysis of differences in percent survivorship among treatments (Sokal and Rohlf 1995). One-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among larval size at each $CO_2$ level.

**Results**

Enriched levels of dissolved $CO_2$ had a pronounced negative effect on the survival of all three species of shellfish larvae. *Mercenaria mercenaria* larvae were greatly affected by ~650 ppm and ~1500 ppm $CO_2$ and the resultant decreases of pH, $CO_3^{2-}$ availability, and $CaCO_3$ saturation state (Fig. 1 and Table 1). There was a significant decrease in survivorship of larvae with increased $CO_2$ compared to larvae receiving ambient levels (*G*-test, $p< 0.001$; Fig. 1). Fewer than 20% of *Mercenaria* larvae survived to fully metamorphose into juvenile clams in the ~650 and 1500 ppm $CO_2$ treatments compared to 76 ± 4 % in the ambient, control treatment (Fig. 1). Larvae within elevated $CO_2$ treatments also experienced delays in metamorphosis. For example, although all surviving clam larvae had fully metamorphosed after 18 days under ambient conditions, 18 ± 6% had still not done so in the highest $CO_2$ treatment (Fig. 1). The mean length of 18 day old *Mercenaria mercenaria* larvae decreased significantly from 220 ± 9.5 $\mu$m at 350 ppm $CO_2$, to 170 ± 6.7 $\mu$m and 170 ± 4.8 $\mu$m at 650 and 1500 ppm $CO_2$, respectively, (ANOVA, $p<0.001$, Tukey $p<0.01$; Fig. 2).

Since the levels of $CO_2$ predicted in the world's oceans later this century (~650 ppm) caused significant increases in mortality and delays in development times for *Mercenaria mercenaria* compared to current $CO_2$ levels, an experiment examining the range of $CO_2$ predicted through this century was conducted (~400, 500, 600, and 700 ppm; Table 2). There were significant differences in larval survival between all of the $CO_2$ treatments (~700, 600, 500, and 400 ppm; *G*-test, $p< 0.001$). *Mercenaria mercenaria* larval survivorship declined from 86 ± 6% to 66 ± 2% with a 100 ppm rise in $CO_2$ (~400 to 500 ppm; Fig. 3.), a change predicted to occur early in the 21$^{st}$ century (Caldeira and Wickett 2003). Survivorship of *Mercenaria* larvae

through the experiment continued to decrease to 63 ± 3% and 20 ± 6% of individuals at ~600 and 700 ppm $CO_2$, respectively (Fig. 3).

*Argopecten irradians* (bay scallop) larvae were also extremely sensitive to higher $CO_2$ concentrations. Only 3 ± 1% and 2 ± 0.5% of *Argopecten* larvae survived to metamorphosis at ~650 and ~1600 ppm $CO_2$ levels respectively (a 7.9% $d^{-1}$ population mortality rate), while 52% survived in the ambient treatment (Fig. 1 and Table 1), a highly significant difference (*G*-test, $p< 0.001$). High $CO_2$ levels also slowed development rates of *Argopecten* larvae. After 16 days, only 54% and 76% of scallop larvae had metamorphosed to the juvenile stage at ~1600 and ~650 ppm $CO_2$, whereas 100% of the surviving individuals under ambient conditions (~350 ppm) had metamorphosed at this time (Fig. 1). At day 19, the lengths of *A. irradians* larvae grown under high $CO_2$ were half the length (200 ± 12 $\mu$m) of individuals grown under ambient $CO_2$ conditions (400 ± 4.6 $\mu$m at ~350 ppm; Tukey $p<0.0001$; Fig. 2).

In contrast to *Mercenaria* and *Argopecten*, *Crassostrea virginica* (Eastern oyster) larvae responded differently to the enrichment of $CO_2$ in seawater. The rate of oyster larvae metamorphosis was significantly delayed by exposure to enrichment of $CO_2$ (*G*-test, $p< 0.001$). After two weeks, one-third of oyster larvae exposed to current $CO_2$ conditions had fully metamorphosed, while only 6 ± 2% and 3 ± 1% had done so at ~650 and 1500 ppm $CO_2$, respectively (Fig. 1). Similarly, after three weeks, metamorphosis had occurred in 89 ± 9, 69 ± 12, and 58 ± 12% of surviving individuals at ~350, 650, and 1500 ppm $CO_2$, respectively, differences which were statistically significant (*G*-test, $p< 0.001$; Fig. 1). Furthermore, *C. virginica* larvae reared under higher $CO_2$ (650 and 1500 ppm) achieved lengths (300 ± 17 $\mu$m and 260 ± 12 $\mu$m) which were significantly smaller than those grown at ambient $CO_2$ (360 ± 14 $\mu$m at 350 ppm; Tukey $p<0.05$; Fig. 2). However, in contrast to these differences in size and the

time required for larval metamorphosis, there was no difference in survivorship between exposure to 350 and 650 ppm $CO_2$ (61 ± 16%) although the highest levels of $CO_2$ (~1500 ppm) yielded significantly lower survival for oyster larvae (35 ± 13%; *G*-test, $p < 0.001$; Fig. 1).

**Discussion**

This study has demonstrated that the levels of $CO_2$ projected to occur in the world's oceans this century and beyond are capable of significantly decreasing the size, rates of metamorphosis, and survivorship of larvae from three species of commercially and ecologically valuable shellfish (*Mercenaria mercenaria, Argopecten irradians,* and *Crassostrea virginica*). The ability of calcifying organisms to synthesize $CaCO_3$ shells is strongly influenced by $CO_3^{2-}$ concentrations and Ω values. Our highest $CO_2$ treatments (1500-1600 ppm) yielded $\Omega_{aragonite}$ values <1.0 (Table 1) indicating that dissolution of aragonite would be predicted and synthesis of aragonite would not be favorable. Since the three shellfish larvae studied have shells which are partly composed of aragonite (Stenzel 1964; Carriker 1996), the enhanced mortality and delayed development of these larvae in these treatments (Fig. 1) may result from their inability to adequately synthesize shell material. However, calcification rates in coccolithophores, foraminifera, and corals have been shown to decrease with reduced $CO_3^{2-}$ concentration even when $\Omega > 1$ (Riebesell et al. 2000; Kleypas et al. 2006), likely in part because biotic aragonite is less crystalline than nonbiogenic aragonite (Weiss et al. 2002). Additionally, the presence of amorphous calcium carbonate, which is substantially more soluble than aragonite (Brecevic and Nielsen 1989), in the shells of larval mollusks (Weiss et al. 2002), likely exacerbates their sensitivity to declining $CO_3^{2-}$ availability. Consistent with this hypothesis, even decreases in aragonite Ω in the saturating range caused by increasing $CO_2$ adversely affected the ability of

shellfish larvae to survive and metamorphose (Fig. 1, Table 1). In the future, such effects will be exacerbated within higher latitudes with colder waters and lower states of $CaCO_3$ saturation (Joos et al. 1999).

It is probable that the negative effects of elevated $CO_2$ on shellfish larvae will extend beyond those observed during our experiments. For example, since pelagic predation pressure on bivalve larvae is high (Andre and Rosenberg 1991), the delayed metamorphosis of shellfish larvae caused by enhanced $CO_2$ (Fig. 1) would likely translate into prolonged predation and hence would further decrease survival of larvae in the field. Similarly, the up to 50% decrease in size of larvae under high $CO_2$ (Fig. 2) could translate into higher mortality rates for these individuals once settled (Marshall et al 2003). Since recently settled bivalves are highly prone to dissolution and shell loss, and mortality loss is size dependent (Green et al. 2004), even surviving larvae may not fully develop in some coastal zones. Finally, it is plausible that rapid evolution of shellfish and other calcifying species will lead to the proliferation of strains which are more resistant to ocean acidification, perhaps circumventing some of the effects described here. However, the current rates of atmospheric $CO_2$ increases are 100 times faster than any recorded in the past one million years, rapidly changing the ocean chemistry to levels not experienced in hundreds of millions of years (Sabine et al. 2004), suggesting this evolutionary challenge is without precedent for many extant calcifying species.

The negative effects of rising levels of $CO_2$ on shellfish larvae are consistent with prior studies of calcifying organisms in the ocean such as coral reefs, coccolithophores, and even juvenile stages of shellfish (Riebesell et al. 2000; Green et al. 2004; Kleypas et al. 2006). Among planktonic species of coccolithophores, some strains have shown decreased levels of calcification with increased levels of $CO_2$ (Riebesell et al. 2000), while others have displayed an

increase in calcification and abundance under such conditions (Iglesias-Rodriguez et al. 2008). Similarly, the levels of $CO_2$ predicted later this century may be more detrimental to some species (*Mercenaria mercenaria, Argopecten irradians*) than others (*Crassostrea virginica*). It has been previously noted that *Crassostrea virginica* eggs and larvae are more tolerant of low pH than *Mercenaria mercenaria* larvae (Calabrese and Davis 1966), a finding consistent with our demonstration that oyster larvae survival was higher than larval hard clams at the $CO_2$ levels of ~650 ppm (Fig. 1).

Larvae survival can greatly affect future adult shellfish population densities (Caley et al. 1996; Gosselin and Qian 1997; Schneider et al. 2003; Arnold 2008). All three shellfish in this study are important ecological and commercial species along the Atlantic coast, with annual US landings of these species being worth hundreds of millions of dollars and their ecosystem services far exceeding that value (Costanza et al. 1997). However, wild populations of shellfish have been under increasing pressure from overfishing, loss of habitat, hypoxia, and eutrophication in recent decades and their populations have experienced precipitous declines (Lotze et al. 2006). Globally, shellfish restoration efforts such as habitat restoration, transplantation, and reduction of fishing pressure are being implemented to enhance stocks of these species (Arnold et al. 2002). In the future, these efforts will need to account for the differential ability of shellfish larvae to persist in a high $CO_2$ environment, with some populations, such as the Eastern oyster, perhaps having a greater success in the future than other species, such as hard clams or bay scallops.

While the high levels of $CO_2$ used during our experiments are predicted to occur within the open oceans during the coming century, they can already be found in some coastal zones. Upwelling of deeper ocean water and riverine discharge into coastal regions can both expose

pelagic shellfish larvae to $CO_2$ levels exceeding 1000 ppm and decreased levels of carbonate ion (Feely et al. 2008; Salisbury et al. 2008). Furthermore, many coastal regions are often 'net heterotrophic' due to terrestrial, riverine, and wetland supplements of allochthonous carbon (Gattuso et al. 1998; Thomas et al. 2004; Koch and Gobler 2009), leading to supersaturated $CO_2$ and lower pH. The degree of net heterotrophy in coastal zones is generally maximal during summer when spring bloom productivity is degraded (Blight et al. 1995; Ram et al. 2003) and shellfish larvae are spawned in temperate estuaries (Kennedy and Krantz 1982; Bricelj et al. 1987; Kraeuter and Castagna 2001). Moreover, as anthropogenic nutrient loading rates rise, the concomitant increase in the intensity of algal blooms and subsequent heterotrophic degradation of bloom-derived organic matter may result in larger declines in pH and increases in $CO_2$. Consistent with these ideas, our cursory examination of $CO_2$ concentrations in a New York estuary during summer months (June – September) revealed levels were frequently above 500 ppm and averaging ~650 ppm (Table 3). Hence, the three species of bivalve mollusks presented here, which are native to temperate estuaries in North American such as Shinnecock Bay, New York, are likely to produce larvae under elevated $CO_2$ conditions found in this and other similar systems. Therefore, the elevated levels of $CO_2$ in coastal zones coupled with the 100 ppm rise in ocean $CO_2$ levels which occurred during the $20^{th}$ century (Caldeira and Wickett 2003) may have already diminished shellfish populations and partly contributed to their global decline (Lotze et al. 2006).

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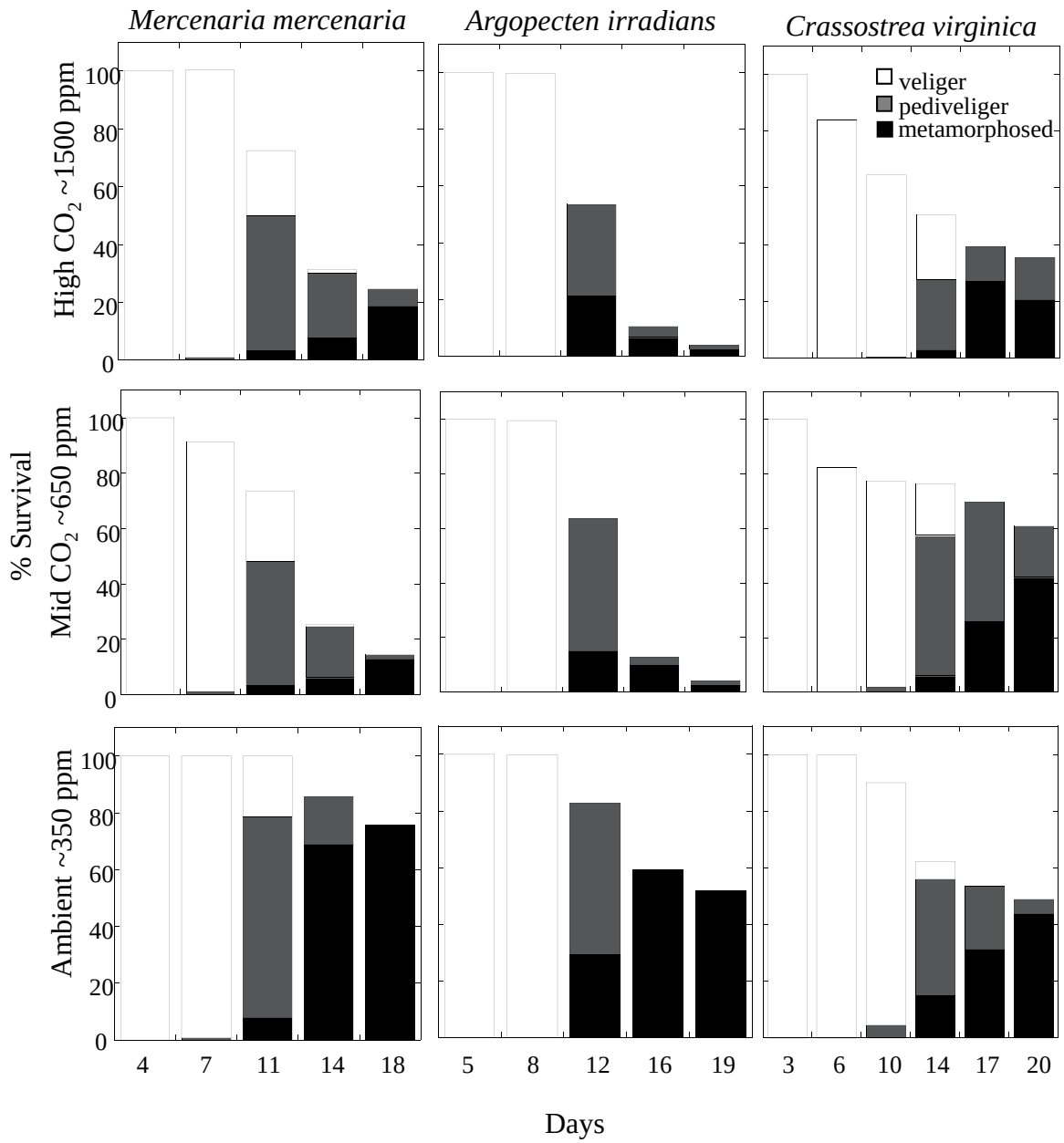


Figure 1: Survival and development of *Mercenaria mercenaria*, *Argopecten irradians*, and *Crassostrea virginica* larvae under three levels of $CO_2$, approximately 350, 650, and 1500 ppm (Table 1). Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated. The relative standard deviation of replicated vessels per treatment for all time points and experiments was 9% ($n$ =4 per treatment).

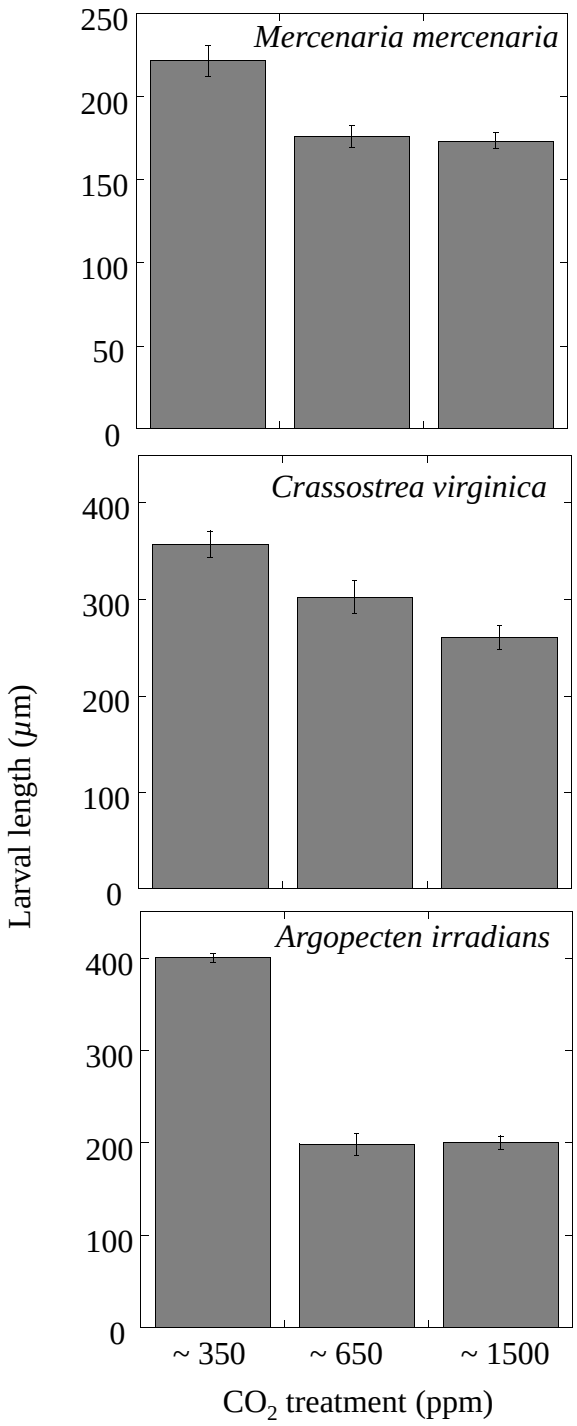


Figure 2: Mean length of larval shellfish ± 1 standard error at three levels of $CO_2$ (*n* =15 per treatment). Measurements were made at day 18 *(Mercenaria mercenaria)*, day 20 (*Crassostrea virginica*), and day 19 (*Argopecten irradians*). Precise $CO_2$ levels administered appear in Table 1.

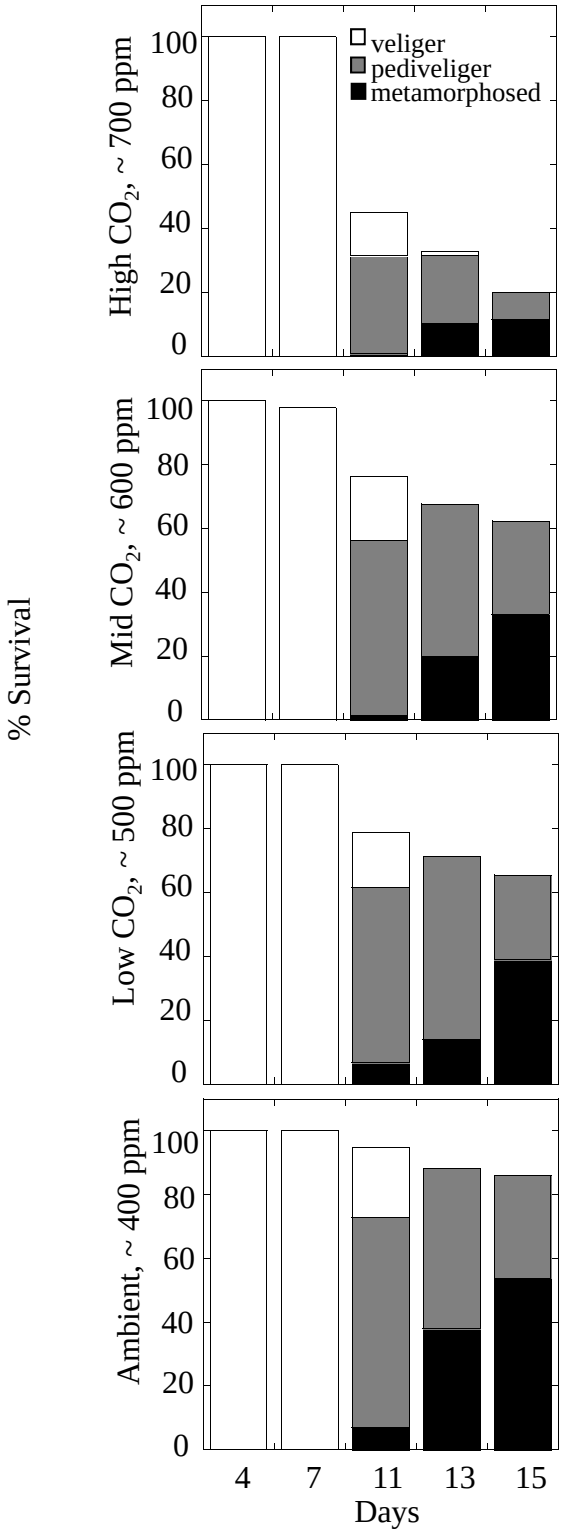


Figure 3: Survival and development of *Mercenaria mercenaria* larvae under four levels of $CO_2$, approximately 400, 500, 600, and 700 ppm (Table 2). Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated. The relative standard deviation of replicated vessels per treatment for all time points was 7% (*n* =4 per treatment)

| Parameter | Year 2200 $CO_2$ | Year 2100 $CO_2$ | Ambient |
|---|---|---|---|
| *Mercenaria mercenaria* | | | |
| Temperature (˚C) | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 7.49 ± 0.021 | 7.84 ± 0.042 | 8.02 ± 0.021 |
| $pCO_2$ (ppm) | 1480 ± 182 | 631 ± 83.2 | 354 ± 38.1 |
| $\Omega_{calcite}$ | 1.33 ± 0.121 | 2.98 ± 0.623 | 3.68 ± 0.222 |
| $\Omega_{aragonite}$ | 0.85 ± 0.12 | 1.92 ± 0.421 | 2.37 ± 0.124 |
| Total DIC ($\mu$mol $L^{-1}$) | 1850 ± 159 | 1850 ± 249 | 1580 ± 109 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 54 ± 8.4 | 121 ± 3.4 | 150 ±5.2 |
| Alkalinity (TA) | 1888 ± 20.1 | 2002 ± 21.4 | 1791.8 ± 79.2 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| % mortality $d^{-1}$ | -6.3 | -6.7 | -1.8 |
| | | | |
| *Crassostrea viginica* | | | |
| Temperature (˚C) | 24 ± 0.51 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 7.50 ± 0.012 | 7.85 ± 0.031 | 8.07 ± 0.012 |
| $pCO_2$ (ppm) | 1480 ± 50.2 | 626 ± 49.4 | 351 ± 17.2 |
| $\Omega_{calcite}$ | 1.43 ± 0.121 | 2.97 ± 0.233 | 4.52 ± 0.241 |
| $\Omega_{aragonite}$ | 0.92 ± 0.032 | 1.91 ± 0.111 | 2.91± 0.123 |
| Total DIC (umol $L^{-1}$) | 1920 ± 41.3 | 1840 ± 18.2 | 1770 ± 67.2 |
| $CO_3^{2-}$ ($\mu$mol $L^{1}$) | 58 ± 7.2 | 121 ± 3.5 | 180 ± 25.1 |
| Alkalinity (TA) | 1961 ± 39.2 | 1996 ± 54.4 | 2014 ±12.3 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| % mortality $d^{-1}$ | -3.9 | -1.9 | -3.5 |
| | | | |
| *Argopecten irradians* | | | |
| Temperature (˚C) | 24 ± 0.53 | 24 ± 0.53 | 24 ± 0.53 |
| pH | 7.48± 0.061 | 7.83 ± 0.032 | 8.08 ± 0.034 |
| $pCO_2$ (ppm) | 1610 ± 268 | 684 ± 45.4 | 355 ± 26.2 |
| $\Omega_{calcite}$ | 1.41 ± 0.123 | 2.9 ± 0.24 | 4.8 ± 0.42 |
| $\Omega_{aragonite}$ | 0.91 ± 0.12 | 1.87 ± 0.121 | 3.06 ± 0.341 |
| Total DIC ($\mu$mol $L^{-1}$) | 1980 ± 54.3 | 1900 ± 15.2 | 1830 ± 66.1 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 57 ± 6.2 | 118 ± 3.4 | 194 ± 26.2 |
| Alkalinity (TA) | 2011 ± 24.3 | 2047 ± 30.2 | 2087 ± 51.0 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| % mortality $d^{-1}$ | -7.9 | -7.8 | -3.8 |

**Table 1**. Temperature, pH, carbonate chemistry, alkalinity, salinity, and percent mortality of larvae per day (± 1 SD) during the three-level carbon dioxide experiments with *Mercenaria mercenaria, Crassostrea virginica,* and *Argopecten irradians* larvae.

| Parameter | Year 2100 $CO_2$, latest | Year 2100 $CO_2$, later | Year 2100 $CO_2$, early | Ambient |
|---|---|---|---|---|
| Temperature (˚C) | 24 ± 0.52 | 24 ± 0.51 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 7.79 ± 0.012 | 7.88 ± 0.021 | 7.97 ± 0.022 | 8.02 ± 0.012 |
| $pCO_2$ (ppm) | 722 ± 17.1 | 592 ± 36.3 | 480 ± 23.4 | 409 ± 15.2 |
| $\Omega_{calcite}$ | 2.57 ± 0.123 | 3.15 ± 0.101 | 4.0 ± 0.222 | 4.14 ± 0.133 |
| $\Omega_{aragonite}$ | 1.66 ± 0.042 | 2.03 ± 0.102 | 2.58 ± 0.122 | 2.67 ± 0.111 |
| Total DIC ($\mu$mol $L^{-1}$) | 1830 ± 12.2 | 1850 ± 8.43 | 1910 ± 61.2 | 1800 ± 16.0 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 105 ± 10.1 | 129 ± 6.2 | 163 ± 4.6 | 169 ± 1.1 |
| Alkalinity (TA) | 1961 ± 18.2 | 2020 ± 34.2 | 2126 ± 34.2 | 2036.3 ± 4.6 |
| Salinity | 28 ± 1.0 | 28 ± 1.0 | 28 ± 1.0 | 28 ± 1.0 |
| % mortality $d^{-1}$ | -8.2 | -3.8 | -3.5 | -1.4 |

**Table 2**. Temperature, pH, carbonate chemistry, alkalinity, salinity, and percent mortality of larvae per day (± 1 SD) during the four-level carbon dioxide experiments with *Mercenaria mercenaria* larvae.

| Date | Temperature (°C) | Salinity | pH | Total $CO_2$ (µmol $L^{-1}$) | $pCO_2$ (ppm) | $\Omega_{calcite}$ | $\Omega_{aragonite}$ |
|---|---|---|---|---|---|---|---|
| 9- Jun | 17.4 | 26.2 | 7.937 | 1523 | 414 | 2.20 | 1.38 |
| 15-Jun | 21.2 | 28.2 | 7.989 | 1506 | 450 | 2.94 | 1.88 |
| 7-Jul | 21.9 | 27.3 | 7.656 | 1756 | 949 | 1.67 | 1.06 |
| 20-Jul | 24.4 | 27.6 | 7.848 | 1695 | 580 | 2.74 | 1.76 |
| 27-Jul | 26.1 | 24.6 | 7.777 | 1253 | 524 | 1.74 | 1.11 |
| 10-Aug | 25.1 | 28.4 | 7.778 | 1926 | 777 | 2.78 | 1.80 |
| 17-Aug | 26.2 | 27.5 | 8.019 | 1814 | 409 | 4.51 | 2.92 |
| 24-Aug | 25.1 | 28.9 | 8.015 | 1892 | 426 | 4.59 | 2.98 |
| 7-Sep | 21.4 | 30.0 | 7.691 | 1642 | 795 | 1.74 | 1.12 |
| 28-Sep | 19.7 | 27.9 | 7.563 | 1826 | 1200 | 1.30 | .83 |

**Table 3**. Measurements of temperature, salinity, pH, total inorganic carbon, carbon dioxide, $\Omega_{calcite}$, and $\Omega_{aragonite}$ in eastern Shinnecock Bay, New York, during summer 2007.

# CHAPTER THREE:

## Effects of past, present, and future ocean carbon dioxide concentrations on the growth and survival of the early life history of bivalves

**(Published in Proceedings of the National Academy of Sciences of the United States of America, October 2010)**

**Abstract**

The combustion of fossil fuels has enriched levels of $CO_2$ in the world's oceans and decreased ocean pH. While the continuation of these processes may alter the growth, survival, and diversity of marine organisms that synthesize $CaCO_3$ shells, the effects of ocean acidification since the dawn of the industrial revolution are not clear. The experiments in this chapter examined the effects of the ocean's past, present, and future ($21^{st}$ and $22^{nd}$ centuries) $CO_2$ concentrations on the growth, survival, and condition of larvae of two species of commercially and ecologically valuable bivalve shellfish (*Mercenaria mercenaria* and *Argopecten irradians*). Larvae grown under near pre-industrial $CO_2$ concentrations (250 ppm) displayed significantly faster growth and metamorphosis as well as higher survival and lipid accumulation rates compared to individuals reared under modern day $CO_2$ levels. Bivalves grown under near pre-industrial $CO_2$ levels displayed thicker, more robust shells than individuals grown at present $CO_2$ concentrations whereas bivalves exposed to $CO_2$ levels expected later this century had shells that were malformed and eroded. These results suggest that the ocean acidification that has occurred during the past two centuries may be inhibiting the development and survival of larval shellfish and contributing to global declines of some bivalve populations.

## Introduction

More than eight petagrams of carbon dioxide ($CO_2$) are annually released into our planet's atmosphere via the combustion of fossil fuels (Le Quere et al. 2009). About one third of anthropogenically derived $CO_2$ has entered the world's oceans during the past two centuries (Sabine et al. 2004) and atmospheric and surface ocean $CO_2$ levels are expected to reach ~750 ppm by 2100 (Caldeira and Wickett 2003; I.P.C.C. 2007). $CO_2$ entering the ocean decreases the availability of carbonate ions ($CO_3^{-2}$) and reduces ocean pH, a process known as ocean acidification. These changes in ocean chemistry may have dire consequences for ocean animals that produce hard parts made from calcium carbonate ($CaCO_3$). The experimental enrichment of $CO_2$ to levels expected in the coming century has been shown to dramatically alter the growth, survival, and morphology of numerous calcifying organisms including coccolithophores, coral reefs, crustose coralline algae, echinoderms, foraminifera, and pteropods (Riebesell et al. 2000; Kleypas et al. 2006; Guinotte and Fabry 2008). Many shellfish also produce calcareous shells and juvenile and adult clams, mussels, and oysters have been shown to be adversely affected by elevated $CO_2$ (Green et al. 2004; Gazeau et al. 2007; Kurihara et al. 2007; Kurihara et al. 2008; Green et al. 2009). The earliest life history stages of shellfish, larvae, have been shown to be especially vulnerable to high $CO_2$, displaying large declines in survival and delays in metamorphosis at levels predicted to occur later this century suggesting recruitment of these populations may be adversely impacted by ocean acidification (Kurihara et al. 2007; Talmage and Gobler 2009; Watson et al. 2009).

While it is clear that calcifying ocean animals such as shellfish are sensitive to the increases in $CO_2$ projected for the future, the extent to which the rise in $CO_2$ that has

occurred since the dawn of the industrial revolution has impacted these populations is poorly understood. Here I present experiments that examined the effects of past (250 ppm), present (390 ppm), and future (> 400 ppm) $CO_2$ concentrations on larvae of two species of shellfish: the Northern quahog or hard clam, *Mercenaria mercenaria* and the bay scallop, *Argopecten irradians*. These bivalves are ecologically and commercially valuable resources: US mollusk harvests are $750M annually (Cooley and Doney 2009) with ecosystem services far exceeding that value (Costanza et al. 1997; Cooley et al. 2009).

**Methods**

*Carbon dioxide treatments and measurements*- A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used to deliver $CO_2$ gas to seawater treatments at multiple rates. The gas proportionator mixed appropriate flow rates of 5% carbon dioxide gas, low carbon dioxide gas, and pressurized air (~ 390 ppm $CO_2$) to yield the concentrations of carbon dioxide desired for experiments at a net flow rate (350 ± 5 mL $min^{-1}$) that turned over the volume of plexi-glass covered experimental beakers >400 times daily. Experiments were repeated with tanked gas premixed at each specific $CO_2$ level and nearly identical seawater chemistry and larval responses were obtained. For experiments, the $CO_2$ gas mixtures from the proportionator system were continuously delivered to the bottom of four replicated, polypropylene 1-liter beakers containing 0.2 $\mu$m filtered seawater from eastern Shinnecock Bay, New York, United States. With continuous bubbling, all treatment beakers remained saturated with respect to oxygen (~8 mg $L^{-1}$). To quantify precise $CO_2$ levels attained in experimental beakers, seawater in

beakers was bubbled for 24 hours and analyzed at the start (immediately prior to the addition of larvae and phytoplankton) and at the end (larvae removed, phytoplankton present) of each experiment using an EGM-4 Environmental Gas Analyzer® (PP Systems) system that quantifies total dissolved inorganic carbon levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). This instrument provided a methodological precision ± 3.6% for replicated measurements of total dissolved inorganic carbon and provided full recovery (102 ± 3%) of Dr. Andrew Dickson's (University of California, San Diego, Scripps Institution of Oceanography) certified reference material for total inorganic carbon in seawater (Batch 102 = 2013 µmol DIC kg seawater$^{-1}$). Levels of $CO_2$ were subsequently calculated based on measured levels of total inorganic carbon, pH (mol kg seawater$^{-1}$; NBS scale), temperature (~24°C), salinity (~28), and first and second dissociation constants of carbonic acid in seawater according to Roy et al (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/). Multiple daily measurements of pH (Thermo Scientific Orion Star Series™ Benchtop pH meter; ± 0.002; calibrated prior each use with NIST traceable standards) indicated experiment beakers maintained a constant pH level throughout all experiments (<0.5% RSD within treatments). Spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson *et al.* (2007) and corrected for scale (Dickson 1993) were never significantly different from those obtained with the high sensitivity microprocessor.

*Experimental design.* The drafted recommendations of the 'best practices' for small microcosm experiments set forth by European Project on Ocean Acidification

(EPOCA) were followed for this project. For example, aeration of seawater was utilized to reach a target $pCO_2$ level, the ideal mechanism to manipulate seawater carbon chemistry (Riebesell et al. 2010). Experiments were conducted using two species of bivalves: *Mercenaria mercenaria* and *Argopecten irradians*. For each experiment, four levels of carbon dioxide were administered: a high level (~1500 ppm $CO_2$), predicted for the year 2250, a moderate level (~750 ppm $CO_2$), predicted for the year 2100 (Caldeira and Wickett 2003; Zeebe et al. 2008), ambient air (~390 ppm $CO_2$), and a near pre-industrial level (~250 ppm $CO_2$; 25, 26; Table 1). *Argopecten irradians* and *Mercenaria mercenaria* larvae were obtained from locally obtained broodstock spawned at the East Hampton Shellfish Hatchery, East Hampton, New York, United States.

A culture of *Isochrysis galbana* (Tahitian strain, T-Iso) was maintained in exponential phase growth using standard culture conditions and added at a density of $2 \times 10^7$ cells daily to each experimental beaker ($2 \times 10^4$ $mL^{-1}$) as a food source. This algal species administered at this density and at this rate is known to produce high growth rates and survivorship of shellfish larvae through metamorphosis (Carriker 2001; Cragg 2006; Padilla et al. 2006). To promote the high survivorship, all containers that were in contact with larvae were never exposed to chemicals or detergents (Padilla et al. 2006). To discourage the growth of bacteria during experiments, an antibiotic solution (Sigma-Aldrich No.4083, 5000 units of Penicillin, 5 mg of Streptomycin, and 10 mg of Neomycin per milliliter of solution) was added to each beaker at 1% its original concentration at the beginning of each experiment and during each water change (approximately 2 times weekly). This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae

(Padilla et al. 2006). For each experiment, approximately 200 larvae were distributed to each experimental beaker, achieving an environmentally realistic abundance of larvae (Carriker 2001). Each treatment began with approximately 900 mL to allow beaker volume for the algal culture to be added daily as a food source. Twice weekly during experiments, larvae were gently poured onto a 64 $\mu$m mesh, and the condition (live or dead) and developmental stage of each larvae (veligers, pediveligers, and metamorphosed) was determined visually under a dissecting microscope; every individual larvae was counted at every water change. Larvae from each beaker ($n$ =4, per treatment) were removed, counted, observed, and transferred into a new beaker with new filtered seawater, food, and antibiotics within a 15 minute period. Throughout experiments, all beakers were submerged in a water bath maintained at 24˚C via the use of commercially available heaters and chillers. This temperature generally yields high growth rates for *Argopecten irradians* and *Mercenaria mercenaria* larvae (Carriker 2001; Cragg 2006). Percent survivorship of all larvae was determined at each of the bi-weekly water changes when the numbers of larvae in each stage of veligers, pediveligers, and metamorphosed juveniles were quantified. Experiments were terminated after all surviving larvae in all treatments had metamorphosed. To statistically evaluate the effect of $CO_2$ treatments on larval survival, goodness of fit tests (G-Tests) were performed (Sokal and Rohlf 1995).

*Scanning electron microscopy.* To document differences in the size and structure of larval and early juvenile shellfish exposed to differing levels of $CO_2$, randomly chosen individuals ($n$ = 4 per treatment) were mounted for scanning electron microscopy images (SEM) in two distinct ways. First, to image the outside of shells, individuals were

attached at 45º relative to a level surface to a conductive substrate using carbon, double-sided tape and were subsequently coated with ~12 nm of gold using an Edwards© 150B rotary pump. To image the thickness and internal dimensions, cross-sections of shellfish were prepared. Individuals were mounted on glass microscope slides using UV-curing adhesive coating (Locite© 4304) and were impregnated with low-vicosity epoxy (Stuers'© Specifix-20) under vacuum outgassing, a step that did not alter the original shape or size of individuals. After curing, the epoxy mount was progressively ground and polished to the centerline (hinge to shell edge) of the shellfish using silicon carbide sandpapers, followed by successively finer diamond polishing grits (15, 6, and 3 micron), 0.05 micron aluminum oxide suspension, and finally with colloidal silica. All individuals were cross-sectioned at the same location (hinge to shell edge) across the shell. This mount was then attached to a conductive substrate using carbon double-sided tape and coated with ~4 nm of gold. SEM images were collected on both types of samples with a Leo (Zeiss) Model # 1550 electron microscope using a high voltage of 20KV and a Robinson back-scatter detector. All components of individual bivalve shells displayed in figures 3 and 4 were probed using advanced EDAX/EDA microanalysis in the LEO (Zeiss) Model # 1550 electron microscope and were confirmed to contain almost exclusively C, O, and Ca.

*Size and lipid analysis.* To estimate the relative lipid content of larvae, Nile Red stain was used to bind to neutral lipids and fluoresce under an FITC filter on an epifluorescent microscope (Castell and Mann 1994; Phillips 2002). A Nile Red stock solution was made of 1.25 mg of Nile Red crystals in 100 ml of acetone. Randomly

selected larvae (*n*= 15) from each treatment were stained with a 1:9 dilution of the stock solution and 0.2 µm filtered seawater. Larvae were exposed to the stain for ~1.5 hours, rinsed with filtered seawater, and digitally photographed with a Roper Scientific Photometrics CoolSNAP ES attached to an epiflorescent microscope. Digital images of each larvae were analyzed for the area of lipid accumulation and the diameter and the area of individuals using Image J® software. A lipid index was estimated by dividing the area of the larvae containing the fluorescing lipids by the total larval area thereby allowing for direct comparisons among treatments. One-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among larval lipid indexes, as well as shell length and thickness, at each $CO_2$ level.

**Results and discussion**

Larvae grown under near pre-industrial levels of $CO_2$ (250 ppm) displayed the highest rates of metamorphosis, growth, and survival. After 36 days of development, 40% of *M. mercenaria* grown under ~250 ppm $CO_2$ had survived while only 20% survived at modern day $CO_2$ levels (~390 ppm), and only 6% survived at ~1500 ppm $CO_2$ ($p< 0.001$; Fig. 1). *A. irradians* displayed similar patterns, with 74% of individuals surviving 38 days under ~250 ppm $CO_2$, 43% surviving at ~390 ppm, and only 5.4% remaining at ~1500 ppm $CO_2$ ($p< 0.001$; Fig. 1). Larvae grown under the lowest $CO_2$ levels displayed remarkably faster rates of metamorphosis compared to individuals grown under present day $CO_2$. For example, after 14 days of development, 51% of *M. mercenaria* larvae had fully metamorphosed at ~250 ppm $CO_2$ while < 7% had done so under higher levels of $CO_2$ ($p< 0.001$; Fig. 1). After 12 days of development, *A. irradians* larvae displayed a somewhat similar trend as 87% of the larvae had

metamorphosed at ~250 ppm $CO_2$, while 68% had done so at ~390 ppm $CO_2$ ($p < 0.001$; Fig. 1). The mean diameters attained by both species of larvae also were strongly affected by $CO_2$. *M. mercenaria* and *A. irradians* larvae grown under 250 ppm $CO_2$ (523 ± 38 and 531 ± 51 µm) were significantly larger than those grown under present day (282 ± 5 and 449 ± 35 µm) and higher (210 ± 9 and 311 ± 26 µm at ~1500 ppm) levels of $CO_2$ ($p<0.001$; Fig. 2.) These trends in the size of individuals were obvious during the examination of individuals under SEM (Fig. 3, 4).

Levels of $CO_2$ strongly influenced the early formation of *M. mercenaria* and *A. irradians* shells. For example, after 17 days of development *M. mercenaria* shells were 17 ± 2 µm thick under ~250 ppm $CO_2$, 6.7 ± 2 µm at ~390 ppm $CO_2$, and 3.8 ± 1 µm at ~750 ppm and ~1500 ppm $CO_2$ ($p < 0.001$; Fig. 2,3). *A. irradians* shells also decreased in thickness with increasing $CO_2$ being 20 ± 3, 12 ± 1, 11 ± 1 and 6.3 ± 1 µm thick under ~250, 390, 750, and 1,500 ppm $CO_2$ ($p < 0.001$, Fig. 2). Beyond impacting shell thickness, elevated levels of $CO_2$ severely altered the development of the hinge structure of early stage bivalves. As $CO_2$ levels increased from ~250 to ~1500 ppm there were dramatic declines in the size, integrity, and connectedness of the hinge (Fig. 3). While the *M. mercenaria* hinge displayed a 'tongue and groove' pattern under low $CO_2$ (250 and 390 ppm), under higher $CO_2$ concentrations the hinge and associated hinge teeth became increasingly separated and detached. Given that the bivalve hinge facilitates opening and closing of shells, allowing for intake of food and the excretion of waste (Eble 2001), the compromised hinges observed under elevated $CO_2$ may hinder the ability of individuals to open both valves to obtain and process suspended particles for nutrition as well as to keep the valves closed during resting phases. This hypothesis is

consistent with changes in lipid stores of larval shellfish exposed to differing $CO_2$ concentrations. For both species, with each increasing level of $CO_2$, the lipid content (as estimated by an index) decreased significantly ($p<0.001$; Fig. 2). Increasing $CO_2$ concentrations also caused marked changes in the morphology of the outer edge of juvenile shells (Fig. 3, 4). With increasing levels of $CO_2$, this region of the shell became increasingly riddled with holes, pockmarks, and crevices, observations consistent with other juvenile and larval shellfish reared under high $CO_2$ (Green et al. 2009; Watson et al. 2009), suggesting $CaCO_3$ shells were malforming and/or dissolving under more acidic conditions. Altered shell morphology was also obvious in juvenile scallops that had distinct ridges, characteristic of later stages of development, under pre-industrial $CO_2$ whereas individuals reared under higher $CO_2$ conditions lacked ridges, a sign of slower development (Fig. 4, (Cragg 2006)).

Shell integrity is one of the most important lines of defense for larval and juvenile bivalve shellfish, as shells provide physical support for soft and delicate internal organs (Carriker 1986) and protection from benthic and pelagic predators and suspended particles (Purcell et al. 1991; Carriker 1996). As such, the thinner, frailer shells displayed by early life history bivalves reared under modern day and elevated $CO_2$ would likely make individuals more vulnerable to predation and/or other environmental stressors. Similarly, within an ecosystem setting, larvae that accumulate fewer lipids (Fig. 2) are generally slower to metamorphose (Cragg 2006) and are more likely to perish once settled (Phillips 2002). Finally, individuals with extended metamorphosis times (Fig. 1) and that are smaller (Fig. 2) would be susceptible to greater rates of predation and natural mortality (Tamburri and Zimmer-Faust 1996; Phillips 2002). Hence, within an

ecosystem setting, mortality rates of early life history bivalves that develop under modern day and higher $CO_2$ levels would be expected to be even greater than the rates observed during our experiments. Given that bivalves in coastal areas naturally experience extremely high mortality rates in the transition from larvae to benthic juveniles (Green et al. 2004), increases in mortality due to elevated $CO_2$ could have profound effects on estuarine bivalve populations (Guinotte and Fabry 2008).

Our findings regarding the effects of future $CO_2$ levels on larval shellfish are consistent with recent investigations of ocean acidification demonstrating calcifying organisms will experience declines in survival and growth, as well as malformed $CaCO_3$ shells and hard parts (Doney et al. 2009). However, our examination of the development of larval shellfish at levels of $CO_2$ present prior to the industrialization of the planet provides important insight regarding the potential effects ocean acidification has had on calcifying organisms during the past two hundred years. Consistent with our findings, larval oysters (*Crassostrea virginica*) have displayed slightly larger shell area when grown under pre-industrial $CO_2$ levels compared to modern levels (Miller et al. 2009).

During the ~24 million years prior to the industrial revolution, atmospheric $CO_2$ levels are estimated to have been relatively static, likely fluctuating in a narrow range significantly below the concentrations present today (Pearson and Palmer 2000; Pagani et al. 2005). Moreover, periods of higher $CO_2$ prior to this era may not have been accompanied by lower pH and carbonate ion concentrations as the oceans may have buffered the more gradual changes in $CO_2$ that have occurred through geological history (Caldeira et al. 1999; Caldeira and Wickett 2003). The evolution of calcification in ocean animals is unknown and the multiple forms of $CaCO_3$ synthesized by modern day

calcifiers (calcite, aragonite, amorphous $CaCO_3$, and high magnesium $CaCO_3$) differ widely in their vulnerabilities to dissolution under lower pH (Mann 2001). Although the precise evolutionary tracks of modern bivalves remain somewhat uncertain (Harper et al. 2000), fossil evidence suggests that 906 of the 958 living genera of bivalve mollusks, including the species presented here, have a record that began in the mid- to late Cenozoic with the greatest continuous increase in genera between ~15 and ~25 Mya (Jablonski et al. 2003), a period of estimated lower $CO_2$ levels compared to today (Pearson and Palmer 2000; Pagani et al. 2005). Together with our results this suggests that ocean acidification since the industrial revolution may be applying selection pressure on modern marine bivalves and may continue to do so in the future.

The shallow marine environments that many marine bivalves occupy can harbor dynamic levels of pH and $CO_2$ (Feely et al. 2008; Salisbury et al. 2008) and the precise degree of phenotypic plasticity of survival among bivalve larvae in the face of higher $CO_2$ has not been established. Adaptation and evolution could promote the proliferation of bivalve strains that are more resistant to the increases in ocean $CO_2$ expected in the coming century and some calcifying organisms may even benefit from higher $CO_2$ levels (Doney et al. 2009; Ries et al. 2009). Importantly, however, the current rates of increase in atmospheric $CO_2$ are significantly faster than any recorded in tens of millions of years (Pearson and Palmer 2000; Pagani et al. 2005), suggesting this evolutionary challenge may be without precedent for extant calcifying species.

A comparison of our two study species may provide insight into future evolutionary pressure of ocean acidification on marine calcifiers. Globally, *M. mercenaria* has a larger, more diverse geographic distribution (Harte 2001) than

*A. irradians* (Brand 2006), an attribute that generally provides resistance to evolutionary pressures (Jablonski 1987) such as increasing $CO_2$ levels. In addition, predicted extinction rates are higher for the marine mollusk family Pectinidae, which includes *A. irradians*, than the Veneridae family which includes *M. mercenaria* (Roy et al. 2009). This information, combined with the more dramatic declines in survival displayed by *A. irradians* under higher $CO_2$ levels compared to *M. mercenaria* (Fig. 1), suggests *A. irradians* may face a greater evolutionary challenge in adapting to future increases in $CO_2$ concentrations.

Precipitous declines in wild populations of bivalves during the twentieth century have been attributed to overfishing, loss of habitat, hypoxia, and harmful algal blooms (Jackson et al. 2001; Gobler et al. 2005). Our results suggest that ocean acidification is another process that may have contributed to the declines of these populations in the recent past, and could further impact bivalve population densities and diversity in the future. Looking forward, marine organisms will be threatened by aspects of climate change beyond elevated $CO_2$ including higher temperatures. Given that the rise in ocean temperatures projected for the coming century (I.P.C.C. 2007) are within a range that could also hinder the growth and survival of bivalve larvae (Carriker 2001; Cragg 2006), future studies should consider the impact of higher $CO_2$ in conjunction with temperature changes in-line with such projections.

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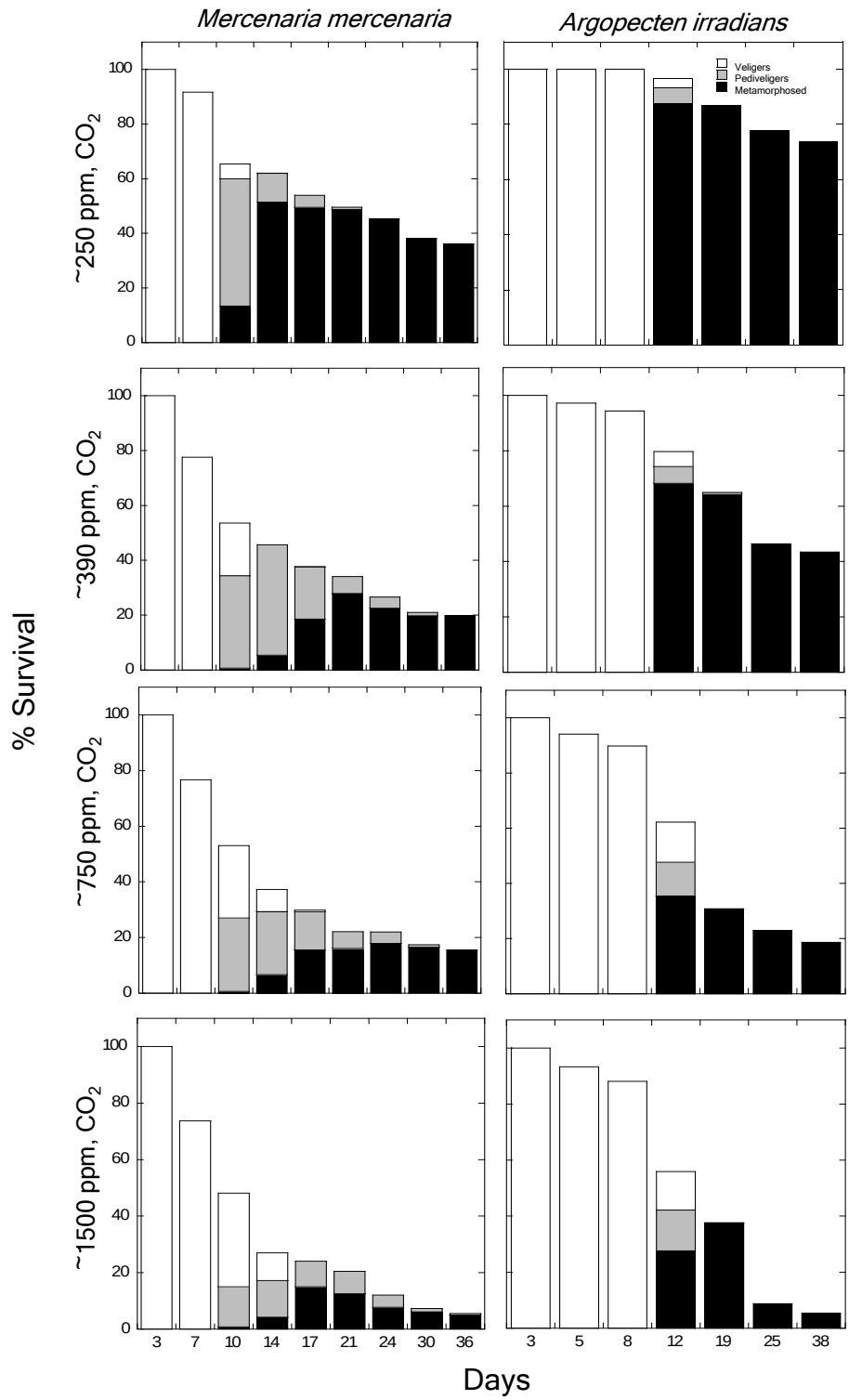


**Figure 1**. Development and survival of *Mercenaria mercenaria* and *Argopecten irradians* larvae. Percent survival and developmental stage (veliger, pediveliger, and metamorphosed) of larvae grown under four levels of $CO_2$, approximately 250, 390, 750, and 1500 ppm (Table 1). The relative standard deviation of larval survival among replicated vessels per treatment for all times points and experiments was 4% ($n$ = 4 per treatment).

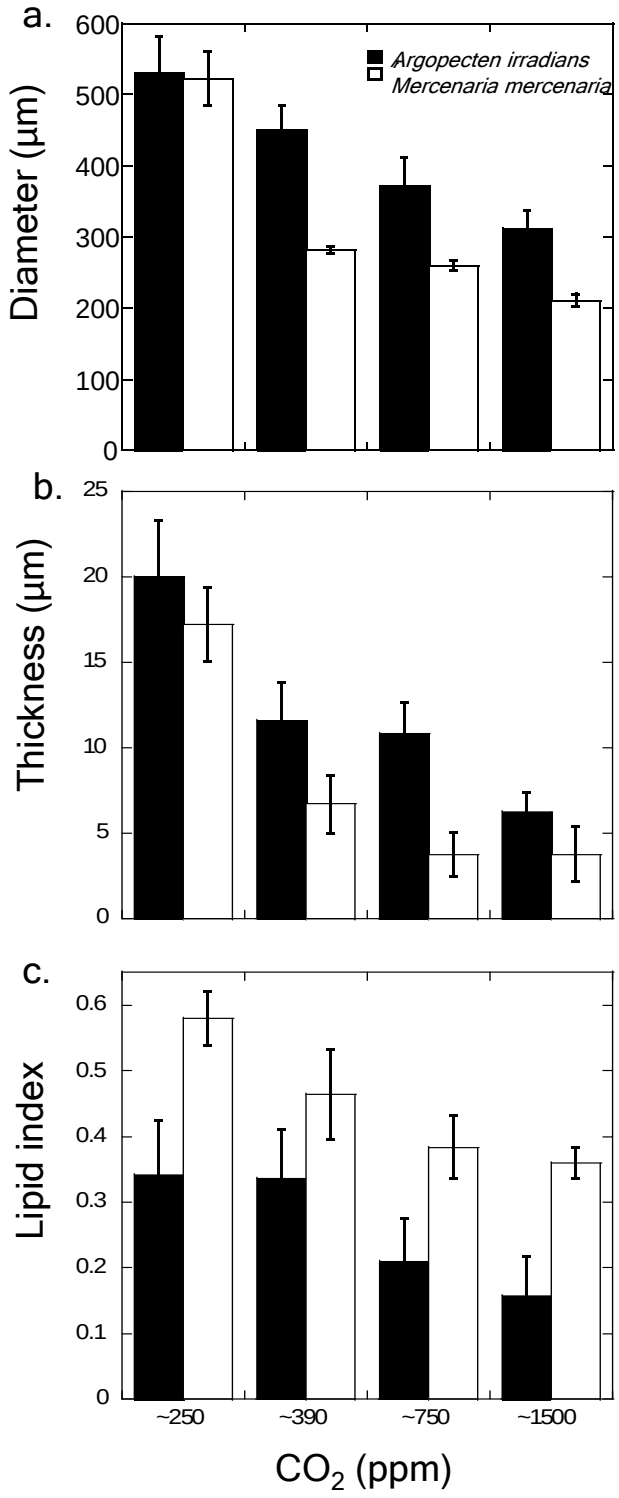


**Figure 2**. Diameters, thickness, and lipid index of bivalves grown under a range of $CO_2$ concentrations. Data are from four levels of $CO_2$, approximately 250, 390, 750, and 1500 ppm. a, Diameters of *Mercenaria mercenaria* (day 24) and *Argopecten irradians* (day 20). b, Thickness of *Mercenaria mercenaria* (day 36) and *Argopecten irradians* (day 52) shells at mid-point between the hinge and valve edge of the upper and lower shell of cross sectioned individuals. c, Lipid index (lipid area / total area) for *Mercenaria mercenaria* (day 24) and *Argopecten irradians* (day 20). Error bars represent standard deviation of replicated vessels per treatment ($n$ = 4 per treatment).

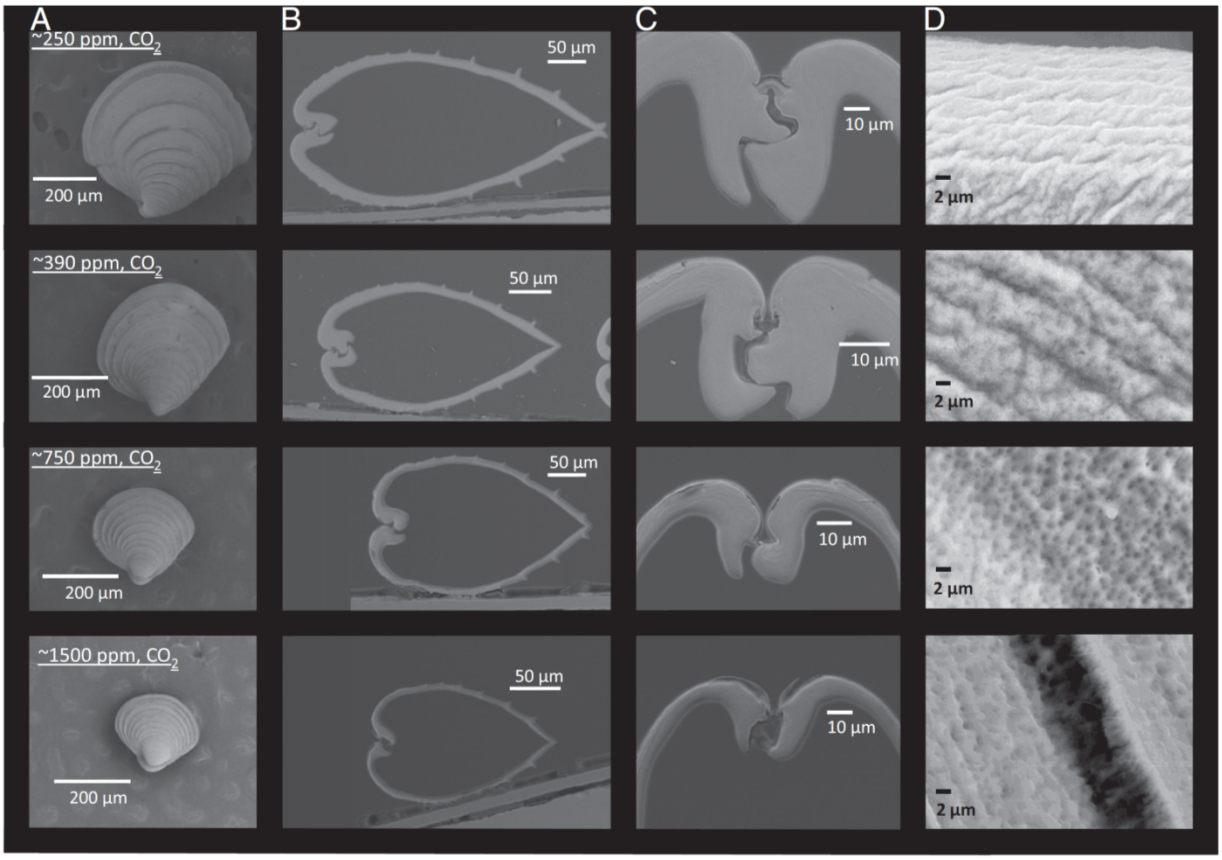


**Figure 3**. Scanning electron microscopy images of *Mercenaria mercenaria* bivalves grown under a range of $CO_2$ concentrations. Scanning electron microscopy images of 36 day old *Mercenaria mercenaria* grown under different levels of $CO_2$:, approximately 250, 390, 750, and 1500 ppm (Table 1). a, Images of individual larvae under each $CO_2$ level. b, Hinge to valve cross sections of individuals under each $CO_2$ level. c, The hinge of individuals under each $CO_2$ level. d, A magnification of the outermost shell of individuals under each $CO_2$ level.

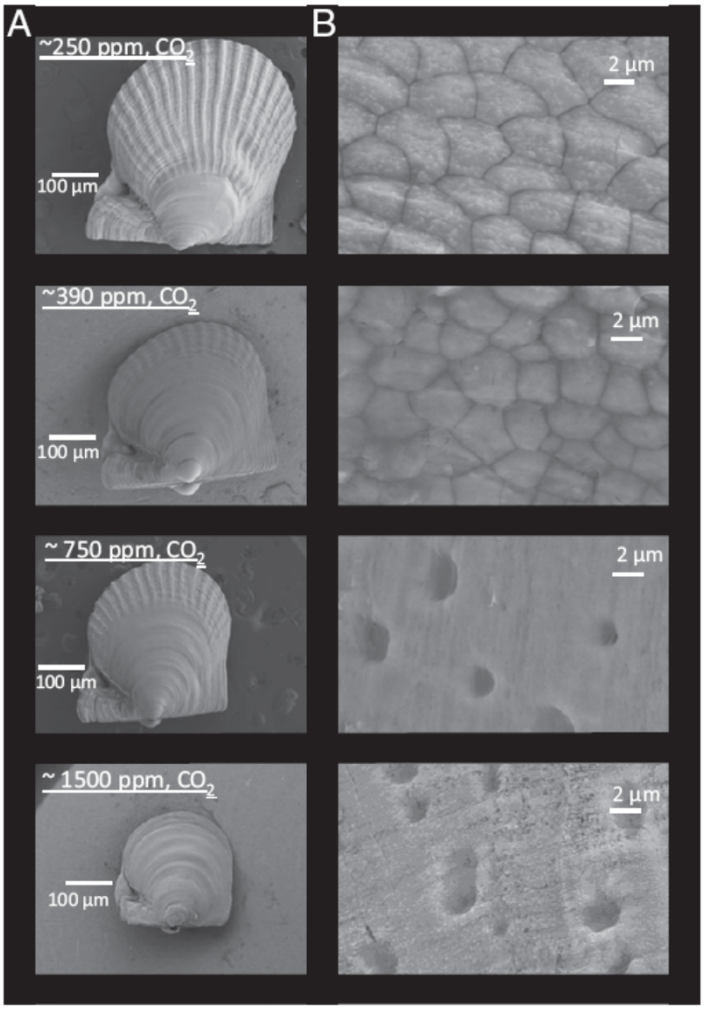


**Figure 4**. Scanning electron microscopy images of 52 day old *Argopecten irradians* grown under different levels of $CO_2$:, approximately 250, 390, 750, and 1500 ppm, (Table 1). a, Image of a full individual larvae under each $CO_2$ level. b, A magnification of the outermost shell of individuals under each $CO_2$ level.

| **Parameter** | **Near preindustrial $CO_2$** | **Ambient, present day $CO_2$** | **Year 2100 $CO_2$** | **Year 2200 $CO_2$** |
|---|---|---|---|---|
| *Mercenaria mercenaria* | | | | |
| Temperature (˚C) | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 8.171 ± 0.022 | 8.052 ± 0.036 | 7.801 ± 0.004 | 7.532 ± 0.021 |
| $pCO_2$ (ppm) | 247.1 ± 6.231 | 380.0 ± 33.02 | 742.3 ± 9.111 | 1516 ± 31.21 |
| $\Omega_{calcite}$ | 5.31 ± 0.47 | 4.53 ± 0.41 | 2.82 ± 0.05 | 1.67 ± 0.05 |
| $\Omega_{aragonite}$ | 3.42 ± 0.30 | 2.92 ± 0.26 | 1.82 ± 0.03 | 1.08 ± 0.03 |
| Total DIC ($\mu$mol $L^{1}$) | 1646 ± 94.21 | 1831 ± 52.34 | 1947 ± 21.33 | 2108 ± 18.06 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 208.0 ± 20.22 | 178.0 ± 16.03 | 111.0 ± 1.806 | 66.0 ± 1.904 |
| Alkalinity (TA) | 1938 ± 117.3 | 2070 ± 66.42 | 2080 ± 22.63 | 2127 ± 49.71 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | | |
| *Argopecten irradians* | | | | |
| Temperature (˚C) | 24 ± 0.51 | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 8.170 ± 0.026 | 8.041 ± 0.044 | 7.801 ± 0.005 | 7.530 ± 0.011 |
| $pCO_2$ (ppm) | 244.1 ± 4.006 | 386.5 ± 40.04 | 738.9 ± 9.941 | 1529 ± 35.05 |
| $\Omega_{calcite}$ | 5.18 ± 0.06 | 4.55 ± 0.47 | 2.81 ± 0.06 | 1.66 ± 0.05 |
| $\Omega_{aragonite}$ | 3.34 ± 0.35 | 2.94 ± 0.30 | 1.81 ± 0.04 | 1.07± 0.03 |
| Total DIC (umol $L^{-1}$) | 1613 ± 53.54 | 1850 ± 30.98 | 1941 ± 25.54 | 2101 ± 9.221 |
| $CO_3^{2-}$ ($\mu$mol $L^{1}$) | 202.0 ± 23.42 | 180.0 ± 18.44 | 111.0 ± 2.341 | 66.02 ± 1.911 |
| Alkalinity (TA) | 1899 ± 35.24 | 2090± 50.01 | 2075± 26.84 | 2146±11.21 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | | |

**Table 1.** Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the four-level carbon dioxide experiments with *Mercenaria mercenaria,* and *Argopecten irradians* larvae.

# CHAPTER 4:

## The short and long term effects of larval stage exposure to elevated carbon dioxide for coastal bivalves

**Abstract:**

Experiments were conducted to resolve the short term (days) and long term (months) effects of larval stage exposure to varying $CO_2$ concentrations on the performance of calcifying bivalves. To resolve the short term physiological effects of elevated $CO_2$ concentrations, calcification rates and RNA:DNA ratios of *Mercenaria mercenaria* and *Argopecten irradians* larvae exposed to varying $CO_2$ concentrations (~250, 390, and 750) were quantified. To determine the duration of high $CO_2$ exposure that inhibits *A. irradians* larval survival, individuals were exposed to high $CO_2$ for 4 -19 days during different portions of the larval development period. Finally, the juvenile-stage performance of *M. mercenaria* and *A. irradians* exposed to differing $CO_2$ levels as larvae was tracked for up to 6 months.

In short term experiments, higher $CO_2$ concentrations depressed both calcification rates and RNA:DNA ratios, with the latter being highly correlated with larval growth rates ($r^2$=0.92 – 0.99). These findings suggested that high $CO_2$ has a cascading negative physiological impact on bivalve larvae stemming from lower calcification rates. Experiments examining varying durations of $CO_2$ exposure demonstrated that only four days of exposure to elevated $CO_2$ during larval development was enough to suppress survival rates of *A. irradians*.

Finally, short- (3 weeks) and long-term (6 month) experiments demonstrated that individuals that survive exposure to high $CO_2$ during larval development grow more rapidly when exposed to normal $CO_2$ as juveniles, compared to individuals reared under ambient $CO_2$ as larvae. These increased growth rates were not enough to compensate for size deficiencies established during larval development, as size deficits among

individuals exposed to even modern levels of $CO_2$ as larvae were not overcome after 8 months of growth under modern $CO_2$ concentrations. This 'legacy effect' emphasizes the central role larval stage $CO_2$ exposure can play in shaping the success of modern day bivalve populations.

## Introduction

The partial pressure of $CO_2$ in the earth's atmosphere has risen by 40% since the Industrial Revolution and concentrations are expected to double this century (I.P.C.C. 2007). The ocean's ability to absorb $CO_2$ has resulted in one third of all anthropogenic emissions being stored in the world's oceans (Sabine et al. 2004). This flux of $CO_2$ has resulted in a reduction of ocean pH, a decrease in carbonate ion availability, and an environment that is challenging calcifying organisms in marine environments around the globe (Orr et al. 2005; Doney et al. 2009).

Ocean acidification can have a wide range of negative effects on marine organisms from minor to severe. The earliest stages of development for numerous marine species can be the most sensitive to decreased pH and carbonate ion availability. For example, larval stages of bivalves (Kurihara et al. 2007; Kurihara et al. 2008; Talmage and Gobler 2009; Talmage and Gobler 2010), corals (Albright et al. 2008), echinoderms (Dupont et al. 2010), pteropods (Comeau et al. 2010), and crustaceans (Walther et al. 2010) have all been shown to be negatively affected by the $CO_2$ concentrations expected later this century in the world's oceans. Despite these findings, studies investigating the impacts of ocean acidification on calcifying organisms are in their infancy. The mechanisms by which $CO_2$ imparts negative effects on calcifying organisms are unknown. There have been few investigations of the biochemical effects of ocean acidification on marine organisms, and calcification rates, the process most likely to be altered by ocean acidification, have been rarely measured.

Most ocean acidification experiments conducted to date have administered a static exposure of specific $CO_2$ concentrations to organisms (Doney et al. 2009). In a coastal

ecosystem setting, however, it is likely that marine organisms would experience dynamic $CO_2$ concentrations. The effects of variable, compared to chronic, exposure to high levels of $CO_2$ on larval stage marine animals has never been investigated. This exposure may be important since $CO_2$ concentrations in estuaries may vary tidally, diurnally, and with the succession of planktonic communities. Finally, longer term implications of larval stage exposure to high $CO_2$ for juvenile bivalves has never been reported.

The objectives of this study were to examine the short term (days) and long term (months) implications of larval stage exposure to high $CO_2$ for calcifying bivalves. *Mercenaria mercenaria* and *Argopecten irradians* larvae were grown under $CO_2$ concentrations of ~250, 390, 750, and 1500 ppm, their growth was estimated from their RNA to DNA ratio, and their uptake of radio-isotope $^{45}Ca$ was quantified to estimate calcification rates. A second set of experiments investigated the effects of varying duration of high $CO_2$ exposure on development of *A. irradians* larvae. A third set investigated the six-month, post-set growth rates of juvenile bivalves exposed to different levels of $CO_2$ during larval development.

**Methods**

This chapter examined the effects of the exposure of larval stage bivalves to variable $CO_2$ on their calcification rates, growth rates, and juvenile stage growth. For all experiments, replicate ($n$=4) experimental beakers with bivalve larvae (described below) were maintained in water baths maintained at 24ºC using commercially available aquarium heaters (Aquatic Eco-systems, Inc., Florida, USA). Temperatures were recorded every 6 minutes throughout experiments using in situ data loggers (Onset©);

temperatures varied within 2.5% of target values. The experimental temperature (24ºC) is optimal for growth and survival of larvae from the two species used here (Kennedy 1996; Kraeuter and Castagna 2001; Cragg 2006).

*Maintenance of $CO_2$ treatment parameters*

A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used to deliver $CO_2$ gas to seawater treatments at different rates. The gas proportionator mixed appropriate flow rates of 5% $CO_2$ gas, low $CO_2$ gas, and pressurized air (~390 ppm $CO_2$) to yield the concentrations of carbon dioxide desired for experiments at a net flow rate that turned over experimental vessels >100 times daily so the treatment seawater never was able to equilibrate with atmospheric $CO_2$. Experiments performed with gases mixed via a proportionator as described here generate nearly identical seawater chemistry and larval responses obtained from tanked gases premixed at specific $CO_2$ levels (Talmage and Gobler 2010). For experiments, the $CO_2$ gas mixtures from the proportionator system were continuously delivered to the bottom of the beakers. With continuous bubbling, all treatment vessels remained saturated with respect to oxygen (~8 mg $L^{-1}$). To quantify precise $CO_2$ levels attained in experimental treatments, aliquots were removed and analyzed during experiments using an EGM-4, Environmental Gas Analyzer® (PP Systems) system that quantified total dissolved inorganic carbon levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). This instrument provided a methodological precision ± 3.6% for replicated measurements of total dissolved inorganic carbon and provided full recovery (102 ± 3%) of Dr. Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography)

certified reference material for total inorganic carbon in seawater (Batch 102 = 2013 µmol DIC kg seawater$^{-1}$). Levels of $CO_2$ were calculated based on measured levels of total inorganic carbon, pH (mol kg seawater$^{-1}$; NBS), temperature, salinity, and first and second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/). Daily measurements of pH with a high sensitivity microprocessor (Thermo Scientific Orion Star Series™ Benchtop pH meter; ± 0.002; calibrated prior each use with NIST traceable standards) indicated experimental vessels maintain a constant pH level throughout experiments (<0.5% RSD within treatments). Random spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson *et al.* (2007) and corrected for scale (Dickson 1993) were never significantly different from those obtained with the high sensitivity microprocessor.

*Effects of varying $CO_2$ exposure on calcification rates*

*M. mercenaria* and *A. irradians* larvae were grown at three levels of $CO_2$: a high level (~750 ppm $CO_2$), predicted for the year 2100, a modern level (~390 ppm $CO_2$), and a near pre-industrial level (~250 ppm $CO_2$). Precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 1 and 2. One liter high density polyethylene beakers were filled with 0.2 $\mu$m filtered seawater from eastern Shinnecock Bay, New York, United States and within hours of fertilization, larvae were distributed to each treatment beaker at a concentration of ~ 400 L$^{-1}$, consistent with post-spawning densities in estuaries (Carriker 2001). Larvae were fed an ideal food source, *Isochrysis galbana*, (Tahitian strain, T-Iso) at a density known to maximize bivalve larval growth

and survivorship through metamorphosis (Castell and Mann 1994; Cragg 2006; Talmage and Gobler 2009). Cultures of *I. galbana* were maintained in exponential phase growth using standard culture conditions. To promote high survivorship, all containers in contact with larvae were never exposed to chemicals or detergents (Talmage and Gobler 2009). To discourage the growth of bacteria during experiments, an antibiotic solution (Sigma-Aldrich No.4083, 5000 units of Penicillin, 5 mg of Streptomycin, and 10 mg of Neomycin per milliliter of solution) was added to each beaker at 1% its original concentration at the beginning of each experiment and at the time of each water change (approximately 2 times weekly). This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae (Talmage and Gobler 2009). Experiments presented here were repeated without antibiotic treatments and yielded no difference in bivalve larval survival suggesting that neither the antibiotics nor the bacteria in seawater altered the results presented here. Every three days, larvae were gently poured onto a 64 $\mu$m mesh, and all larvae from each beaker ($n$ =6, per treatment, 3 beakers for veligers, and 3 beakers for pediveligers at each $CO_2$ level) were removed and transferred into a new beaker with new filtered seawater, food, and antibiotics within a 15 minute period.

At the veliger and pediveliger for *Mercenaria mercenaria* larvae (3 and 10 days post-fertilization) and *Argopecten irradians* larvae (12 and 15 days post-fertilization), 250 larvae per treatment were randomly selected, removed, and placed into 125 ml Nalgene bottles with 100 ml new filtered seawater; temperature was maintained at 24ºC, and the bottles were continuously bubbled to achieve the same $CO_2$ levels as the larvae were grown under. Differences in the rate of calcification of larvae exposed to differing

levels of $CO_2$ was assessed using a $^{45}Ca$ isotope tracer method (Ho and Zubkoff 1980). High specific activity, 0.25 mCi, $^{45}Ca$ was added to ~100 ml of filtered seawater with 250 larvae in 4 replicated, polypropylene 125 ml Nalgene bottles with one hole caps (allowing for bubbling of treatment $CO_2$ concentrations) resulting in a final concentration of 0.1 µCi $ml^{-1}$. A killed-control bottle was established at each level of $CO_2$ via the addition of glutaraldehyde to a final concentration of 2%. After 24h, bottles were gently gravity filtered onto 20 µm polycarbonate membranes. For *A. irradians* larvae, time series uptake experiments were also conducted during the veliger and pediveliger stages when sub-samples of larvae were removed at hour 1, 2, 4, 6, and 12 to obtain an estimate of calcium uptake over time. All larvae were retained on filters and were transferred to scintillation vials, and digested with 1 ml of concentrated $HNO_3$ (15.8 N) for one hour after which 10 ml of UltimaGold™ scintillation cocktail was added. Beta activity of the samples was counted on a Perkin Elmer Tri-Carb 1600 liquid scintillation counter with the discriminator window optimized for $^{45}Ca$ detection. The weight-specific calcium uptake rate per individual was determined by the following equation:

$$\mathbf{CaU = ([(dpm)_l - (dpm)_d]\ Ca_m / (dpm)_m) / t / L}$$

CaU = calcium uptake rate (ng Ca/larva/hr)
$(dpm)_l$ = radioactivity of live larvae
$(dpm)_d$ = radioactivity of killed larvae which represents nonbiological factors including background, ion-exchange and isotope absorption
$Ca_m$ = calcium content of unit medium water in mg $L^{-1}$
$(dpm)_m$ = radioactivity of unit medium water
t = the length of the incubation (days)
L = the number of larvae per beaker

This equation is based on three assumptions: 1) There is no significant discrimination by larvae for $^{45}Ca$ and $^{40}Ca$ uptake, 2) the shell dissolution by metabolites was negligible compared to shell deposition (Ho and Zubkoff 1980), and 3) all calcium uptake immediately gets allocated to shell deposition. Calcium content of water was estimated by the ratio of its salinity to that of typical oceanic water of 35‰ which has 408 ppm of $Ca^{2+}$ (Sverdrup et al. 1942). Precise $CO_2$ levels and complete carbonate chemistry from these experiments appear in Tables 1 and 2. All calcification data met the assumption of normality and homogeneity. One-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among calcification rates at each $CO_2$ level. Statistical analyses were performed with SYSTAT 13 © Copyright, 2009, Systat Software, Inc. Representation of the degrees of freedom for all ANOVAs in this chapter are presented as: *df*= x, y; where x represents the degrees of freedom for the factor being described, and y represents the degrees of freedom for error in the ANOVA analysis of that factor.

*Effects of varying $CO_2$ on RNA:DNA ratios (a proxy for short term growth)*

To assess RNA:DNA ratios of *M. mercenaria* and *A. irradians* larvae, individuals were grown at four levels of $CO_2$: a high level (~1500 ppm $CO_2$), predicted for the year 2200, a mid level (~750 ppm $CO_2$), predicted for the year 2100, a modern level (~390 ppm $CO_2$), and a near pre-industrial level (~250 ppm $CO_2$). The general experimental set-up followed the description above; precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 3. The overall growth and ribonucleic acid synthesis rate of larval shellfish was assessed by analysis of the ratio between

ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content in the organisms (Clemmesen 1994). This approach provides an RNA:DNA ratio which has been used in other marine organisms, especially fish larvae, to estimate growth and nutritional condition, with elevated ratios being associated with fast growing individuals (Malzahn et al. 2003; Malzahn et al. 2007). Larvae ($n$ =15) were removed from each treatment beaker poured onto a sieve, and were then macerated using a Pellet Pestle® Motor, and heated to 50 ºC for 15 minutes, and then frozen in –80ºC for at least 90 minutes. Nucleic acids from groups of larvae ($n$=15) were extracted using a modified CTAB technique, and quantified using Quant-iT™ RiboGreen RNA ® and Quant-iT™ PicoGreen® DNA assay kits (Invitrogen), according to manufacturer's protocol. RiboGreen® RNA and PicoGreen® DNA are ultra sensitive fluorescent nucleic acid stains for quantifying RNA and DNA respectively in solution. RiboGreen also binds DNA, therefore complete DNAse digestion of the sample preceded analysis of RNA. RNA and DNA concentrations in the extracted samples were determined by measuring fluorescence using an Applied Biosystems 7300 Real-Time PCR system-genetic analyzer and compared to a standard curve of nucleic acids. All RNA:DNA data met the assumption of normality and homogeneity. One-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among RNA:DNA and daily growth at each $CO_2$ level. Statistical analyses were performed with SYSTAT 13 © Copyright, 2009, Systat Software, Inc.

*Effects of varying $CO_2$ exposure on survival of larval bivalves*

An experiment was conducted to investigate the effects of two levels of $CO_2$ (~390 and ~750 ppm) exposure on the growth and survival of *A. irradians* larvae. The general experimental set-up followed the description above; precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 4. There was a total of 15 treatment vessels at each $CO_2$ level (~390 and ~750 ppm) and every 3 days, for the first 12 days of a 19 day experiment, 3 treatment vessels were switched from ~750 to ~390 ppm and 3 treatment vessels were switched from ~390 to ~750 ppm $CO_2$ so that *A. irradians* larvae experienced exposure time to each $CO_2$ level which ranged from 0 – 19 days. In addition, some larvae were exposed to high $CO_2$ early in their development only while others were exposed later in their development.

To assess the juvenile stage implications of larval stage $CO_2$ exposure for *M. mercenaria*, larvae were grown at two $CO_2$ concentrations: ~390 and 1500 ppm. The general experimental set-up followed the description above; precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 5. After 24 days of development, 40 individuals that had metamorphosed into early juvenile stages were transferred from ~390 to ~1500 and from ~1500 to ~390 ppm $CO_2$; individuals were pooled from replicated treatment beakers. Early stage juveniles were cared for as described above for bivalve larvae and were monitored until day 36. To meet the assumption of normality and homogeneity, survival data were arc-sin square root transformed after which one-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among RNA:DNA and daily growth

survival at each $CO_2$ level. Statistical analyses were performed with SYSTAT 13 © Copyright, 2009, Systat Software, Inc.

*Long term growth experiment*

To assess the longer term (months) implications of larval stage exposure to high $CO_2$, *Argopecten irradians* larvae (*n* =1000) were grown at three $CO_2$ concentrations (~250, 390, and 750 ppm) for their entire larval cycle and early days as a juvenile. The general experimental set-up followed the description above; precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 6. After 35 days of development, all metamorphosed juveniles were quantified and placed on 64 µm mesh sieves submerged in seawater where they received a continuous flow of coarsely filtered (100µm nylon) seawater from Shinnecock Bay, New York, United States, that had normal $CO_2$ levels. At 47 days, all surviving individuals were moved into Three Mile Harbor, East Hampton, New York, where they were placed in mesh bags in a cage so as to receive ample water flow and food but to exclude predators. Size of all individuals was recorded monthly over an eight month period and specific growth rates were estimated using the equation: [ln(final size) – ln(initial size)] / change in time. A one-way ANOVA was performed to examine the differences between specific growth rates and $CO_2$ levels. Statistical analyses were performed with SYSTAT 13 © Copyright, 2009, Systat Software, Inc. Representation of the degrees of freedom for all ANOVAs in this chapter are presented as: *df*= x, y; where x represents the degrees of freedom for the factor being described, and y represents the degrees of freedom for error in the ANOVA analysis of that factor.

**Results**

*Mercenaria mercenaria*, and *Argopecten irradians* calcium uptake

For *Mercenaria mercenaria* larvae grown under three levels of $CO_2$ (~250, 390, and 750 ppm), there was a significant effect of $CO_2$ concentration on calcium uptake for the veliger stage ($F$= 503.890, $df$ =2, 6, $p$<0.001; ANOVA) and for the pediveliger stage ($F$= 10.327, $df$ =2, 6, $p$<0.05; ANOVA), there was no statistical difference between developmental stage and also no interactive effect of both $CO_2$ and temperature. For *M. mercenaria* day 3, veliger larvae calcium uptake rates were 0.72 ± 0.001, 0.44 ± 0.005, and 0.35 ± 0.03 ng Ca larvae$^{-1}$ hour$^{-1}$ ± standard deviation under ~250, 390, and 750 ppm $CO_2$ respectively (Fig. 1a). *M. mercenaria* day 10, pediveligers under ~250, 390, and 750 ppm $CO_2$ had decreasing calcium uptake rates of 0.72 ± 0.002, 0.53 ± 0.005, and 0.33 ± 0.002 ng Ca larvae$^{-1}$ hour$^{-1}$ ± standard deviation (Fig. 1a).

*Argopecten irradians* larvae demonstrated a similar pattern of decreased calcium uptake under increasing $CO_2$ concentrations. For *A. irradians* there was a significant effect of $CO_2$ on calcium uptake ($F$= 10,714.690, $df$ =2, 6, $p$<0.001; ANOVA) for day 12, veliger larvae as well as for day 15, pediveliger larvae ($F$= 769.852, $df$ =2, 6, $p$<0.001; ANOVA). There was also a significant effect of $CO_2$ ($F$(1)), developmental stage ($F$(2)), and an interactive effect of $CO_2$ and developmental stage ($F$(3)) on *A. irradians* calcium uptake ($F$(1)= 1,923.442, $df$= 2, 4; $F$(2)= 649.843, $df$= 1, 4; $F$(3)= 163.849, $df$= 1, 4; $df$ =23, $p$<0.001; two-way ANOVA)(Fig.1b). With an increase in $CO_2$ concentrations from ~250 to 390 and 750, *A. irradians* veliger larvae decreased calcium uptake rates from 0.61 ± 0.003, to 0.42 ± 0.001 and to 0.44 ± 0.002 ng Ca larvae$^{-1}$ hour$^{-1}$ ± standard deviation (Fig. 1b). *A. irradians* pediveliger larvae also decreased calcium uptake with

increasing $CO_2$ concentrations from 0.76 ± 0.02, 0.53 ± 0.001, and 0.44 ± 0.004 ng Ca larvae$^{-1}$ hour$^{-1}$ ± standard deviation at ~250, 390, and 750 ppm $CO_2$ (Fig. 1b). A separate experiment was conducted to estimate *A. irradians* veliger larvae uptake of calcium over time (12 hours; Fig 2). At ~250 ppm $CO_2$, *A. irradians* veliger larvae at day 12 had the greatest slope of 0.90 ng Ca larvae$^{-1}$ hour$^{-1}$ (Fig.2). At ~390 and ~750 ppm $CO_2$, *A. irradians* larvae decreased calcium uptake rates with corresponding slopes of 0.52 and 0.29 ng Ca larvae$^{-1}$ hour$^{-1}$, respectively (Fig. 2).

*Mercenaria mercenaria* and *Argopecten irradians* RNA:DNA estimates of growth

To understand the effects of $CO_2$ on larval growth, proxies of growth were estimated using two methods: RNA:DNA calculations and measurement of larval diameters. *Mercenaria mercenaria* larvae (day 24) and *Argopecten irradians* larvae (day 20) both displayed step-wise decreases in both measurements of growth with increasing $CO_2$ concentrations. $CO_2$ concentrations had a significant effect on RNA:DNA for *M. mercenaria* ($F$= 218.883, $df$ =3, 12, $p$<0.001; ANOVA). At ~250 ppm $CO_2$, the RNA:DNA ratio was 1.43 ± 0.07 for *M. mercenaria* larvae and decreased to 0.21 ± 0.06 at ~1500 ppm $CO_2$ (Fig. 3). *A. irradians* also displayed decreasing RNA:DNA with increasing $CO_2$ (Fig. 3). When $CO_2$ concentrations increased from ~250 to 390 to 750 to 1500 ppm, RNA:DNA for *A. irradians* changed from 1.02 ± 0.07 to 0.49 ± 0.17 to 0.20 ± 0.03 to 0.25 ± 0.04 respectively (Fig. 3).

The second proxy for growth, changes in shell diameter followed a similar pattern as RNA:DNA. $CO_2$ concentrations had a significant effect on *M. mercenaria* larval shell growth ($F$= 194.271, $df$ =3, 12, $p$<0.001; ANOVA) and *A. irradians* larval shell growth

($F$= 18.471, $df$ =3, 12, $p$<0.001; ANOVA; Fig. 3). With increasing $CO_2$ from ~250, 390, 750 to 1500 ppm, *M. mercenaria* larvae growth decreased from 21.77 ± 1.58, 11.74 ± 0.20, 10.85 ± 0.28, to 8.76 ± 0.38 µm $day^{-1}$ ± standard deviation, respectively (Fig. 3). *A. irradians* larvae followed the same pattern of decreasing growth from 26.52 ± 2.53, 22.47 ± 1.75, 18.55 ± 2.02, and 15.56 ± 1.32 µm $day^{-1}$ under $CO_2$ concentrations of ~250, 390, 750 and 1500 respectively (Fig. 3). Finally, there was the high degree of correlation between RNA:DNA ratios and shell-based growth rates of *M. mercenaria* and *A. irradians* larvae ($r^2$ =0.92 ($p$=0.08, n.s.) and 0.99 ($p$<0.01), respectively; Fig 4).

<u>*Argopecten irradians* survival under varying exposure to increased $CO_2$</u>

To determine the minimum $CO_2$ exposure period needed to reduce larval survival, *Argopecten irradians* larvae were either begun at present $CO_2$ (~390 ppm) or high $CO_2$ (~750 ppm) and then every three days, for the first 12 days, a subset of treatment vessels was switched to either the higher (for vessels at 390 ppm $CO_2$) or present day (for vessels at 750 ppm $CO_2$) $CO_2$ concentration. The duration of exposure to high $CO_2$ significantly affected larval survival ($F$= 20.137, $df$ =1, 22, $p$<0.001; ANOVA)(Fig. 5). *A. irradians* larvae which developed exclusively under 390 and 750 ppm $CO_2$ displayed survival rates of 77.8 ± 1.0% and 56.1 ± 1.0%, respectively (Fig 5). In contrast, individuals which began their development at high $CO_2$ and were switched to lower $CO_2$ after 4, 7, 10, and 13 days displayed survival rates of 73 ± 3.0, 71.5 ± 2.3, 63.5 ± 3.1, and 61.7 ± 2.2%, respectively (Fig. 5). Importantly, even three days of exposure to 750 ppm $CO_2$ significantly reduced larval survival rates compared to chronic exposure to 390 ppm (Fig. 5). Similarly, *A. irradians* larvae that began at ~390 ppm $CO_2$ and were switched to

750ppm $CO_2$ after 7, 10, 13, and 16 days displayed survival rates of 76.8 ± 1.3, 75.3 ± 0.25, and 57.25 ± 0.9, and 56.7 ± 0.8%, respectively (Fig. 5).

*Mercenaria mercenaria* survival under varying exposure to increased $CO_2$

An experiment was conducted to investigate the effects of changing $CO_2$ exposure on survival by moving veliger larvae from higher to lower concentrations of $CO_2$ and from lower to higher concentrations. For *Mercenaria mercenaria* exposed to ~390 and 1500 ppm $CO_2$ for the first 24 days of development there was a significant effect of $CO_2$ on total survival ($F$= 50.489, $df$ =1, 6, $p$<0.001; ANOVA) where survival at ~390 was 36.60 ± 3.25% and at ~1500 was 23.34 ± 1.84% (Fig. 6a). At day 24, a subset of individuals were moved from low $CO_2$ (~390 ppm) to high $CO_2$ (~1500 ppm) and from high $CO_2$ (~1500 ppm) to low $CO_2$ (~390 ppm). Survival from day 24-36 was also significantly affected by $CO_2$ ($F$= 38.398, $df$ =1, 14, $p$<0.001; ANOVA)(Fig. 6b). Total survival of *M. mercenaria* individuals at day 36 at corresponding $CO_2$ treatments of ~390, ~390 switched to ~1500, ~1500 switched to ~390, and ~1500 ppm were 70.31 ± 9.02, 62.5 ± 10.21, 97.5 ± 2.04, and 43.09 ± 4.72%, respectively (Fig. 6b).

Juvenile *Argopecten irradians* growth following larval stage exposed to high $CO_2$

To assess the longer term effects of elevated $CO_2$ exposure during larval development, the growth of post-set larvae was measured over an six month period. For *A. irradians* growth during the first 12 weeks post-spawning, $CO_2$ had a significant effect on specific growth rates ($F$= 658.431, $df$ =2, 1, $p$<0.001; ANOVA; Fig. 7). With increasing $CO_2$ treatments during the larval stage development from ~250 to 390 to 750,

specific growth rates for week 0-12 (± standard error) decreased from 0.25 ± 0.0008 to 0.23 ± 0.008 to 0.21 ± 0.0005 mm week$^{-1}$ respectively (Fig. 7). For weeks 13-26, these trends reversed as individuals reared under ~250, 390, and 750 ppm as larvae displayed specific growth rates of 0.009 ± 0.001, 0.014 ±0.001, and 0.02308 ± 0.001 mm week$^{-1}$ respectively (Fig. 7). While shell diameters of individuals metamorphosed under ~250 and 750 ppm $CO_2$ were 21.19 ± 0.24 and 13.07 ± 0.08 mm in September 2010, they were similar in size by February 2011 (26.96 ± 0.79 mm and 23.59 ± 0.77; Fig. 8).

**Discussion**

Through the first two chapters of my dissertation, I established the decline in survival and delays in development experienced by three species of bivalve larvae exposed to elevated concentrations of $CO_2$ (Talmage and Gobler 2009; Talmage and Gobler 2010). In this chapter, experiments examining calcification rates and RNA:DNA ratios provided insight regarding the specific physiological processes that may be impacted by elevated $CO_2$. Other experiments identified the minimal high $CO_2$ exposure period, of four days, required to inhibit the growth of larval growth. Finally, experiments established the juvenile-stage implications of larval-stage exposure to elevated concentrations of $CO_2$ for the bivalves tested. Collectively, this data set provides novel insight regarding the short and long term implications of larval stage $CO_2$ exposure for calcifying bivalves.

At $CO_2$ concentrations exceeding ~250 ppm, both *M. mercenaria* (hard clam) and *A. irradians* (bay scallop) larvae displayed significant declines in calcium uptake and presumably rates of calcification. This observation is consistent with the thinner shells

displayed by these bivalve larvae when exposed to higher $CO_2$ concentrations (Talmage and Gobler 2010). The integrity of the bivalve shell may be one of the most important lines of defense for larval shellfish. Bivalve larvae depend on shells to provide physical support for soft and delicate internal organs, for protection from impact with suspended particles and physical stress (Carriker 1986) and for protection from some benthic and pelagic predators (Purcell et al. 1991; Carriker 1996). Therefore, the declines in calcification observed with increasing carbon dioxide levels likely contributes to larval bivalve shellfish mortality, and thus could be considered a primary impact of ocean acidification on these organisms.

Reductions in calcification under acidified ocean conditions have already been described for post-larval hard clam *Mercenaria* spp. that displayed decreased calcification rates with decreases in sea water pH (Waldbusser et al. 2010). Juvenile bivalves and larvae from the oyster species, *Saccostrea glomerata*, have displayed compromised shell integrity with decreasing calcium carbonate saturation states (Green et al. 2009; Watson et al. 2009). Juveniles spending only four days at undersaturated levels of calcium carbonate displayed signs of dissolution or shell pitting of the ostracum, with most dissolution of the surface shell in the umbonal region or the part of the shell that was deposited first (Green et al. 2009). These declines in calcification by juvenile bivalves coupled with the current findings of decreased bivalve larvae calcification with increasing $CO_2$ collectively support the 'death by dissolution' hypothesis proposed as the underlying mechanism for how acidified waters and sediments lead to decreased survival of calcifying bivalves (Green et al. 2009). Different forms of calcium carbonate are secreted by bivalve larvae as they develop. Larval shells begin as amorphous calcium

carbonate (ACC) which is significantly more soluble than aragonite and the combinations of aragonite and calcite synthesized by later stage bivalves (Weiss et al. 2002). Even the most resistant forms of calcite dissolve under high levels of $CO_2$ and leave organisms with shell loss (Harper 2000). Hence, the dissolution and/or inhibition of calcification during larval stages contribute towards thin and frail shells which, in turn, leave larvae vulnerable to enhanced mortality rates.

Calcification is a significant metabolic cost for marine organisms, with other metabolic costs including, but not limited to the energy committed to the production of the organic matrix, somatic growth, and gamete production (Palmer 1992). If the shells of bivalve larvae are more difficult to synthesize under increasing $CO_2$ levels and thus synthesized at a slower rates, this may make less energy available for growth and development for larvae and, thus, could contribute toward later stage mortality. This would represent a secondary effect of high $CO_2$ for surviving larvae: individuals that do not perish from high $CO_2$ are under enhanced physiological stress since more energy may be allocated to calcification and less is available for maintenance and growth.

The hypothesis that reduced calcification rates have 'trickle down' effects on larval physiology and performance was supported by measurements of RNA:DNA ratios. RNA transcribes the genetic material stored in DNA and is subsequently translated by ribosomes to synthesize proteins. Hence, high levels of RNA compared to DNA are indicative of an organism in an active state of transcribing RNA, synthesizing proteins and growth (Malzahn et al. 2003). Under high concentrations of $CO_2$, the ratio of RNA:DNA was reduced for both species of larvae, suggesting that rates of transcription and growth rates were compromised. This conclusion was supported by the high degree

of correlation between RNA:DNA ratios and shell-based growth rates of *M. mercenaria* ($r^2$ =0.92; p=0.08, n.s.) and *A. irradians* larve ($r^2$ = 0.99, p<0.01, respectively; Fig 4). The reduced RNA:DNA ratios displayed by individuals developing under high levels of $CO_2$ indicates the systemic negative impact of ocean acidification on these organisms.

The negative, secondary effects of elevated $CO_2$ on larval bivalves included declines in lipid content, RNA:DNA ratios, and size, possibly suggesting that hypercapnia within the larvae may have impacts independent of calcification. Higher metabolic rates in juvenile oysters, *Crassostrea virginica*, have been measured under elevated $CO_2$ treatments indicating a higher energy cost of homeostasis under these conditions (Beniash et al. 2010). Similar rises in energy costs for bivalves under elevated $CO_2$ concentrations would support the hypothesis that ocean acidification has secondary negative effects on larvae beyond calcification.

Nearly all ocean acidification experiments conducted to date have administered a static exposure of specific $CO_2$ concentrations to organisms (Doney et al. 2009). In an ecosystem setting, however, it is likely that marine organisms, in general, and estuarine larvae, in particular, would experience varying $CO_2$ concentrations due to diurnal and seasonal fluctuations in photosynthesis and respiration. To identify the duration of $CO_2$ exposure that begins to reduce larval bivalve survival, *A. irradians* larvae were grown at either ~390 or ~750 ppm and then every three days a subset of individuals were exposed to higher or lower $CO_2$ (Fig. 5). Individuals beginning at ~390 ppm $CO_2$ were able to withstand a longer exposure period at higher $CO_2$ (approximately 10 days) before significant declines in survival occurred, while individuals that began their development at higher $CO_2$ experienced significant declines in survival after only four days (Fig. 5).

This suggests that the first days of development are the most sensitive exposure period for *A. irradians* larvae, perhaps because more soluble forms of calcium carbonate are secreted by larvae during this period (Weiss et al 2002). This result further suggests that aquaculturists and restoration efforts should focus efforts into ensuring ideal chemical conditions for the first week of bivalve larval development. Importantly, however, the benefits of initial development under normal $CO_2$ levels are not enough to buffer against extended later exposure to high $CO_2$ as exposure to 13 days 750ppm $CO_2$ after six days of optimal $CO_2$ caused a significant decline in larval survival (Fig. 5). Given the in-situ variability of $CO_2$ concentrations, this experimental increase in $CO_2$ from ambient (~390 ppm) to elevated $CO_2$ concentrations (~750 ppm) mimics what these larvae may experience as they develop from pediveligers into juveniles and settle onto the seafloor.

While larval exposure to high $CO_2$ can have strong negative impacts on growth and survival for many marine animals (Kurihara et al. 2007; Kurihara et al. 2008; Talmage and Gobler 2009; Dupont et al. 2010; Talmage and Gobler 2010), the post-larval stage implications of this exposure have yet to be established. After 24 days of exposure to ambient and high $CO_2$ concentrations, *M. mercenaria* larvae displayed significantly higher survival under lower $CO_2$ concentrations (~390 ppm; Fig. 6). When a subset of the surviving individuals were moved from high to low and low to high $CO_2$ concentrations, however, the highest survival rate over the next two weeks was displayed by individuals which developed under high $CO_2$ (~1500 ppm) and then were reared at ~390 ppm $CO_2$ (Fig. 6b). As broadcast spawners, bivalves produce cohorts of larvae derived from multiple parents that are likely to display plasticity in their general fitness (Kraeuter and Castagna 2001; Cragg 2006). Although high $CO_2$ eliminated 80% of the

larval cohort, individuals that survived this treatment displayed superior survival rates as early stage juveniles suggesting high $CO_2$ eliminated generally weaker individuals.

The superior performance of individuals exposed to high $CO_2$ as larvae and reared under normal conditions as juveniles was also evident in *A. irradians* potentially evidencing a physiological plasticity. Although *A. irradians* bivalves had the greatest specific growth at the lowest $CO_2$ concentration during the larval stage, individuals surviving the highest $CO_2$ level experienced the highest specific growth rates as juveniles (Fig 7). This finding supports the hypothesis that individuals that survive high $CO_2$ as larvae are, on average, more fit as juveniles than individuals exposed to normal $CO_2$ levels as larvae. The compensatory, juvenile-stage growth displayed by individuals reared under high $CO_2$ as larvae resulted in their larval stage size differences being eliminated after two months of growth as juveniles under normal $CO_2$ conditions. Interestingly, while the growth rate of the individuals reared at ambient and high $CO_2$ (~390 and 750ppm) outpaced those of the lowest $CO_2$ treatment (~250ppm) as early stage juveniles, they did not overcome the deficit in size established during the larval stage after six months of growth under normal $CO_2$. Given that smaller juvenile bivalves are more susceptible to predators than larger individuals (Kraeuter 2001), this finding suggests the negative effects of larval stage exposure to even modern day levels of $CO_2$ represents a legacy that can persist for at least eight months in an ecosystem setting. This further emphasizes the critical role larval stage $CO_2$ can play in influencing the success of modern day bivalve populations.

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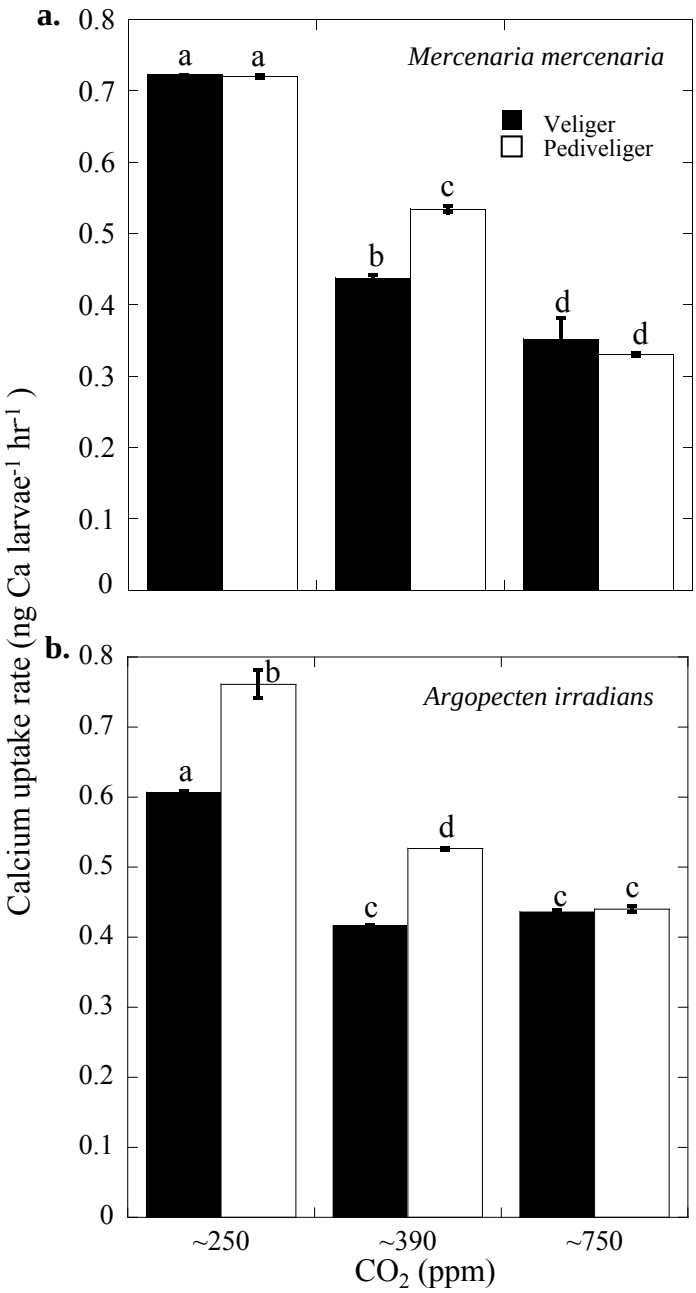


Figure 1. Mean calcium uptake ± 1 standard deviation for larvae from two developmental stages of *Mercenaria mercenaria* (veligers from day 3, and pediveligers from day 10*)* and *Argopecten irradians* larvae (veligers from day 12, and pediveligers from day 15*).* Larvae were grown under $CO_2$ concentrations of approximately 250, 390, and 750 ppm $CO_2$ (Table 1). Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

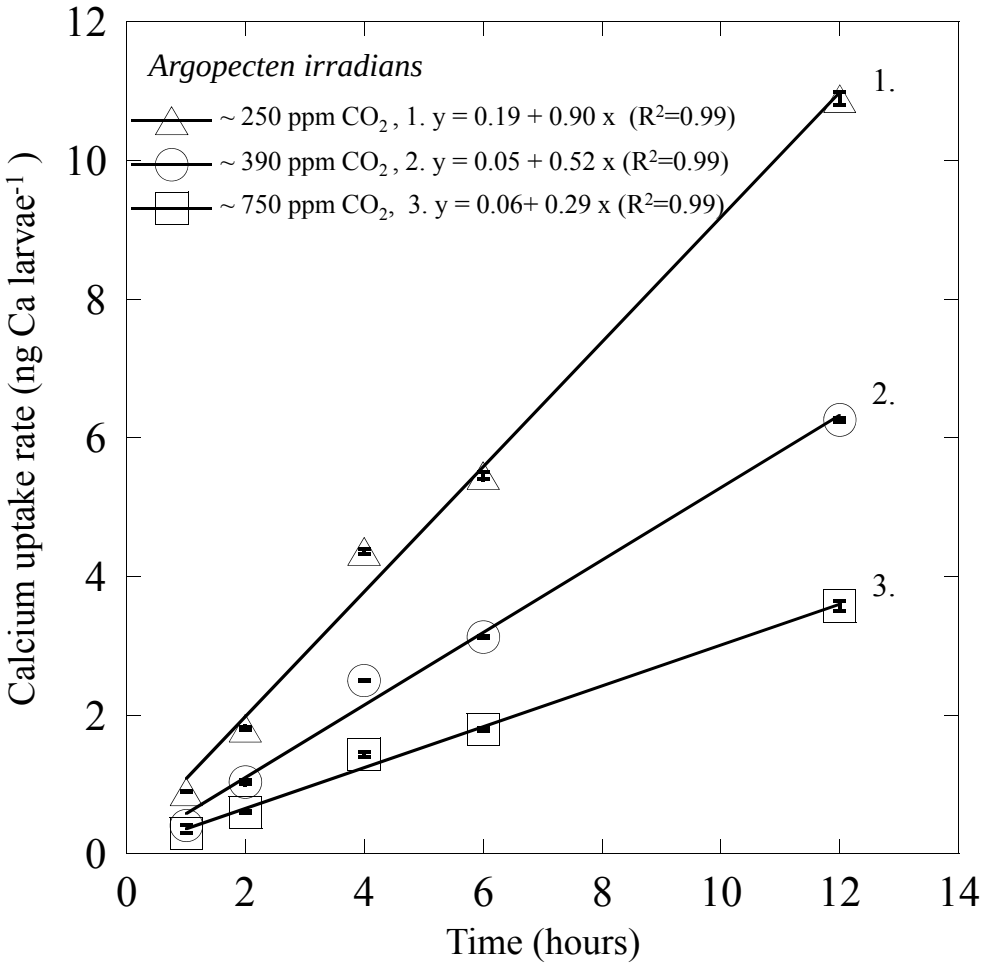


Figure 2. Mean (± 1 SD) calcium uptake rates over 12 hours for *Argopecten irradians* larvae (veligers from day 12). Larvae were grown under $CO_2$ concentrations of approximately 250, 390, and 750 ppm $CO_2$ (Table 2), $n = 4$ per treatment. Regressions corresponding to each treatment are listed in legend.

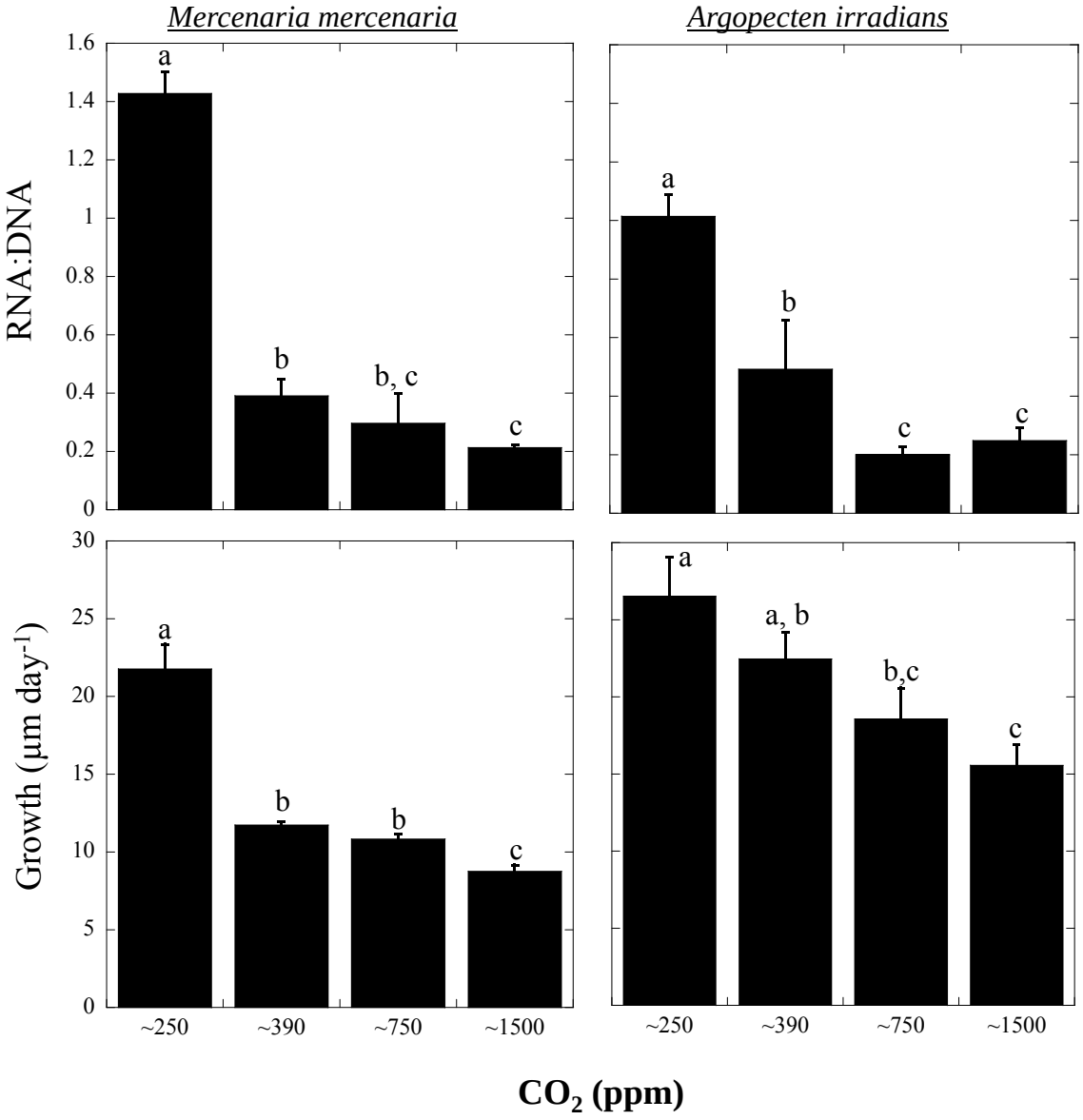


Figure 3. Mean RNA:DNA (± 1 SD) and mean, shell-based, specific growth rates (± 1 SD) for *Mercenaria mercenaria* (day 24) and *Argopecten irradians* (day 20) larvae $n = 15$ larvae per treatment. Larvae were grown under $CO_2$ concentrations of approximately 250, 390, and 750 ppm $CO_2$ (Table 3). Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

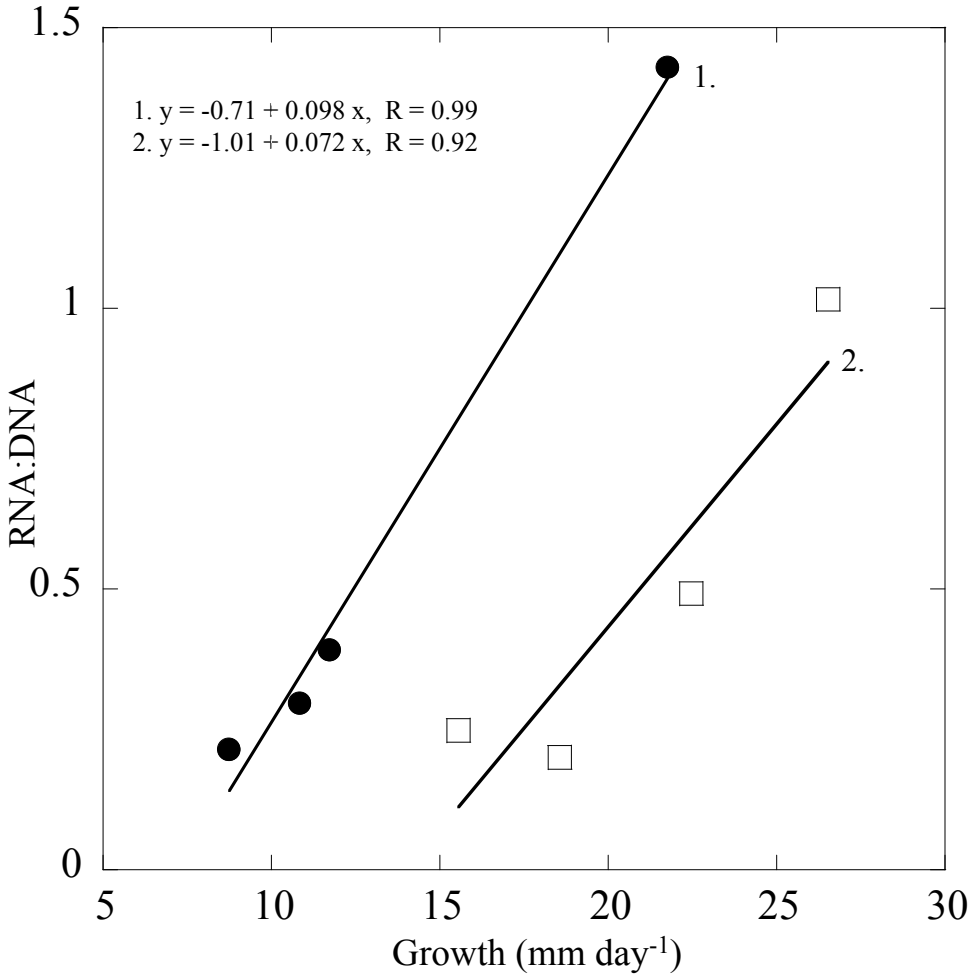


Figure 4. RNA:DNA ratios and shell-based growth rates of *Mercenaria mercenaria* larvae and *Argopecten irradians* larvae as depicted in Fig 3. Regressions corresponding to each comparison are listed in legend. Regression 1. is for *Mercenaria mercenaria* larvae and 2. represents *Argopecten irradians* larvae.

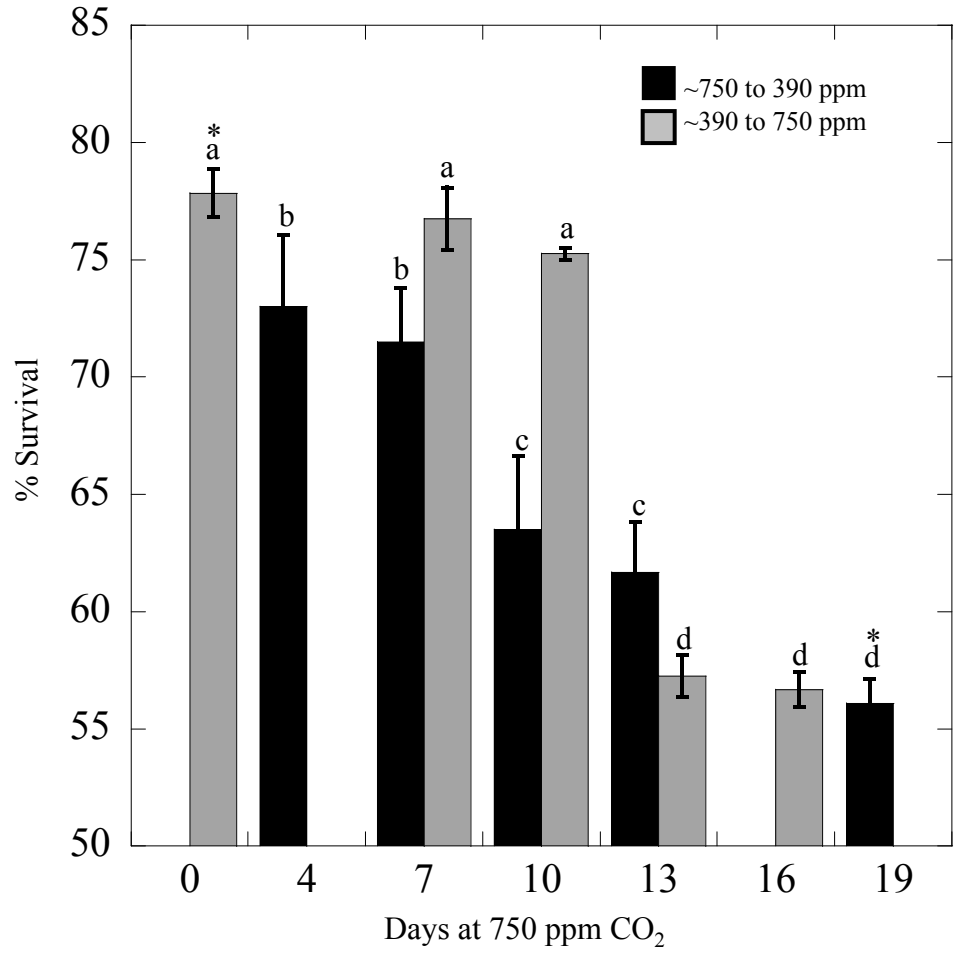


Figure 5. Survival ± standard deviation of *Argopecten irradians* larvae under varying days of exposure to approximately 750 ppm $CO_2$ (Table 4). Different colors indicate the $CO_2$ exposure the larvae experienced first and then were switched to ($n$ =4 per treatment). Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all, and * indicated these individuals only experienced either 390 ppm or 750 ppm and were never switched.

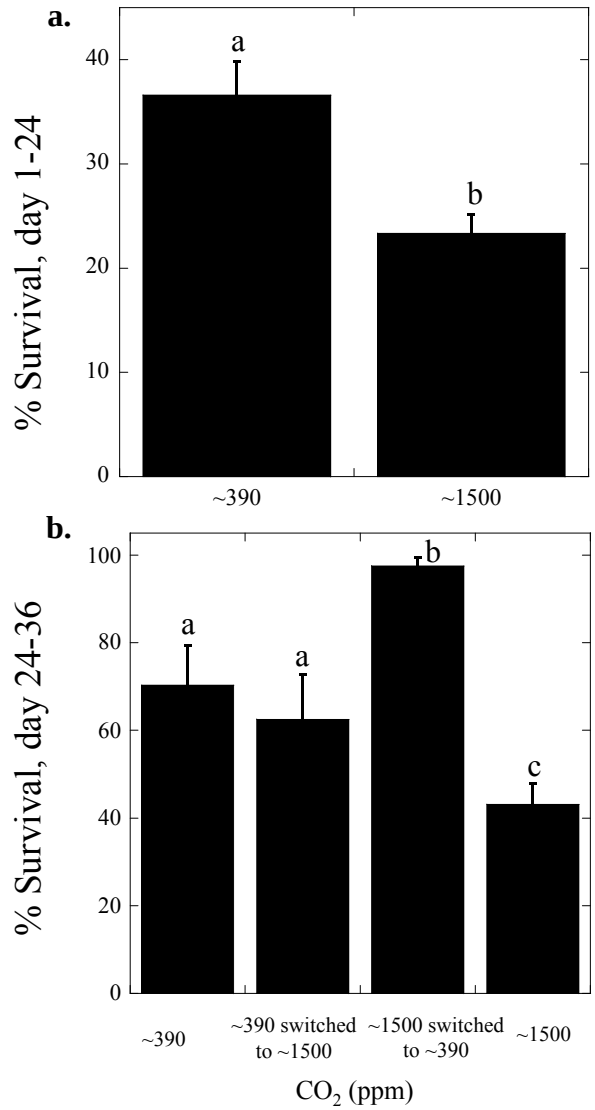


Figure 6.**a.)** Percent survival of *Mercenaria mercenaria exposed to* 390, and 1500 ppm $CO_2$ during larval development, (Table 5) **b.)** Percent survival of post-set, juvenile *M. mercenaria* under four treatments of $CO_2$: 1. Individuals exposed to 390ppm since fertilization, 2. Individuals exposed to 390ppm as larvae and increased to 1,500 at day 24, 3. Individuals exposed to 1,500ppm as larvae and decreased to 390ppm at day 24, 4. Individuals exposed to 1,500ppm since fertilization. Values are means ± 1. SD; $n$ =4 per treatment. Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

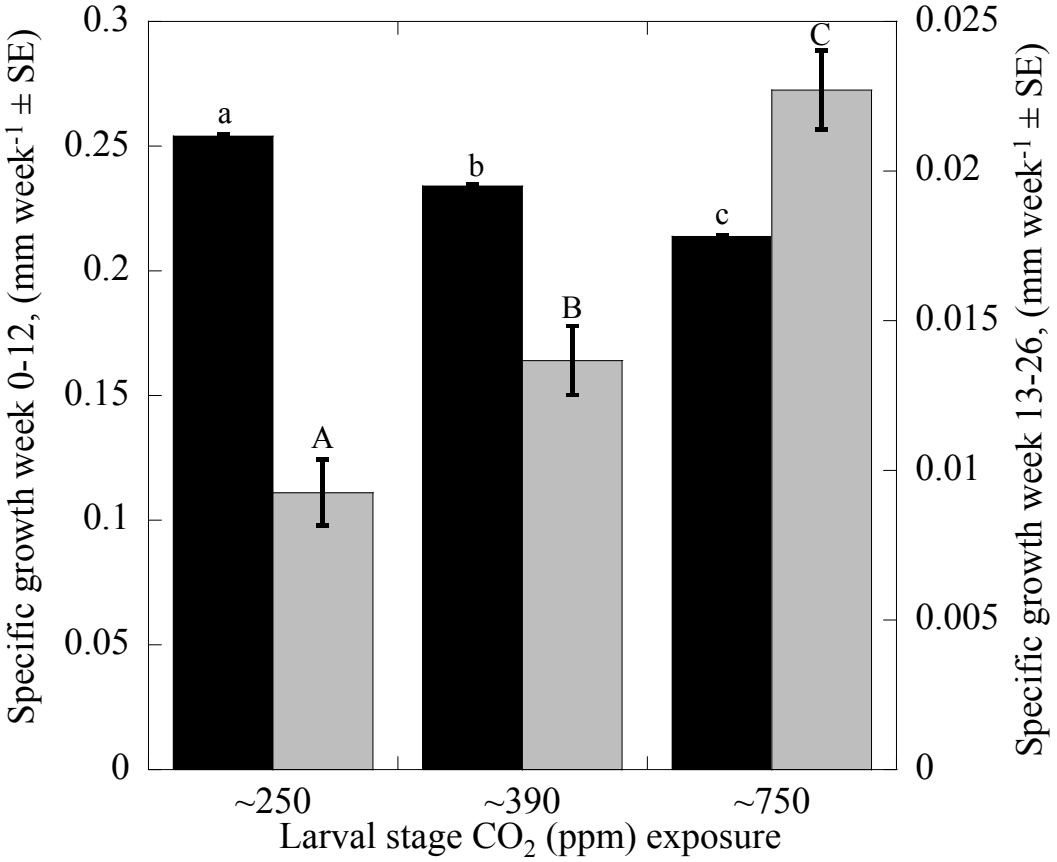


Figure 7. Specific growth rates of *Argopecten irradians* for 0-12 weeks (black bars) and for 13-26 weeks (gray bars). Larvae ($n$ = 1000) were grown under three $CO_2$ concentrations (250, 390, and 750 ppm $CO_2$; Table 6) during the larval stages before introduced into the field as juveniles. For Tukey multiple comparisons, $p \leq 0.05$ for all and capitalized letters indicate a separate analysis.

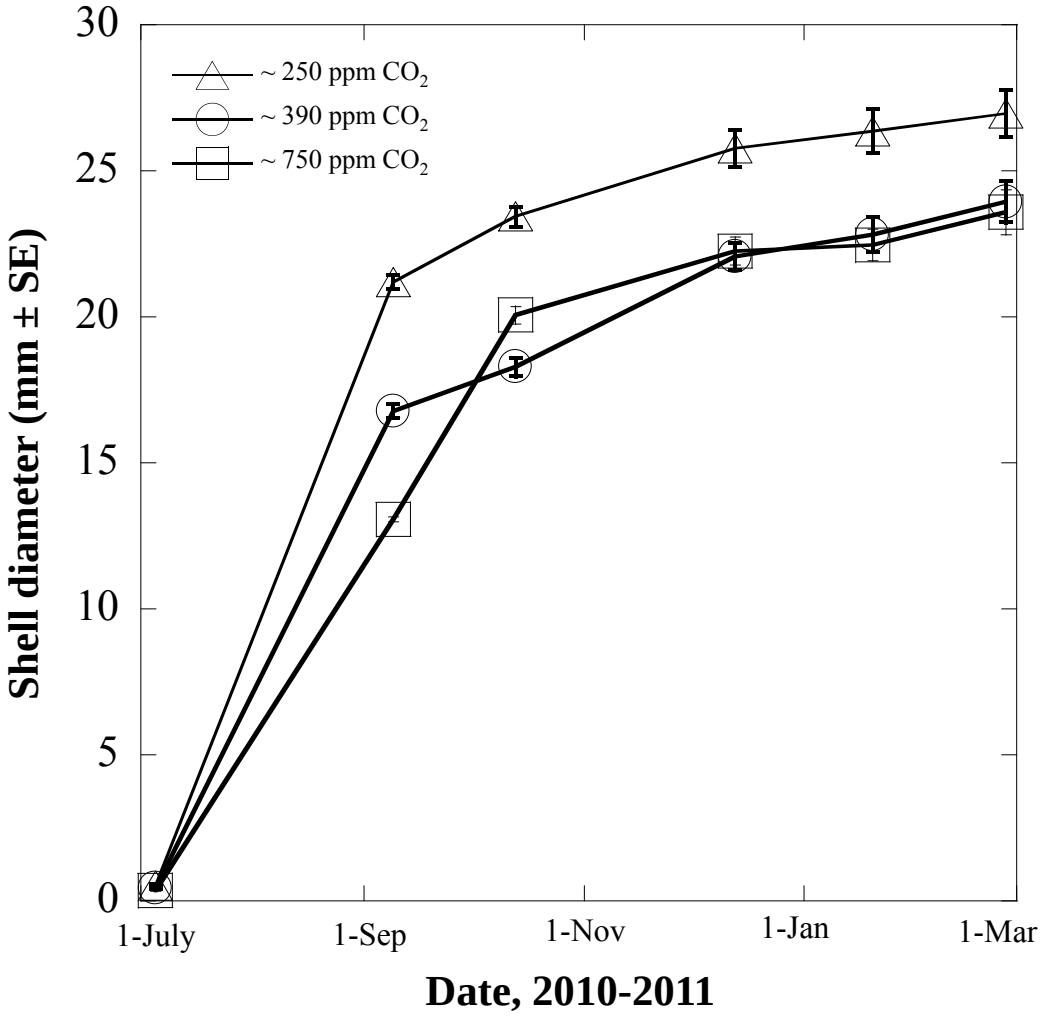


Figure 8. Shell diameter for *Argopecten irradians* juveniles over a 26 week period in an estuary in East Hampton, NY. Larvae ($n$ = 1000) were first grown under $CO_2$ concentrations of approximately 250, 390, and 750 ppm $CO_2$ (Table 6) during the larval stages before introduced into the field as juveniles. Estimates for first time point are from Chapter 3. Values are means ± 1. SD.

| **Parameter** | **Near pre-industrial $CO_2$** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|---|
| *Mercenaria mercenaria* | | | |
| Temperature (˚C) | 24 ± 0.8 | 24 ± 0.8 | 24 ± 0.8 |
| pH | 8.2 ± 0.051 | 8.08 ± 0.076 | 7.800 ± 0.013 |
| $pCO_2$ (ppm) | 248.3 ± 21.03 | 372.6 ± 39.48 | 781.6 ± 29.99 |
| $\Omega_{calcite}$ | 3.10 ± 0.13 | 2.59 ± 0.21 | 1.53 ± 0.13 |
| $\Omega_{aragonite}$ | 1.98 ± 0.39 | 1.69 ± 0.21 | 0.99 ± 0.13 |
| Total DIC ($\mu$mol $L^1$) | 1117 ± 37.95 | 1365 ± 54.22 | 1458 ± 23.33 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 117.8 ± 3.96 | 104.8 ± 35.78 | 60.5 ± 15.632 |
| Alkalinity (TA) | 1412.7 ± 112.5 | 1516 ± 74.56 | 1528 ± 32.44 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | |
| *Argopecten irradians* | | | |
| Temperature (˚C) | 24 ± 0.8 | 24 ± 0.8 | 24 ± 0.8 |
| pH | 8.2 ± 0.047 | 8.08 ± 0.061 | 7.810 ± 0.009 |
| $pCO_2$ (ppm) | 237.3 ± 5.32 | 396.5 ± 21.43 | 753.2 ± 19.04 |
| $\Omega_{calcite}$ | 2.94 ± 0.25 | 2.60 ± 0.24 | 1.55 ± 0.11 |
| $\Omega_{aragonite}$ | 1.89 ± 0.40 | 1.71 ± 0.18 | 1.01 ± 0.11 |
| Total DIC (umol $L^{-1}$) | 1220 ± 42.22 | 1327 ± 26.56 | 1438.6 ± 15.45 |
| $CO_3^{2-}$ ($\mu$mol $L^1$) | 121.3 ± 5.98 | 101.9± 48.55 | 61.0 ± 17.843 |
| Alkalinity (TA) | 1352.7 ± 42.76 | 1518± 55.56 | 1511 ± 36.44 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |

**Table 1**. Mean temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the three-level carbon dioxide experiments for calcium uptake with *Mercenaria mercenaria,* and *Argopecten irradians* larvae.

| **Parameter** | **Near pre-industrial $CO_2$** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|---|
| *Argopecten irradians* | | | |
| Temperature (˚C) | 24 ± 0.8 | 24 ± 0.8 | 24 ± 0.8 |
| pH | 8.201 ± 0.022 | 8.041 ± 0.042 | 7.801 ± 0.013 |
| $pCO_2$ (ppm) | 242.4 ± 19.221 | 402 ± 21.268 | 764.7 ± 19.658 |
| $\Omega_{calcite}$ | 3.01 ± 0.14 | 2.38 ± 0.32 | 1.51 ± 0.16 |
| $\Omega_{aragonite}$ | 1.94 ± 0.46 | 1.54 ± 0.31 | 0.97 ± 0.26 |
| Total DIC (umol $L^{-1}$) | 1196 ± 45.76 | 1334 ± 35.62 | 1430 ± 28.45 |
| $CO_3^{2-}$ ($\mu$mol $L^1$) | 118.4 ± 26.32 | 94.2 ± 28.65 | 59.5 ± 20.01 |
| Alkalinity (TA) | 1381.1 ± 30.05 | 1470.2± 43.02 | 1501± 43.88 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |

**Table 2.** Mean temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the three-level carbon dioxide for calcium uptake over time for *Argopecten irradians* larvae.

| **Parameter** | **Near preindustrial $CO_2$** | **Ambient, present day $CO_2$** | **Year 2100 $CO_2$** | **Year 2200 $CO_2$** |
|---|---|---|---|---|
| *Mercenaria mercenaria* | | | | |
| Temperature (˚C) | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 8.171 ± 0.022 | 8.052 ± 0.036 | 7.801 ± 0.004 | 7.532 ± 0.021 |
| $pCO_2$ (ppm) | 247.1 ± 6.231 | 380.0 ± 33.02 | 742.3 ± 9.111 | 1516 ± 31.21 |
| $\Omega_{calcite}$ | 5.31 ± 0.47 | 4.53 ± 0.41 | 2.82 ± 0.05 | 1.67 ± 0.05 |
| $\Omega_{aragonite}$ | 3.42 ± 0.30 | 2.92 ± 0.26 | 1.82 ± 0.03 | 1.08 ± 0.03 |
| Total DIC ($\mu$mol $L^{1}$) | 1646 ± 94.21 | 1831 ± 52.34 | 1947 ± 21.33 | 2108 ± 18.06 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 208.0 ± 20.22 | 178.0 ± 16.03 | 111.0 ± 1.806 | 66.0 ± 1.904 |
| Alkalinity (TA) | 1938 ± 117.3 | 2070 ± 66.42 | 2080 ± 22.63 | 2127 ± 49.71 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | | |
| *Argopecten irradians* | | | | |
| Temperature (˚C) | 24 ± 0.51 | 24 ± 0.52 | 24 ± 0.52 | 24 ± 0.52 |
| pH | 8.170 ± 0.026 | 8.041 ± 0.044 | 7.801 ± 0.005 | 7.530 ± 0.011 |
| $pCO_2$ (ppm) | 244.1 ± 4.006 | 386.5 ± 40.04 | 738.9 ± 9.941 | 1529 ± 35.05 |
| $\Omega_{calcite}$ | 5.18 ± 0.06 | 4.55 ± 0.47 | 2.81 ± 0.06 | 1.66 ± 0.05 |
| $\Omega_{aragonite}$ | 3.34 ± 0.35 | 2.94 ± 0.30 | 1.81 ± 0.04 | 1.07± 0.03 |
| Total DIC (umol $L^{-1}$) | 1613 ± 53.54 | 1850 ± 30.98 | 1941 ± 25.54 | 2101 ± 9.221 |
| $CO_3^{2-}$ ($\mu$mol $L^{1}$) | 202.0 ± 23.42 | 180.0 ± 18.44 | 111.0 ± 2.341 | 66.02 ± 1.911 |
| Alkalinity (TA) | 1899 ± 35.24 | 2090± 50.01 | 2075± 26.84 | 2146±11.21 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | | |

**Table 3.** Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the four-level carbon dioxide experiments with *Mercenaria mercenaria,* and *Argopecten irradians* larvae. (Same as Table 1 from Chapter 3, since these individuals were taken from that experiment and later analyzed for RNA:DNA).

| **Parameter** | **Ambient, present day $CO_2$** | **Elevated$CO_2$** |
|---|---|---|
| *Argopecten irradians* | | |
| Temperature (˚C) | 24 ± 0.6 | 24 ± 0.6 |
| pH | 8.08 ± 0.049 | 7.82 ± 0.015 |
| $pCO_2$ (ppm) | 372.6 ± 23.21 | 732.5 ± 29.02 |
| $\Omega_{calcite}$ | 2.66 ± 0.47 | 1.58 ± 0.17 |
| $\Omega_{aragonite}$ | 1.71 ± 0.28 | 1.02 ± 0.38 |
| Total DIC ($\mu$mol $L^{1}$) | 1364.1 ± 32.44 | 1432.6 ± 46.87 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 104.7 ± 23.24 | 62.2 ± 20.03 |
| Alkalinity (TA) | 1516.3 ± 66.54 | 1508 ± 52.56 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 |

**Table 4.** Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the two-level carbon dioxide varying exposure experiment with *Argopecten irradians* larvae.

| **Parameter** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|
| *Mercenaria mercenaria* | | |
| Temperature (˚C) | 24 ± 0.4 | 24 ± 0.4 |
| pH | 8.08 ± 0.037 | 7.58 ± 0.054 |
| $pCO_2$ (ppm) | 407.5 ± 27.832 | 1750.4 ± 17.996 |
| $\Omega_{calcite}$ | 2.93 ± 0.32 | 1.25 ± 0.08 |
| $\Omega_{aragonite}$ | 1.87 ± 0.39 | 0.80 ± 0.12 |
| Total DIC ($\mu$mol $L^{1}$) | 1492 ± 42.22 | 1956 ± 23.28 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 114.5 ± 24.3 | 49.2 ± 15.4 |
| Alkalinity (TA) | 1652.8 ± 36.56 | 1973.4 ± 37.81 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 |

**Table 5.** Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the four-level carbon dioxide experiments with *Mercenaria mercenaria* larvae for varying $CO_2$ exposure experiment.

| **Parameter** | **Near pre-industrial $CO_2$** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|---|
| *Argopecten irradians* | | | |
| Temperature (˚C) | 24 ± 0.7 | 24 ± 0.7 | 24 ± 0.7 |
| pH | 8.20 ± 0.067 | 8.08 ± 0.072 | 7.81 ± 0.009 |
| $pCO_2$ (ppm) | 231.3 ± 34.538 | 373.1 ± 29.78 | 755.9 ± 14.226 |
| $\Omega_{calcite}$ | 2.86 ± 0.53 | 2.66 ± 0.55 | 1.55 ± 0.09 |
| $\Omega_{aragonite}$ | 1.94 ± 0.37 | 1.71 ± 0.50 | 1.00 ± 0.43 |
| Total DIC ($\mu$mol $L^{1}$) | 1141 ± 98.33 | 1365.7 ± 56.79 | 1443.8 ± 38.92 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 112.9 ± 25.35 | 104.8 ± 37.37 | 61.3 ± 14.54 |
| Alkalinity (TA) | 1320.9 ± 119.3 | 1518.1 ± 43.36 | 1516.4 ± 47.54 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |

**Table 6.** Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the three-level carbon dioxide experiments for *Argopecten irradians* larvae growth before deployment in the estuary for juvenile growth.

# CHAPTER FIVE:

## Effects of elevated temperature and carbon dioxide on the growth and survival of larvae and juvenile stages of three species of Northwest Atlantic bivalves

**Abstract**

Rising $CO_2$ concentrations and water temperatures are likely to have transformative effects on many coastal marine organisms in this century. Here, we compared the responses of two life history stages (larval, juvenile) of three species of calcifying bivalves (*Mercenaria mercenaria*, *Crassostrea virginica*, and *Argopecten irradians*) to present and future temperatures (24 and 28ºC) and $CO_2$ concentrations (~250, 390, and 750 ppm) representative of past, present, and future summer conditions in temperate estuaries. Results demonstrated that these increases in temperature and $CO_2$ each significantly depressed rates of survival, development, growth, and lipid synthesis of *M. mercenaria* and *A. irradians,* larvae and that the effects were additive. Juvenile *M. mercenaria* and *A. irradians* were negatively impacted by higher temperatures while *C. virginica* juveniles were not. *C. virginica* and *A. irradians* juveniles were negatively affected by higher $CO_2$ concentrations, while *M. mercenaria* was not. Larvae were substantially more vulnerable to elevated $CO_2$ than juvenile stages. These findings suggest that current and future increases in temperature and $CO_2$ are likely to have substantial consequences for the persistence of coastal bivalve populations.

## Introduction

The combustion of fossil fuels during the past two centuries has promoted increases in atmospheric carbon dioxide and global temperatures, trends that are projected to continue in the coming decades (I.P.C.C. 2007). Global temperatures are expected to increase 2 to 5ºC this century (Houghton et al. 2001). Atmospheric $CO_2$ concentrations that had increased at a rate of 1% per year in the 20$^{th}$ century are now increasing ~3% per year and may exceed 800 ppm by the end of this century (I.P.C.C. 2007; Fussel 2009). Ocean chemistry will be altered by this rising $CO_2$ as levels of both pH and carbonate ions will decline (Cao et al. 2007). These simultaneous increases in temperature and $CO_2$ levels and decreases in pH and carbonate may have transformative effects on ocean life.

Coastal zones are likely to be the first regions to experience high levels of temperature and $CO_2$ predicted for the open ocean in the future. Upwelling can introduce water with high concentrations of $CO_2$ (800-1100 ppm) along large sections of the continental shelf (Feely et al. 2008). Acidic river water can depress carbonate ion concentrations in coastal marine environments (Salisbury et al. 2008). Furthermore, many coastal regions can be net heterotrophic due to decomposition of anthropogenic, terrestrial, riverine, and wetland loadings of organic carbon (Gattuso et al. 1998; Paerl et al. 1998; Thomas et al. 2004; Koch and Gobler 2009), processes that collectively promote supersaturated $CO_2$ concentrations and lower pH. Coastal water temperatures are more sensitive to extreme and rapid increases in air temperature, and increases in estuarine water temperatures have outpaced those observed in the surface ocean (Levitus et al. 2000; Nixon et al. 2004).

Many marine organisms, in particular those with calcified parts, can be negatively affected by acidification of ocean waters (Doney et al. 2009). Enrichment of $CO_2$ can have negative impacts across a wide range of calcifying marine taxa from corals (Hoegh-Guldberg et al. 2007), to coccolithophores (De Bodt et al. 2010), echinoderms (Brennand et al. 2010), and coralline algae (Martin and Gattuso 2009). Sediments with high levels of $CO_2$ and low levels of carbonate ion have been shown to promote mortality of juvenile mollusks (*Mercenaria mercenaria* and *Mya arenaria*) (Green et al. 2004; Green et al. 2009). Elevated $CO_2$ can cause decreased calcification in mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*; (Gazeau et al. 2007)), as well as decreased growth in mussels (*M. edulis*; (Berge et al. 2006)). Seawater enriched in $CO_2$ can also depress the survival, growth, and metamorphosis of larval stages of calcifying bivalves (Gazeau et al. 2007; Kurihara et al. 2007; Green et al. 2009; Miller et al. 2009; Talmage and Gobler 2009; Gazeau et al. 2010).

The increases in ocean temperatures projected to occur this century will impact marine life. Higher temperatures in marine ecosystems can alter primary productivity, stratification, and organismal physiology (Scavia et al. 2002). The current rate of warming in ocean waters will likely apply thermal stress to a wide range of marine organisms as the limits of their temperature tolerances are approached or exceeded (Hoffman and Todgham 2010). Temperature is a vital factor that influences the spawning and development of invertebrate larvae (Thorson 1950; Eversole 2001; Barber and Blake 2006). Since most bivalve gametes are spawned at temperatures specific to their species, ocean warming will affect the timing of this spawning (Thorson 1950; Eversole 2001; Barber and Blake 2006). While larval bivalves experience maximal growth and survival

rates under ideal temperature conditions (e.g. ~24ºC for many northwestern Atlantic species), small increases in temperature beyond that range will depress these rates (Loosanoff 1965; Fritz 2001; Cragg 2006). In addition, higher temperatures can make larval bivalves more vulnerable to other environmental stressors (Cherkasov et al. 2010) such as ocean acidification.

Concurrent, future increases in $CO_2$ and water temperatures in marine environments will likely have multiplicative effects on ocean life, in general, and invertebrate larvae in particular. In organically-enriched coastal ecosystems, higher temperatures may promote higher rates of microbial respiration and $CO_2$ production. Negative impacts of high $CO_2$ are often the greatest for early life stages of many organisms, while thermal stress can affect all life stages (Pörtner 2008). For the tropical sea urchin, *Tripneustes gratilla*, higher temperatures increased the growth and size of larvae, while higher $CO_2$ concentrations reduced calcification and negated the positive effect of higher temperatures when both temperature and $CO_2$ were increased (Brennand et al. 2010). One week old barnacles, *Semibalanus balanoides*, displayed a significant reduction in calcification and decreases in survival under simultaneously elevated temperature and $CO_2$ (Findlay et al. 2010). Red abalone larvae, *Haliotis rufescens*, displayed significant reductions in survivorship with increased $CO_2$ and a brief thermal stress compared to ambient $CO_2$ levels at the same thermal stress level (Zippay and Hofmann 2010). To date, no study has examined the simultaneous effects of varying $CO_2$ levels and temperature on any life history stage of marine bivalves.

Here I present experiments investigating the effects of higher seawater temperatures and past, present, and future $CO_2$ concentrations on the growth and survival

of the larvae of two species and juveniles of three species of $CaCO_3$ synthesizing bivalves native to the east coast of North America: the hard clam or northern quahog, *Mercenaria mercenaria* (*Linnaeus*, 1758), the Eastern oyster, *Crassostrea virginica* (*Gmelin*, 1791), and the bay scallop, *Argopecten irradians* (*Lamarck*, 1819). These shellfish are vitally important economic resources and ecosystem engineers in shallow coastal waters (Newell 2004), and performance of these early life history stages have a profound effect on the population dynamics of these animals (Andre and Rosenberg 1991; Caley et al. 1996; Arnold 2008). By simultaneously investigating the impacts of high temperature and increased $CO_2$ concentrations, I was able to determine which stages were most vulnerable to these multiple environmental stressors.

**Methods**

This study examined the effects of multiple $CO_2$ and temperature levels on juvenile and larval stages of bivalves. All experimental vessels with bivalves (described below) were maintained in water baths at 24 and 28ºC using commercially available aquarium heaters (Aquatic Eco-systems, Inc., Florida, USA). Temperatures were recorded every 6 minutes during theexperiments using in situ data loggers (Onset©); that demonstrated temperatures varied within 2.5% of target values. The two experimental temperatures (24 and 28ºC) were chosen to represent normal and above average temperatures in northeast U.S. estuaries during summer months (S.C.D.H.S. 1976-2008; Nixon et al. 2004) when larvae are spawned and juvenile stages are most likely to experience thermal stress. A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used to deliver $CO_2$ gas to seawater treatments at multiple

rates. The gas proportionator mixed appropriate flow rates of 5% $CO_2$ gas, low $CO_2$ gas, and pressurized air (~ 390 ppm $CO_2$) to yield the concentrations of carbon dioxide desired for experiments at a net flow rate that turned over experimental vessels >100 times daily so that the treatments never had the opportunity to equilibrate with the atmosphere. We have found that experiments performed with gases mixed via a proportionator as described here generate nearly identical seawater chemistry and larval responses obtained from tanked gases premixed at specific $CO_2$ levels. For experiments, the $CO_2$ gas mixtures from the proportionator system were continuously delivered to the bottom of replicated (*n*=3 or 4) experimental vessels (detailed below). With continuous bubbling, all treatment carboys remained saturated with respect to oxygen (~8 mg $L^{-1}$). To quantify precise $CO_2$ levels attained in experimental treatments, aliquots were removed and analyzed during experiments using an EGM-4 Environmental Gas Analyzer® (PP Systems) system that quantified total dissolved inorganic carbon levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). This instrument provided a methodological precision of ± 3.6% for replicated measurements of total dissolved inorganic carbon and provided full recovery (102 ± 3%) of Dr. Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography) certified reference material for total inorganic carbon in seawater (Batch 102 = 2013µmol DIC kg $seawater^{-1}$). Levels of $CO_2$ were calculated based on measured levels of total inorganic carbon, pH (mol kg $seawater^{-1}$; NBS scale), temperature, salinity, and first and second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/). Daily measurements of pH (Thermo Scientific Orion Star Series™ Benchtop pH meter; ±

0.002; calibrated prior to each use with NIST traceable standards) indicated experimental vessels maintained a constant pH level throughout experiments (<0.5% RSD within treatments). Random spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson *et al.* (2007) and corrected for scale (Dickson 1993) were never significantly different from those obtained with the high sensitivity microprocessor.

*Larvae experiments*

The recommendations of the 'best practices' for small microcosm experiments set forth by European Project on Ocean Acidification (EPOCA) were followed for this project. For example, aeration of seawater was used to reach a target $pCO_2$ level, which is noted as the ideal mechanism to manipulate seawater carbon chemistry (Riebesell et al. 2010). *M. mercenaria* and *A. irradians* larvae were grown at three levels of $CO_2$: a high level (~750 ppm $CO_2$), predicted for the year 2100, a modern level (~390 ppm $CO_2$), and a near pre-industrial level (~250 ppm $CO_2$), while at two different temperatures (24 and 28$^o$ C). Precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 1. One-liter high-density polyethylene beakers were filled with 0.2 $\mu$m filtered seawater from eastern Shinnecock Bay, New York, United States. *M. mercenaria* larvae were obtained from Cornell Cooperative Extension, Southold, NY, and *A. irradians* larvae were from the East Hampton Shellfish Hatchery, East Hampton, NY, within hours of fertilization and were distributed to each treatment beaker at a concentration of ~ 350 $L^{-1}$, consistent with post-spawning densities in estuaries (Carriker 2001). Twice weekly during experiments, larvae were gently poured onto a 64 $\mu$m mesh, and the condition (live or dead) and developmental stage of each larvae (veligers,

pediveligers, and metamorphosed) were determined visually under a dissecting microscope; every individual larvae was counted at every water change. Larvae from each beaker ($n$ =4, per treatment) were removed, counted, observed, and transferred into a new beaker with new filtered seawater, food, and antibiotics within a 15 minute period. Percent survivorship of all larvae was determined at each of the bi-weekly water changes when the numbers of larvae in each stage of veligers, pediveligers, and metamorphosed juveniles were quantified. Experiments were terminated after all surviving larvae in all treatments had metamorphosed.

Larvae were fed an ideal food source at a density known to maximize bivalve larval growth and survivorship through metamorphosis (Castell and Mann 1994; Cragg 2006; Talmage and Gobler 2009). Cultures of *Isochrysis galbana* (Tahitian strain, T-Iso) were maintained in exponential phase growth using standard culture conditions and added as a food source at a density of $2 \times 10^4$ $mL^{-1}$ daily to each experimental beaker. To promote high survivorship, all containers in contact with larvae were never exposed to chemicals or detergents (Talmage and Gobler 2009). To discourage the growth of bacteria during experiments, an antibiotic solution (Sigma-Aldrich No.4083, 5000 units of Penicillin, 5 mg of Streptomycin, and 10 mg of Neomycin per milliliter of solution) was added to each beaker at 1% its original concentration at the beginning of each experiment and at the time of each water change (approximately 2 times weekly). This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae (Talmage and Gobler 2009). Experiments presented here were repeated without antibiotic treatments and yielded no difference in bivalve larval survival suggesting that neither the antibiotics nor the bacteria in seawater

altered the results presented here. To meet the assumption of normality and homogeneity, survival and percent metamorphosed data were arcsin square root transformed after which a two-way ANOVAs was performed where temperature and $CO_2$ were the main effects. Sizes of larvae and lipid content were also examined via two-way ANOVAs. Post-hoc Tukey multiple comparison tests were performed to examine the differences among survival , metamorphosis, and size at each temperature and $CO_2$ level. Statistical analyses were performed with SYSTAT 13 © Copyright, 2009, Systat Software, Inc. Representation of the degrees of freedom for all ANOVAs in this chapter are presented as: *df*= x, y; where x represents the degrees of freedom for the factor being described, and y represents the degrees of freedom for error in the ANOVA analysis of that factor.

To estimate the relative lipid content of larvae, Nile Red dye was used to stain neutral lipids (Castell and Mann 1994; Phillips 2002). A Nile Red stock solution was made of 1.25 mg of Nile Red crystals in 100 ml of acetone. Randomly selected larvae ($n$ = 15) from each treatment were stained with a 1:9 dilution of the stock solution and 0.2 µm filtered seawater. Larvae were exposed to the stain for ~1.5 hours during which larval motion ceased, permitting the orientation of individuals for microscopic imaging using an FITC filter on an epifluorescent microscope. Larvae were then rinsed with filtered seawater, and digitally photographed with a Roper Scientific Photometrics CoolSNAP ES camera attached to the top of an epiflorescent microscope. Digital images of each larvae were analyzed for the area of lipid accumulation and the diameter and the area of individuals using Image J® software (Przeslawski et al. 2008). A lipid index was estimated by dividing the area of the larvae containing the fluorescing lipids by the total

larval area thereby allowing for direct comparisons among treatments. Two-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among larval lipid indexes, as well as shell length at each $CO_2$ level.

*Juvenile experiments*

Juvenile bivalves were obtained during early summer from the East Hampton Shellfish Hatchery, East Hampton, NY. Starting mean lengths and ash-free, dry weights (± standard deviation) of individuals were 6.09 ± 0.65 mm and 1.36 ± 0.048g for *M. mercenaria*, 11.48 ± 3.60 mm and 1.48 ± 0.221g for *C. virginica*, and 15.93 ± 1.59 mm and 1.74 ± 0.172g for *A. irradians*. Ten individuals of each species were placed into triplicate, 10-liter, high-density polyethylene vessels that were maintained in water baths of 24 or 28ºC (Table 2). $CO_2$ was continuously delivered as described above at ~400 and 1700 ppm representing ambient, pelagic $CO_2$ found today and a level expected in the 22$^{nd}$ century surface ocean (Caldeira and Wickett 2003), but only slightly above levels found in and near the seabed which is frequently undersaturated with regarding to carbonate (Green et al. 2004; Salisbury et al. 2008; Green et al. 2009). Precise $CO_2$ levels and complete carbonate chemistry from this experiment appear in Table 2. To ensure treatment concentrations were met, experimental vessels were bubbled with appropriate $CO_2$ levels for 24 h prior to commencing experiments and prior to addition of the organisms. Each of the 10 juveniles introduced into each treatment was identified with a dot of colored paint, allowing growth of individuals to be assessed through the 45 day experiment, a duration consistent with peak, hot, summer temperature in temperate estuaries (S.C.D.H.S. 1976-2008; Nixon et al. 2004). Every three days, water was

exchanged with ambient sea water from Old Fort Pond, Southampton, NY, USA, or Northwest Harbor in East Hampton, NY, USA (salinities = 28 ± 3). Newly collected water was pre-bubbled for 12 hours prior to use. Nutrients (10 µM nitrate and 0.63 µM orthophosphate) were added immediately and daily to experimental vessels that were held under a bank of fluorescent lights that were on an ~ 12:12h light:dark cycle and delivered a light intensity of ~10 µmol quanta $m^{-2}$ $s^{-1}$ to encourage phytoplankton growth. Chlorophyll *a* was measured using standard methods at the start and end of each water change during experiments (Welschmeyer 1994); and averaged 9.8 ± 3.7 µg $L^{-1}$, a level generally deemed adequate for maximal growth rate of juvenile bivalves (Nixon and Buckley 2002; Carmichael et al. 2004; Weiss et al. 2007).

Tissue and shell weight of juvenile bivalves was quantified by weighing individuals before and after drying for 72 hours at 60ºC and then combustion for 4 hours at 450ºC. The post-combustion weight represented the shell weight whereas the difference between the dry and combusted weights represented organic tissue weight. Tissue and shell weight-based growth was calculated by dividing the change in weight by the duration of the experiment in days. Growth was compared by means of two-way ANOVAs where temperature and $CO_2$ were the main effects. Post-hoc Tukey multiple comparison tests were performed to examine the differences among juvenile growth at each temperature and $CO_2$ level. Survival of individuals was assessed daily and dead individuals (*A. irradians* only during the final weeks of the experiment) were removed within < 24 hr of expiring. The percent mortality of *A. irradians* within each treatment was arc-sin square root transformed after which a two-way ANOVA was performed where temperature and $CO_2$ were the main effects.

**Results**

Carbon dioxide, $F(1)$, and temperature, $F(2)$, both significantly affected the percent of larvae that had metamorphosed ($F(1) = 277.661$, $df$=2, 18, $F(2)= 60.088$, $df$ =1, 18, $p<0.001$; two-way ANOVA), survival ($F(1) = 232.680$, $df$=2, 18, $F(2)= 627.477$, $df$ =1, 18, $p<0.001$; two-way ANOVA), growth ($F(1) = 703.999$, $df$= 2, 18, $F(2)= 24.676$, $df$ =1, 18, $p<0.001$; two-way ANOVA) and lipid synthesis ($F(1) = 40.386$, $df$= 2,18, $F(2)= 26.845$, $df$ =1, 18, $p<0.001$; two-way ANOVA) in *M. mercenaria* larvae; temperature and $CO_2$ had a significant, slightly antagonisitic interactive effect, $F(3)$, on *M. mercenaria* metamorphosis and survival ($F(3) = 24.990$, 29.300, $df$ = 2, 18, $p<0.001$ for all; two-way ANOVA). The percentage of individuals that had metamorphosed and survived, as well as individual sizes were all highest for individuals grown under 250 ppm and at 24ºC and were lowest for individuals grown at 750 ppm $CO_2$ and 28ºC (Fig 1). For example, 18 days post-fertilization, 45 ± 2.6, 16 ± 2.0, and 8 ± 5.3% of individuals (± standard deviation) at 24ºC had metamorphosed under ~250, 390, 750 ppm $CO_2$, whereas 27 ± 0.6, 13 ± 0.4, and 5 ± 0.3% (± standard deviation) had done so at ~250, 390, 750 ppm $CO_2$ and 28º C (Fig.1a). With increasing $CO_2$ values (~250, ~390, and ~750 ppm), larval survival decreased from 44 ± 3.1 to 30 ±2.1 and 20 ± 0.3% at 24ºC compared to 20 ± 0.9 to 14 ± 0.5 and 8 ± 1.5% at 28º C (Fig.1b). For *M. mercenaria* larvae, there was a synergistic interaction ($p<0.001$; two-way ANOVA) between $CO_2$ and temperature, as survival percentages in this combined treatment were lower than expected from the individual treatments. Regarding size, *M. mercenaria* larvae at 24ºC and ~250 ppm $CO_2$ had mean diameters of 553 ± 38 $\mu$m while increasing temperatures and $CO_2$ level progressively depressed sizes; with individuals grown at 28ºC and ~750 ppm $CO_2$

having mean diameters of 325 ± 22 $\mu$m (Fig. 1c). Lipid indices for *M. mercenaria* were always higher at 24ºC (0.23 ± 0.09) compared to larvae grown at 28ºC (0.15 ± 0.07*;* $F(2)=26.845$, $df = 1, 18$, $p<0.001$, two-way ANOVA; Fig.1d). The lipid content for *M. mercenaria* larvae also decreased with increasing $CO_2$ levels ($F(1)=40.386$, $df = 1, 18$, $p<0.001$, two-way ANOVA; Fig.1d). While there was no significant differences in lipid indices between ~250 and ~390 ppm at either temperature, there was a significant decrease in lipid indices when the $CO_2$ level was enriched from ~250 or ~390 to ~750 ppm for both temperatures (Fig.1d).

Responses of *A. irradians* larvae to temperature and $CO_2$ levels were similar to *M. mercenaria* and in some cases were larger. There was a significant decrease in the percent of individual *A. irradians* larvae that had developed into metamorphosed juveniles with increasing $CO_2$, $F(1)$, and increasing temperature, $F(2)$, ($F(1) = 609.495$, $df= 2, 18$, $F(2)= 21{,}991.384$, $df =1, 18$, $p<0.001$; two-way ANOVA), as well as synergistic interaction between both temperature and $CO_2$ concentrations, $F(3)$, for larval metamorphosis ($F(3)=371.009$, $df = 2, 18$, $p<0.001$; two-way ANOVA). Fewer than 10% of individuals at any $CO_2$ level had metamorphosed at 28ºC compared to 87 ± 0.8 and 71 ± 0.9 and 53± 2.3% individuals at 24ºC and ~250, ~390, and ~750 ppm, respectively (Fig. 2a). There was also a significant decline in larval survival with each increased $CO_2$ and temperature level ($F(1) = 1{,}004.938$, $df= 2, 18$, $F(2)= 5{,}679.239$, $df =1, 18$, $p<0.001$; two-way ANOVA; Fig. 2b). There was also a slightly antagonisitc interactive effect of $CO_2$ and temperature on the percentage of *A. irradians* survival ($F(3)= 129.801$, $df= 2, 18$, $p<0.001$; two-way ANOVA). At 24ºC, 91 ± 0.9, 74 ± 1.1, and 54 ± 2.3% of individuals survived at 250, ~390, and ~750 ppm, respectively, whereas at 28ºC, 45 ±

0.8, 35 ± 0.4, and 27 ± 0.9 ±% of individuals survived, respectively (Fig. 2b). Higher $CO_2$, *F*(1), and temperature, *F*(2), depressed the size attained by *A. irradians* larvae (*F*(1) = 1,120.054, *df*= 2, 18, *F*(2)= 57.072, *df* =1, 18, $p<0.001$; two-way ANOVA). Mean diameters of *A. irradians* larvae at 24ºC and ~250 ppm were 530 ± 33 $\mu$m while sizes progressively decreased with higher temperature and $CO_2$ levels to 309 ± 33 $\mu$m at 28ºC and ~750 ppm (Fig. 2c). For *A. irradians* larvae, there were significant differences in lipid indices among $CO_2$ levels (*F*(1)=74.069, *df* = 2, 18, $p<0.001$, two-way ANOVA), and between the two temperatures (*F*(2)=4.468, *df* = 1, 18, $p<0.05$, two-way ANOVA). At both temperatures, lipid indices in *A. irradians* larvae decreased from 0.21 ± 0.04 to 0.18 ± 0.03 to 0.08 ± 0.01 as $CO_2$ levels increased from ~250 to ~390 and ~750 ppm (Fig.2d). At 28ºC, lipid indices decreased from 0.19 ± 0.007 to 0.15 ± 0.01 to 0.07 ± 0.004 as $CO_2$ levels increased from ~250 to ~750 pm (Fig. 2d).

Unlike the larvae, juvenile *M. mercenaria* were unaffected by even higher levels of $CO_2$, but were affected by temperature differences. For example, the shell growth of juvenile *M. mercenaria* was significantly greater at 24ºC (1.03 ± 0.06 mg $d^{-1}$) compared to 28ºC (0.66 ± 0.07 mg $d^{-1}$; *F*(2)=7.762, *df* = 29, $p<0.01$, two-way ANOVA; Fig. 3a). Tissue growth for *M. mercenaria* juveniles was not significantly altered by temperature, and $CO_2$ did not significantly alter shell or tissue growth of *M. mercenaria* juveniles (Fig. 3b).

Unlike *M. mercenaria*, shell growth of *C. virginica* juveniles was significantly lower at 1700ppm $CO_2$ (2.88 ± 0.10 mg $d^{-1}$) compared to 400 ppm $CO_2$ (4.57 ± 0.17 mg $d^{-1}$; *F*(1) = 4.279, *df* = 1, 116, $p<0.05$; two-way ANOVA; Fig. 4a). Tissue growth for *C. virginica* juveniles was not significantly affected by temperature or $CO_2$ (Fig. 4b).

Juvenile *A. irradians* were sensitive to both the elevated $CO_2$, $F(1)$, and elevated temperatures, $F(2)$, used in this study. With increasing temperature from 24 to 28ºC, *A. irradians* juvenile shell growth decreased from 4.75 ± 0.17 mg $d^{-1}$ to 3.30± 0.13 mg $d^{-1}$ while tissue growth decreased from 0.14 ± 0.02 mg $d^{-1}$ to 0.03 ± 0.002 mg $d^{-1}$ ($F(1) = 7.347$, $F(2) = 9.362$, $df = 1, 116$, $p<0.05$; two-way ANOVA; Fig.5a). Although $CO_2$ did not significantly alter shell or tissue based growth in juvenile *A. irradians*, the higher $CO_2$ and temperature yielded a significant interactive, $F(3)$, decline in juvenile *A. irradians* survival from 73.3 ± 15% and 53.3 ± 15.3% for 24 and 28ºC, respectively, at 400 ppm $CO_2$, to 43.3 ± 5.8% and 33.3 ± 13.0% for 24 and 28ºC, respectively, at 1700 ppm ($F(3) = 5.625$, $df = 1, 116$, $p<0.05$; two-way ANOVA) (Fig.5c). Survival of juvenile *M. mercenaria* and *C. virginica* juveniles was very high (97 ± 6 and 93 ± 6%, respectively) and was not significantly altered by temperature or $CO_2$ (data not shown).

**Discussion**

Global climate change has made our oceans warmer and more acidic, trends that are projected to continue this century. Coastal ecosystems are currently vulnerable to temperature and $CO_2$ increases due to their shallow nature and proximity to terrestrial and anthropogenic carbon loading, and these increases may not be seen in open ocean waters for many decades. This study demonstrates that elevated levels of $CO_2$ and temperature negatively impact both juvenile and larval stages of bivalves. Larvae were found to be more sensitive to elevated levels of $CO_2$ and temperature than juvenile stages. The high temperature (28ºC) and high $CO_2$ (~750 ppm) treatment yielded the lowest percent survival and metamorphosis, size, and lipid accumulation for these species. Collectively,

these results provide novel insight regarding the effects of $CO_2$ and temperature on the success of bivalve populations in coastal ecosystems.

Larvae represent a critical life stage for shellfish populations as reductions in the growth and survival of larvae have the potential to translate into substantial declines in adult populations (Caley et al. 1996; Gosselin and Qian 1997; Schneider et al. 2003; Arnold 2008). Temperature has a major influence on the spawning, growth, and development of bivalve larvae. *M. mercenaria* adults from New York to Connecticut waters are known to spawn when summer water temperatures reach 23-25ºC (Eversole 2001), while in temperate populations of *A. irradians*, spawning is typically triggered by water temperatures close to 23ºC (Tettelbach et al. 1999; Barber and Blake 2006). Once spawned, these larvae grow optimally at temperatures around 24-25ºC, but may experience slowed growth and even enhanced mortality at higher temperatures (Fritz 2001). Consistent with this finding, *M. mercenaria* and *A. irradians* larvae experienced significant declines in rates of survival, growth, and metamorphosis at 28ºC compared to 24ºC during this study. In the future, hotter summer water temperatures (I.P.C.C. 2007; Fussel 2009) may lead to a smaller optimal temperature window for bivalve larval growth.

Prior studies have demonstrated that increases in $CO_2$ concentrations beyond levels found in today's surface oceans have negative impacts on juvenile (Green et al. 2004; Gazeau et al. 2007) and larval bivalves (Kurihara et al. 2007; Kurihara et al. 2008; Talmage and Gobler 2009; Talmage and Gobler 2010). The present study revealed similar trends and confirmed that pre-industrial $CO_2$ levels provide maximal performance in larval clams and scallops (Talmage & Gobler 2010) as there were declines in survival,

metamorphosis, diameter, and lipid indices for both *M. mercenaria* and *A. irradians* larvae at $CO_2$ concentrations above ~250 ppm (Fig. 1 and 2). Exposure of shellfish larvae to higher temperatures can make them more vulnerable to other stressors such as pollutants (Cherkasov et al. 2010) and consistent with this, the simultaneous increase in temperature and $CO_2$ depressed survival, metamorphosis, growth and lipid content of larvae beyond the effect of either individual treatment (Fig. 1 and 2). This was most dramatically represented by *A. irradians* larvae that displayed 10% mortality under 24ºC and 250 ppm $CO_2$ compared to >70% morality of individuals exposed to 28ºC and ~750 ppm $CO_2$ (Fig 2). *A. irradians* populations are known to display sharp decreases in populations that have been previously attributed to disease (McGladdery et al. 2006), overfishing (Orensanz et al. 2006), and/or harmful algae (Gobler et al. 2005). Our results demonstrate that interannual variability in temperature and $CO_2$ may also promote such cycles. Within an ecosystem setting the net effects of higher temperature and $CO_2$ on bivalve larval survival may be more profound than measured during our experiments since larvae with extended metamorphosis times, that are smaller, and/or that accumulate fewer lipids are more likely to perish once settled (Wikfors et al. 1992; Phillips 2002; Wacker and von Elert 2002; Cragg 2006). In the past decade, temperate coastal waters have experienced extended periods of hot temperatures (three weeks > 28ºC in NY in 2010; C. Flagg, Stony Brook University, unpublished data) and $CO_2$ levels exceeding 1,000 ppm (Feely et al. 2008; Talmage and Gobler 2009) suggesting the negative effects of climate change on marine bivalves may be already occurring (Talmage and Gobler 2010).

Adult populations of the three bivalve species examined in this study exist over a range of temperature regimes. *C. virginica* adults are tolerant of temperatures from –2 to 36º C and the geographical distribution of this species extends from the Gulf of St. Lawrence to the Gulf of Mexico (Carlton and Mann 1996) with growth being most rapid in the warmer waters found at its southern extent (Shumway 1996). *M. mercenaria* distribution extends from the Gulf of St. Lawrence south to the Florida Keys, and this species can survive from 0 to 30ºC (Harte 2001). Water temperatures between 20 and 24ºC, however, have proven to provide maximal growth rates for *M. mercenaria* (Pratt and Campbell 1956; Ansell 1968) with levels above 24ºC yielding reduced pumping rates (Grizzle et al. 2001) and depressed growth rates of juvenile, estuarine populations (Weiss et al. 2007). *A. irradians* and populations of *A. irradians* subspecies can be found from Cape Cod, Massachusetts into the Gulf of Mexico (Brand 2006) and prolonged exposure of all life stages to 30 ºC can promote mortality in this species (Castagna 1975; Brun et al. 2008). With global warming, shallow water habitats are experiencing extended periods of high temperature that heighten physiological stress for bivalves (Helmuth et al. 2002). During this study, temperatures of 28ºC decreased the growth and survival of juvenile *M. mercenaria* and *A. irradians,* respectively (Fig. 3 and 5), a temperature known to be detrimental to juvenile stages of these species (Grizzle et al. 2001; Brand 2006). In contrast, juvenile *C. virginica* growth was not reduced at 28ºC (Fig. 4), a finding consistent with this species' ability to thrive in warmer waters (Shumway 1996).

Some of the differential susceptibility to high $CO_2$ and temperature among bivalve species seems consistent with their position in the benthos. *M. mercenaria* juveniles and adults are infaunal being commonly burrowed in sediments that can be

undersaturated with respect to carbonate (Green et al. 2004). Consistent with being well-adapted to such exposure, *M. mercenaria* growth was unaffected by the high levels of $CO_2$ administered during our experiment, despite aragonite being slightly undersaturated during the experiment ($\Omega = 0.92$). In contrast, high $CO_2$ significantly depressed growth and survival of juvenile *C. virginica* and *A. irradians*, respectively (Fig. 4 and 5), two epifaunal species that are less likely to encounter carbonate undersaturated environments than infaunal species. Consistent with this hypothesis, decreased calcification rates under elevated $CO_2$ values has been observed for blue mussels (*Mytilus edulis*) and the Pacific oysters (*Crassostrea gigas*; (Berge et al. 2006; Gazeau et al. 2007), two other epifaunal bivalves. With regard to temperature, epifaunal species may be less sensitive to high temperatures since they are commonly exposed to warmer temperatures within shallow estuaries whereas infaunal species avoid high temperatures by burrowing in cooler sediment (Carriker 2001). This could partly account for the significant decline in growth for the normally infaunal, *M. mercenaria* at higher temperatures but an absence of a temperature affect on the epibenthic eastern oysters, *C. virginica*. This trend did not hold for juvenile *A. irradians*, however, which was highly sensitive to prolonged exposure to both high temperature and high $CO_2$, demonstrating that factors beyond life history facilitated adaptations influence the vulnerability of bivalves to climate change. Importantly, a more prolonged exposure to elevated temperatures and/or $CO_2$ or extended adaptation periods may have yielded results that differed from our experiment which was specifically designed to mimic the maximal summer heat experienced by bivalves.

For *M. mercenaria* and *A. irradians*, the responses of the larval and juvenile stages to increased temperature and increased $CO_2$ concentrations may be compared. For

*M. mercenaria* larvae, survival declined by 82% as conditions changed from low temperature and $CO_2$ to 28ºC and 750ppm $CO_2$. In contrast, survival for juvenile *M. mercenaria* was unaffected by 28ºC and even higher levels of $CO_2$ (~1700 ppm). *A. irradians* larval survival declined by 70% as conditions changed from low temperature and $CO_2$ to 28ºC and 750 ppm $CO_2$, while juvenile *A. irradians* displayed a 50% reduction in survival when 24º C, ~400 ppm $CO_2$ treatments were compared to 28º C, and ~1700 ppm $CO_2$ treatments, a level more than two-fold higher than the concentration larvae were exposed to. Therefore, the larval stages of both species were substantially more sensitive to high temperature and $CO_2$ than juvenile stages. The greater sensitivity of bivalve larvae compared to juveniles at higher $CO_2$ may be partly related to the types of $CaCO_3$ each stage synthesizes. The first $CaCO_3$ secreted by bivalve larvae is likely amphorous calcium carbonate, which is 50-fold more susceptible to carbonate dissolution compared to the forms of $CaCO_3$ (aragonite, calcite) produced by juvenile stage bivalves (Weiss et al. 2002).

The sum of environmental stressors that may affect marine organisms in the coming decades, particularly in coastal ecosystems, is substantial. Exactly how increased temperature and $CO_2$ concentrations will combine to affect bivalve populations is still not entirely understood. This study demonstrates the negative consequences of developing in a thermally stressed and acidified environment for larval and juvenile bivalves. These effects may have serious implications for the future of these bivalves and other marine calcifying organisms faced with global climate change.

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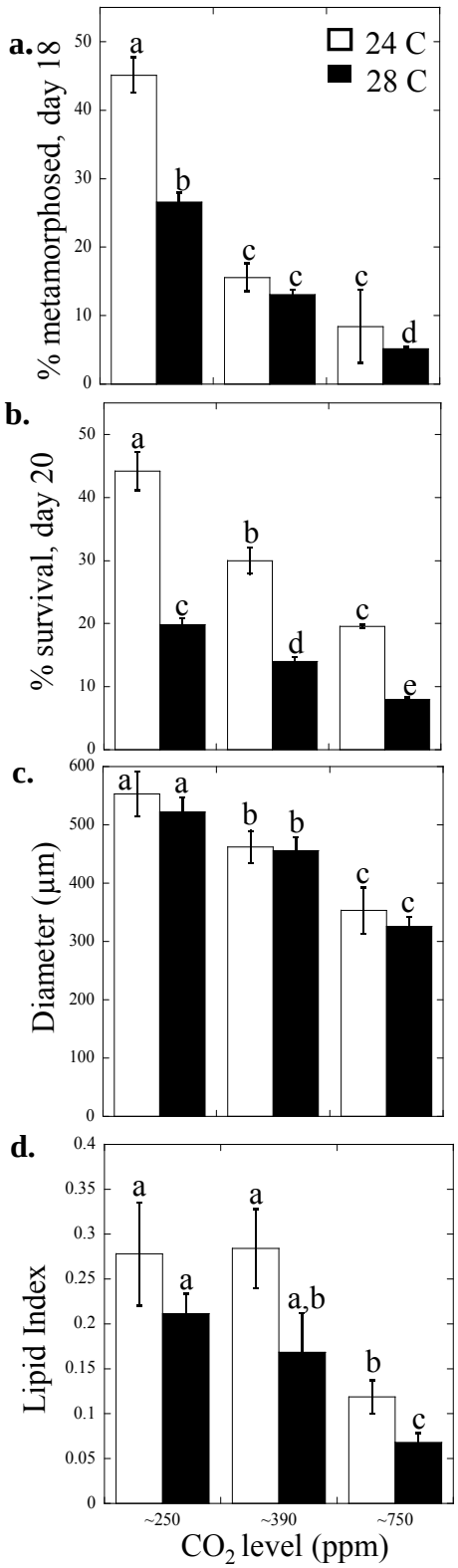


Figure 1.Performance of *Mercenaria mercenaria* larvae grown under three levels of $CO_2$, approximately 250, 390, and 750 ppm, and two temperatures 24º C (white bars) and 28º C (black bars) (Table 1). a. Percent metamorphosed of individuals 18 days post- fertilization, b. Percent larval survival (20 days post-fertilization), c. Diameters of larvae (20 days post-fertilization), and d. Lipid index (lipid area / total area) (20 days post-fertilization). Error bars represent standard deviation of replicated vessels per treatment ($n$ = 4 per treatment), and for Tukey multiple comparisons, $p \leq 0.05$ for all .

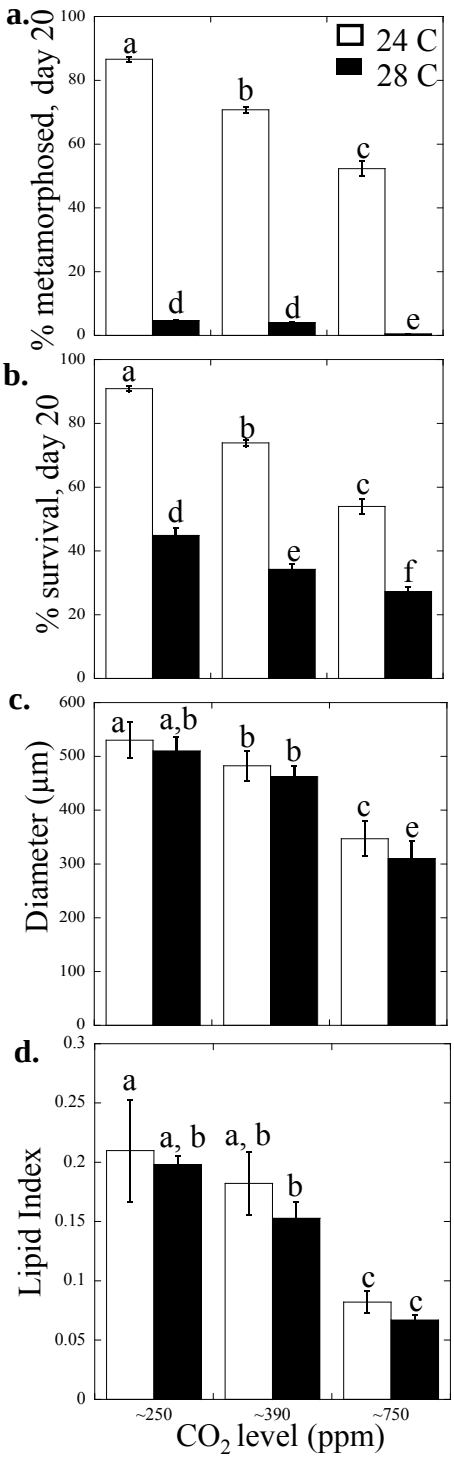


Figure 2. Performance of *Argopecten irradians* larvae grown under three levels of $CO_2$, approximately 250, 390, and 750 ppm, and two temperatures 24º C (white bars) and 28º C (black bars) (Table 1). a. Percent metamorphosed of individuals 18 days post- fertilization, b. Percent larval survival (20 days post-fertilization), c. Diameters of larvae (20 days post-fertilization), and d. Lipid index (lipid area / total area) (20 days post-fertilization). Error bars represent standard deviation of replicated vessels per treatment ($n$ = 4 per treatment), and for Tukey multiple comparisons, $p \leq 0.05$ for all .

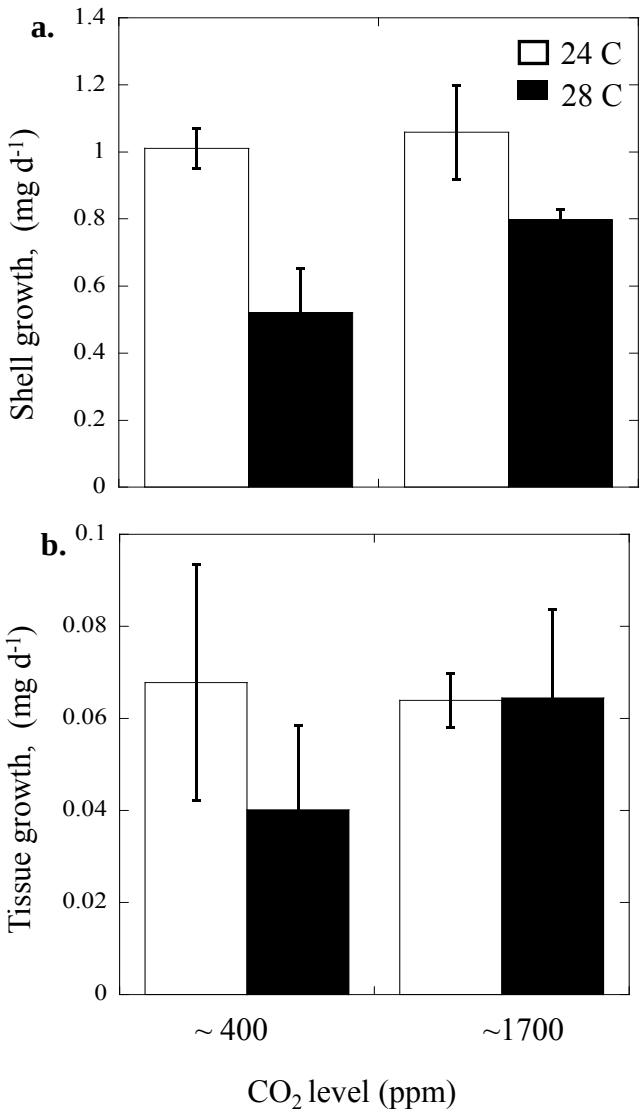


Figure 3. Growth of *Mercenaria mercenaria* juveniles at two levels of $CO_2$, approximately 400 and 1700 ppm, and two temperatures 24°C (white bars) and 28°C (black bars, Table 2), a. Shell growth and b. Tissue growth. Error bars represent standard deviation of replicated vessels per treatment ($n$ = 3 per treatment).

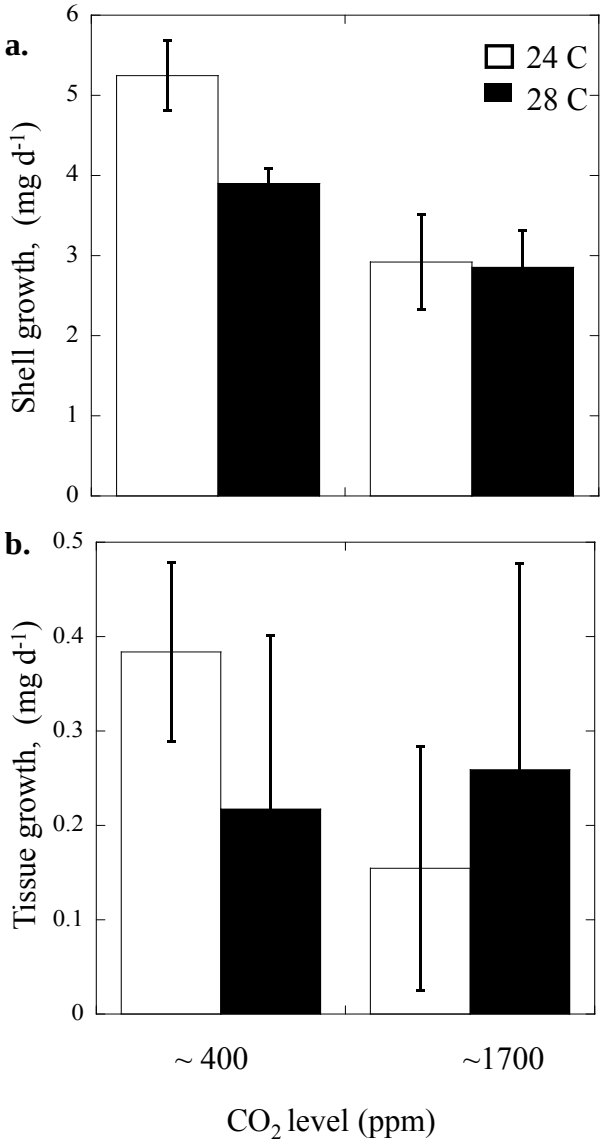


Figure 4. Growth of *Crassostrea virginica* juveniles at two levels of $CO_2$, approximately 400 and 1700 ppm, and two temperatures 24°C (white bars) and 28°C (black bars, Table 2), a. Shell growth and b. Tissue growth. Error bars represent standard deviation of replicated vessels per treatment ($n$ = 3 per treatment).

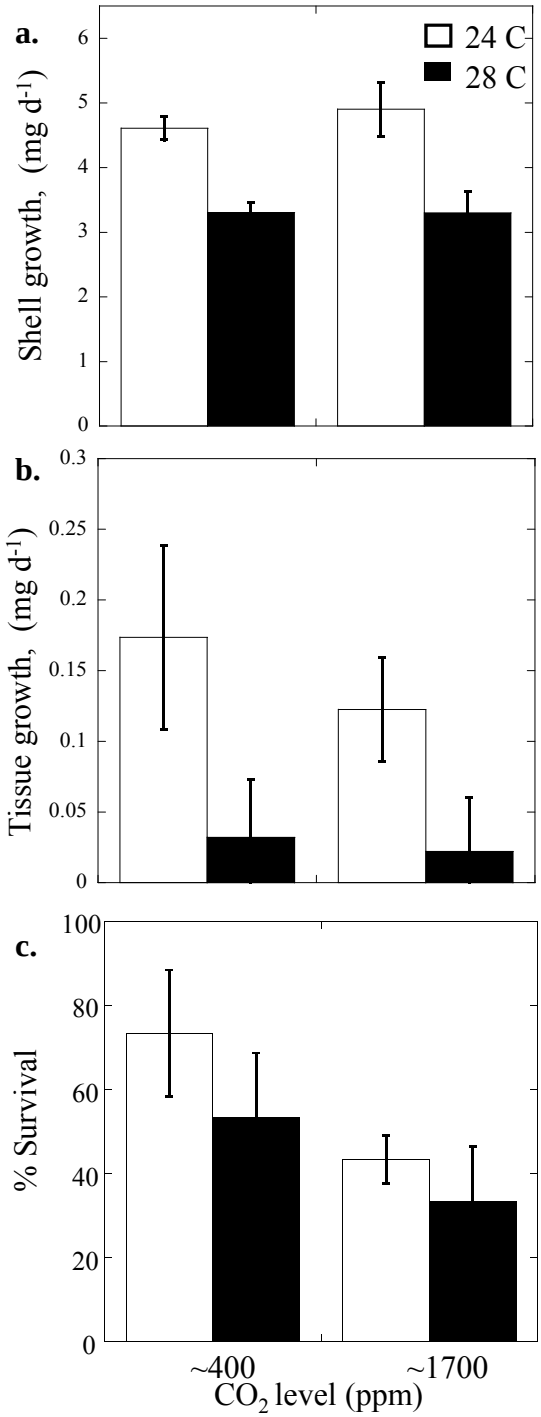


Figure 5. Growth of *Argopecten irradians* juveniles at two levels of $CO_2$, approximately 400 and 1700 ppm, and two temperatures 24°C (white bars) and 28°C (black bars, Table 2), a. Shell growth and b. Tissue growth. Error bars represent standard deviation of replicated vessels per treatment ($n$ = 3 per treatment).

| **Parameter** | **Near pre-industrial $CO_2$** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|---|
| *Mercenaria mercenaria* | | | |
| Temperature (˚C) | 24 ± 0.7 | 24 ± 0.7 | 24 ± 0.7 |
| pH | 8.210 ± 0.032 | 8.081 ± 0.042 | 7.8 ± 0.012 |
| $pCO_2$ (ppm) | 220.4 ± 24.235 | 375.3 ± 36.45 | 771.6 ± 29.113 |
| $\Omega_{calcite}$ | 2.86 ± 0.50 | 2.68 ± 0.51 | 1.51 ± 0.15 |
| $\Omega_{aragonite}$ | 1.84 ± 0.37 | 1.72 ± 0.36 | 0.98 ± 0.13 |
| Total DIC ($\mu$mol $L^{1}$) | 1115.3 ± 95.67 | 1374.1 ± 62.89 | 1439.4 ± 31.38 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 112.7 ± 21.24 | 105.5 ± 26.23 | 59.7 ± 9.806 |
| Alkalinity (TA) | 1296.8 ± 121.3 | 1527.3 ± 86.56 | 1509.3 ± 27.69 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | |
| *Argopecten irradians* | | | |
| Temperature (˚C) | 24 ± 0.7 | 24 ± 0.7 | 24 ± 0.7 |
| pH | 8.200 ± 0.026 | 8.080 ± 0.059 | 7.810 ± 0.016 |
| $pCO_2$ (ppm) | 238.4 ± 25.012 | 373.9 ± 41.540 | 756.2 ± 19.986 |
| $\Omega_{calcite}$ | 2.95 ± 0.16 | 2.66 ± 0.57 | 1.55 ± 0.12 |
| $\Omega_{aragonite}$ | 1.9 ± 0.42 | 1.72 ± 0.45 | 1.00 ± 0.24 |
| Total DIC (umol $L^{-1}$) | 1176 ± 56.27 | 1368.7 ± 36.99 | 1517 ± 35.45 |
| $CO_3^{2-}$ ($\mu$mol $L^{1}$) | 133.7 ± 22.32 | 105.1 ± 28.52 | 61.3 ± 12.321 |
| Alkalinity (TA) | 1359.6 ± 35.98 | 1521.4± 55.06 | 1517.1± 46.66 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | |
| *Mercenaria mercenaria* | | | |
| Temperature (˚C) | 28 ± 0.7 | 28 ± 0.7 | 28 ± 0.7 |
| pH | 8.200 ± 0.040 | 8.090 ± 0.046 | 7.8 ± 0.012 |
| $pCO_2$ (ppm) | 247.4 ± 16.241 | 379.0 ± 43.12 | 794.6 ± 29.113 |
| $\Omega_{calcite}$ | 3.43 ± 0.53 | 3.17 ± 0.56 | 1.75 ± 0.15 |
| $\Omega_{aragonite}$ | 2.24 ± 0.99 | 2.07 ± 0.45 | 1.14 ± 0.13 |
| Total DIC ($\mu$mol $L^{1}$) | 1196 ± 76.24 | 1389.2 ± 53.45 | 1439.6 ± 31.38 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 133.7 ± 20.34 | 123.4 ± 36.42 | 68.1 ± 9.806 |
| Alkalinity (TA) | 1404.3 ± 123.61 | 1568.2 ± 66.49 | 1522.8 ± 27.69 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | |
| *Argopecten irradians* | | | |
| Temperature (˚C) | 28 ± 0.7 | 28 ± 0.7 | 28 ± 0.7 |
| pH | 8.210 ± 0.029 | 8.08 ± 0.054 | 7.810 ± 0.026 |
| $pCO_2$ (ppm) | 239.8 ± 13.078 | 386.7 ± 44.23 | 772.7 ± 29.951 |
| $\Omega_{calcite}$ | 3.48 ± 0.17 | 3.09 ± 0.57 | 1.78 ± 0.16 |
| $\Omega_{aragonite}$ | 2.27 ± 0.78 | 2.01 ± 0.42 | 1.16 ± 0.14 |
| Total DIC (umol $L^{-1}$) | 1189.1 ± 53.57 | 1557.1 ± 32.88 | 1433.6 ± 30.21 |
| $CO_3^{2-}$ ($\mu$mol $L^{1}$) | 135.7 ± 43.2 | 120.3 ± 28.46 | 69.3 ± 12.321 |
| Alkalinity (TA) | 1400.8 ± 65.25 | 1557.1± 70.21 | 1519.4± 36.45 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |

Table. 1 Mean temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the three-level carbon dioxide and two-level temperature experiments with *Mercenaria mercenaria,* and *Argopecten irradians* larvae.

| **Parameter** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|
| *Mercenaria mercenaria, Crassostrea virginica,* and *Argopecten irradians* juveniles | | |
| Temperature (˚C) | 24 ± 0.65 | 24 ± 0.65 |
| pH | 8.091 ± 0.001 | 7.620 ± 0.060 |
| $pCO_2$ (ppm) | 400 ± 12.34 | 1665 ± 25.60 |
| $\Omega_{calcite}$ | 2.99 ± 0.10 | 1.42 ± 0.18 |
| $\Omega_{aragonite}$ | 1.93 ± 0.07 | 0.92 ± 0.12 |
| Total DIC ($\mu$mol $L^{1}$) | 1502 ± 48.47 | 2023 ± 29.01 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 117.9 ± 3.95 | 56.1 ± 7.16 |
| Alkalinity (TA) | 1667.5 ± 52.15 | 2052.2 ± 19.39 |
| Salinity | 28.0 ± 3.0 | 28.0 ± 3.0 |
| | | |
| Temperature (˚C) | 28 ± 0.65 | 28 ± 0.65 |
| pH | 8.092 ± 0.002 | 7.617 ± 0.047 |
| $pCO_2$ (ppm) | 399.5 ± 1.68 | 1737 ± 18.71 |
| $\Omega_{calcite}$ | 3.38 ± 0.04 | 1.64 ± 0.19 |
| $\Omega_{aragonite}$ | 2.20 ± 0.03 | 1.07 ± 0.12 |
| Total DIC ($\mu$mol $L^{1}$) | 1473 ± 11.61 | 2039 ± 4.10 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 131.5 ± 1.64 | 64.0 ± 7.16 |
| Alkalinity (TA) | 1659.4 ± 13.52 | 2080.8 ± 17.67 |
| Salinity | 28.0 ± 3.0 | 28.0 ± 3.0 |
| | | |
| | | |

Table 2. Mean temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the two-level carbon dioxide and two-level temperature experiments with *Mercenaria mercenaria, Crassostrea virginica,* and *Argopecten irradians* juveniles.

# CHAPTER SIX:

## Effects of carbon dioxide and a harmful alga (*Aureococcus anophagefferens*) on the growth and survival of larval Eastern oysters (*Crassostrea virginica*) and bay scallops (*Argopecten irradians*)

**Abstract**

Globally, the frequency of harmful algal blooms is increasing and $CO_2$ concentrations are rising. These factors represent serious challenges to a multitude of estuarine organisms, particularly those that cannot escape these environmental changes. More frequent harmful algal blooms and rising $CO_2$ concentrations can also hamper efforts to restore depleted stocks of filter feeding bivalves. For this study, we compared physiological responses of larval bivalves (*Crassostrea virginica* and *Argopecten irradians*) to the brown tide alga, *Aureococcus anophagefferens* (250 x $10^6$ cells $L^{-1}$, and 1 x $10^9$ cells $L^{-1}$) and a gradient of $CO_2$ concentrations (~240, 390, and 850 ppm). Results indicated that the presence of *A. anophagefferens* and higher levels of $CO_2$ significantly depressed survival, development, growth, and lipid synthesis of *A. irradians* larvae with the combination of both factors having the largest effects. *C. virginica* larvae were also negatively impacted by the harmful alga and elevated $CO_2$, but displayed higher survival when exposed to these combined stressors. For both species, high densities of *A. anophagefferens* ($10^9$ cells $L^{-1}$) elicited a stronger negative effect on larval survival than high levels of $CO_2$ concentrations (850 ppm). Veliger stage clearance rates for both species were elevated when exposed to *A. anophagefferens* compared to a control diet suggesting that later stage mortality was promoted by rapid consumption of the toxic alga in the first days of development. Collectively, these results demonstrate that the concurrent occurrence of harmful algal blooms and high $CO_2$ concentrations have negative implications for bivalve populations and further demonstrate that some species of larval bivalves are more resistant to these stressors than others.

## Introduction

Bivalves are keystone organisms within estuarine ecosystems. The ecosystem services provided by bivalve filtration in marine habitats include control of natural and cultural eutrophication as well as phytoplankton growth caused by increased nutrient loads (Officer et al. 1982; Cerco and Noel 2007) as well as reducing turbidity (Newell and Koch 2004). With increased water clarity caused by bivalve filtration, growth of submerged aquatic vegetation may be facilitated (Carroll et al. 2008; Wall et al. 2008). Beyond filtration, many bivalves also provide physical habitat structure for smaller marine organisms (Jackson 2001). For all of these reasons, estuarine bivalves are often considered ecosystem engineers (Newell 2004). Bivalve populations throughout the world have declined significantly during the past century due loss of habitat, hypoxia, eutrophication, and overfishing (Jackson et al. 2001; Lotze et al. 2006; Beck et al. 2011). Two additional pressures on bivalve populations that have received relatively lesser attention but have progressively intensified in recent decades are harmful algal blooms (HABs) and coastal ocean acidification.

Harmful algal blooms have become increasingly common in recent decades and have had negative ecological and economical consequences on many marine ecosystems (Hoagland et al. 2002; Heisler et al. 2008; Jin et al. 2008). HABs can have negative consequences for bivalve populations (Shumway 1990; Bricelj and Shumway 1998; Landsberg 2002) and can be particularly detrimental to bivalve larvae. Successful larval stages of bivalves are instrumental in the survival and success of the adult cohorts as small decreases in larval survival can have large, bottleneck impacts on future populations (Caley et al. 1996; Gosselin and Qian 1997; Schneider et al. 2003; Arnold

2008). Several HAB-forming dinoflagellates have been shown to cause mortality in multiple species of bivalve larvae including Eastern oysters (*Crassostrea virginica*; (Springer et al. 2002; Leverone et al. 2006; Stoecker et al. 2008; Tang and Gobler 2009), Northern quahogs (*Mercenaria mercenaria*; (Leverone et al. 2006; Tang and Gobler 2009), bay scallops (*Argopecten irradians irradians*, *Argopecten irradians concentricus*; (Springer et al. 2002; Yan et al. 2003; Leverone et al. 2006; Tang and Gobler 2009) and Japanese scallops (*Chlamys farreri*; (Yan et al. 2001). In addition, the brown tide-forming pelagophyte *Aureococcus anophagefferens* has been shown to reduce survival, growth, and the lipid content of Northern quahog larvae (Padilla et al. 2006; Bricelj and MacQuarrie 2007) and can slow the growth of bay scallop larvae, *Argopecten irradians* (Gallager et al. 1989).

Elevated concentrations of carbon dioxide ($CO_2$) are also a significant threat to coastal bivalve populations. $CO_2$ levels have risen by 40% since the Industrial Revolution (Caldeira and Wickett 2003) and the rate of increase has tripled since the mid-20$^{th}$ century (Fussel 2009; Friedlingstein et al. 2010). These increases may be even larger in coastal zones (Miller et al 2009) which receive additional $CO_2$ inputs from upwelling (Feely et al. 2008), freshwater (Salisbury et al. 2008), as well as anthropogenic and terrestrial carbon sources (Gattuso et al. 1998; Paerl et al. 1998; Thomas et al. 2004; Koch and Gobler 2009). High levels of $CO_2$ depress levels of both pH and carbonate ion concentration and thus cause 'ocean acidification' (Cao et al. 2007), a process which negatively affects marine organisms with calcified parts (Doney et al. 2009). Marine bivalve larvae are particularly vulnerable to high $CO_2$ levels, displaying reduced rates of calcification, growth, and survival under levels of $CO_2$ found today (Talmage and Gobler

2010) or projected for later this century (Gazeau et al. 2007; Kurihara et al. 2007; Miller et al. 2009; Talmage and Gobler 2009). No study to date has investigated the concurrent effects of harmful algae and $CO_2$ levels on bivalve larvae development and survival.

The goal of this research was to investigate the combined effects of a HAB, *Aureococcus anophagefferens* (brown tide), and past (~240 ppm), present (~390 ppm), and future (~850 ppm), $CO_2$ levels on larvae of *Argopecten irradians* (bay scallop) and *Crassostrea virginica* (Eastern oyster), a species whose vulnerability to *A. anophagefferens* has never been investigated. Larvae were exposed to bloom and moderate levels of *A. anophagefferens* or a control algae and the survival and development of larvae through metamorphosis was monitored along with larval size, lipid content, and clearance rates. Results provided important information regarding the vulnerability of these organisms to these stressors as well as insight regarding prospects for future success of these populations.

**Methods**

This study examined the effects of multiple $CO_2$ and *A. anophagefferens* (brown tide) levels on the larval stages of two bivalves, *Argopecten irradians* (bay scallop) and *Crassostrea virginica* (Eastern oyster). For all experiments, experimental vessels with bivalves (described below) were maintained in water baths set maintained at 24ºC using commercially available aquarium heaters (Aquatic Eco-systems, Inc., Florida, USA). Temperatures were recorded every 6 minutes throughout experiments using in situ data loggers (Onset©); that demonstrated temperatures varied within 2.5% of target values. The experimental temperature (24ºC) is optimal for growth and survival of larvae of these

two species (Kraeuter and Castagna 2001; Cragg 2006). A gas proportionator system (Cole Parmer® Flowmeter system, multitube frame) was used to deliver $CO_2$ gas to seawater within treatment vessels at multiple rates. The gas proportionator mixed appropriate flow rates of 5% $CO_2$ gas, low $CO_2$ gas, and pressurized air (~ 390 ppm $CO_2$) to yield the concentrations of carbon dioxide desired for experiments at a flow rate that turned over experimental vessels >100 times daily so that the treatments never had the opportunity to equilibrate with the atmosphere. We have found that experiments performed with gases mixed via a proportionator as described here generate seawater chemistry and larval responses nearly identical to those obtained from tanked gases premixed at specific $CO_2$ levels (Talmage and Gobler 2010). For experiments, the $CO_2$ gas mixtures from the proportionator system were continuously delivered to the bottom of replicated ($n$=4) experimental vessels (detailed below). With continuous bubbling, all treatment vessels remained saturated with respect to oxygen (~8 mg $L^{-1}$). To quantify precise $CO_2$ levels attained in experimental treatments, aliquots were removed and analyzed during experiments using an EGM-4 Environmental Gas Analyzer® (PP Systems) system that quantified total dissolved inorganic carbon levels after separating the gas phase from seawater using a Liqui-Cel® Membrane (Membrana). This instrument provided a methodological precision of ± 3.6% for replicated measurements of total dissolved inorganic carbon and provided full recovery (102 ± 3%) of Dr. Andrew Dickson's (University of California San Diego, Scripps Institution of Oceanography) certified reference material for total inorganic carbon in seawater (Batch 102 = 2013 µmol DIC kg seawater$^{-1}$). Levels of $CO_2$ were calculated based on measured levels of total inorganic carbon, pH (mol kg seawater$^{-1}$; NBS), temperature, salinity, and first and

second dissociation constants of carbonic acid in seawater according to Roy et al. (1993) using the program CO2SYS (http://cdiac.ornl.gov/ftp/co2sys/). Daily measurements of pH with a high sensitivity microprocessor (Thermo Scientific Orion Star Series™ Benchtop pH meter; ± 0.001; calibrated prior to each use with NIST traceable standards) indicated experimental vessels maintained a constant pH level throughout experiments (<0.5% RSD within treatments). Random spectrophotometric measurements of pH made using *m*-cresol purple as described by Dickson *et al.* (2007) and corrected for scale (Dickson 1993) were never significantly different from those values obtained with the high sensitivity microprocessor.

Larvae were grown at either two (for *C. virginica*) or three (for *A. irradians*) levels of $CO_2$: a high level (~850 ppm $CO_2$), in the range predicted for the year 2100, a modern level (~390 ppm $CO_2$), and a near pre-industrial level (~240 ppm $CO_2$) which provides maximal survival and growth rates for *A. irradians* (Talmage and Gobler 2010). Precise $CO_2$ levels and complete carbonate chemistry from experiments appear in Table 1 and 2. One-liter high-density polyethylene beakers were filled with 0.2 $\mu$m filtered seawater from eastern Shinnecock Bay, New York, United States. Larvae from locally collected broodstock were obtained from Cornell Cooperative Extension, Southold, NY, and the East Hampton Shellfish Hatchery, East Hampton, NY, within hours of fertilization and were distributed to each treatment beaker at a concentration of ~ 400 $L^{-1}$, consistent with post-spawning densities in estuaries (Mackenzie 1996; Cragg 2006) . Every three days, larvae from each beaker ($n$ =4, per treatment) were gently poured onto a 64 $\mu$m mesh and immediately transferred to a petri dish with filtered seawater. The condition (live or dead) and developmental stage (veligers, pediveligers, and

metamorphosed) of every individual larva were determined under a dissecting microscope. Individuals were transferred into a new beaker with new filtered seawater, food, and antibiotics (see later details) within a 15 minute period. Experiments were terminated after all surviving larvae in all treatments had metamorphosed or when there was ~15 larvae per treatment remaining for post-experiment analyses.

Brown tide blooms caused by *Aureococcus anophagefferens* typically occur during the months of May, June, and July in Long Island waters (Gobler et al. 2005), a period co-incident with the spawning period of Eastern oysters and bay scallops (Thompson et al. 1996; Cragg 2006). To simulate post-spawning conditions, larvae of both bivalve larval species were fed two concentrations of brown tide (*A. anophagefferens* clone CCMP1850): 1) 2.5 x $10^8$ cells $L^{-1}$, representing a low density bloom and, 2) 1.0 x $10^9$ cells $L^{-1}$, representing a high density bloom (Gobler et al. 2005). *A. anophagefferens* cultures were grown in GSe medium (Doblin et al. 1999) made with 0.2 µm filtered seawater collected from Shinnecock Inlet, Southampton, NY, during flood tide (salinity ~30) and maintained in an incubator at 21 ºC on a 14:10 light:dark cycle (Gobler et al. 1997). The strain of brown tide used for all experiments was Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP)1850, which was isolated from Great South Bay, NY, in 1998. This strain has been shown to be highly inhibitory to the growth of copepod nauplii (Smith et al. 2008) and adult bivalves (Harke et al. *In press*). As control treatments, larvae were fed a food source *Isochrysis galbana* (Tahitian strain, T-Iso) at a density known to maximize bivalve larval growth and survivorship through metamorphosis (Castell and Mann 1994; Cragg 2006; Talmage and Gobler 2009). Cultures of *I. galbana* were grown as described for

*A. anophagefferens* and both cultures were maintained in exponential phase growth via daily supplementation with media. Larvae were fed equivalent algal biovolumes (one, *I. galbana* cell = ~eight, *A. anophagefferens* cells) for all experiments. Since the lower food concentration was slightly less than optimal (Talmage and Gobler 2009), it was added to experimental vessels daily whereas the higher density was added every third day during water changes. To promote high survivorship, all containers in contact with larvae were new and never exposed to chemicals or detergents (Talmage and Gobler 2009). To discourage the growth of bacteria during experiments, an antibiotic solution (Sigma-Aldrich No.4083, 5000 units of Penicillin, 5 mg of Streptomycin, and 10 mg of Neomycin per milliliter of solution) was added to each beaker at 1% its original concentration at the beginning of each experiment and at the time of each water change (approximately 2 times weekly). This antibiotic mixture at this concentration has been shown to have no negative effects on the growth and survivorship of shellfish larvae (Talmage and Gobler 2009). Experiments presented here were repeated without antibiotic treatments and yielded no difference in bivalve larval survival suggesting that neither the antibiotics nor the bacteria in seawater altered the results presented here.

To meet the assumption of normality and homogeneity, survival and percent metamorphosed data were arc-sin square root transformed after which two-way ANOVAs were performed where phytoplankton species and $CO_2$ were the main effects. Sizes of larvae, lipid indices, and clearance rates were also statistically examined via two-way ANOVAs. Post-hoc Tukey multiple comparison tests were performed to examine the differences among survival, metamorphosis, and size at each phytoplankton species and $CO_2$ level. The use of different batches of larvae from different broodstock for each

experiment prohibited statistical comparisons across experiments (different concentrations of *A. anophagefferens* or the two species of larvae). Statistical analyses were performed with SYSTAT 13© Systat Software, Inc. Representation of the degrees of freedom for all ANOVAs in this chapter are presented as: *df*= x, y; where x represents the degrees of freedom for the factor being described, and y represents the degrees of freedom for error in the ANOVA analysis of that factor.

To estimate the relative lipid content of larvae, Nile Red dye was used to stain neutral lipids (Castell and Mann 1994; Phillips 2002). A Nile Red stock solution was made of 1.25 mg of Nile Red crystals in 100 ml of acetone. Randomly selected larvae (*n* = 15) from each treatment were stained with a 1:9 dilution of the stock solution and 0.2 µm filtered seawater. Larvae were exposed to the stain for ~1.5 hours, during which larval motion ceased, permitting the orientation of individuals for microscopic imaging using an FITC filter on an epifluorescent microscope. Larvae were then rinsed with filtered seawater, and digitally photographed with a Roper Scientific Photometrics CoolSNAP ES camera attached to the top of an epiflorescent microscope. Digital images of each larvae were analyzed for the area of lipid accumulation and the diameter and the area of individuals using Image J® software (Przeslawski et al. 2008). A lipid index was estimated by dividing the area of the larvae containing the fluorescing lipids by the total larval area thereby allowing for direct comparisons among treatments. Two-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among larval lipid indexes, as well as shell length at each phytoplankton and $CO_2$ level.

To estimate feeding rates of larvae, samples were removed before and after two days of feeding for all larval species when larvae were approximately two days old. A 4.5 mL sample of experimental water from treatment vessels ($n$=3) was placed in individual glass culture tubes, preserved in Lugol's iodine solution (Throndsen 1978), and stored in the dark until quantification. Samples were removed before and after two days of feeding for all larval species when larvae were approximately 2 days old. Samples of both *A. anophagefferens* and *I. galbana* were quantified using a Beckman Coulter Multisizer™ 3 Coulter Counter® with a 70µm aperture which allowed for distinct peaks in cell densities to be resolved for both species (Bricelj et al. 2001). Size ranges resolved were 1.4 - 2.4 µm for *A. anophagefferens* and 2.8-5.8 µm for *I. galbana*. Selected samples of both species were quantified microscopically with a hemacytometer and yielded clearance rates statistically identical to those obtained with the Coulter Counter. Cell densities within experimental vessels were determined before and after a known amount of time were used to quantify clearance rates for each individual larvae under the assumption that the initial cell concentrations in the experimental container were less than the critical maximum concentration (Marin et al. 1986) using the following formulas:

(1) $g = \ln (C_{(0)} / C_{(t)}) (1/t) + k$

(2) $F = Vg / N$

Grazing coefficients (g) were estimated using equation (1) where $C_{(0)}$ = initial cell concentration in the experimental container (cells/ml), $C_{(t)}$ = cell concentrations at time $t$ in the experimental container (cells/ml), $t$ = duration of experiment (minutes), and $k$ = cell growth coefficient. Clearance rates ($F$) by larvae in each treatment on each algal

food source were calculated using equation (2) where $V$ = volume of experimental container (µl), $g$= grazing coefficient, and $N$ = number of herbivores (larvae) in the experimental container (Marin et al. 1986). Two-way ANOVAs and post-hoc Tukey multiple comparison tests were performed to examine the differences among clearance rates at each phytoplankton and $CO_2$ level.

**Results**

***Crassostrea virginica*** larvae exposed to low levels of ***Aureococcus anophagefferens***

For *Crassostrea virginica* larvae exposed to the lower biomass level of *Aureococcus anophagefferens*, $CO_2$ concentrations, $F(1)$, and food source, $F(2)$, both significantly affected metamorphosis ($F(1) = 6.570$, $df= 2, 18$, $F(2)= 63.189$, $df =1, 18$, $p<0.05$; two-way ANOVA), survival ($F(1) = 8.143$, $df= 2, 18$, $F(2)= 5.243$, $df =1, 18$, $p<0.05$; two-way ANOVA), and size ($F(1) = 96.5289$, $df= 2, 18$, $F(2)= 12.204$, $df =1, 18$, $p<0.01$; two-way ANOVA). There was no interaction, $F(3)$, between $CO_2$ concentrations and food type for metamorphosis ($F(3)= 3.416$, $df =2, 18$, $p=0.089$; two-way ANOVA), survival ($F(3)= 0.899$, $df =2, 18$, $p=0.362$; two-way ANOVA), or size ($F(3)= 0.303$, $df =2, 18$, $p=0.592$; two-way ANOVA). The percent of larvae that had metamorphosd and survived, as well as overall larval size was greatest for *C. virginica* individuals grown under ambient $CO_2$ concentrations (390ppm) and with the control food source (*I. galbana*) (Figs. 1 and 5a). At 20 days post-fertilization, to 21 ± 4.9 and 14 ± 5.4% of larvae had metamorphosed at 390 and 850 ppm $CO_2$ when fed *I. galbana*, compared to only 3.7 ± 0.31 and 2.4 ± 0.32% metamorphosed when exposed to 390 and 850 ppm $CO_2$ and fed *A. anophagefferens* (Fig. 1). *C. virginica* larval survival at day 20 was 26 ± 7.8

and 17 ± 5.6% at 390 and 850 ppm $CO_2$ while consuming *I. galbana* compared to 18 ± 0.32 and 14 ± 0.82% at 390 and 850 ppm $CO_2$ respectively when fed *A. anophagefferens* (Fig. 1). Mean diameters for *C. virginica* larvae fed *I. galbana* were 363.9 ± 33.7 and 252.9 ± 6.7 µm at 390 and 850 ppm $CO_2$ respectively but decreased to 328.7 ± 31.3 and 204.5 ± 11.5 µm at 390 and 850 ppm $CO_2$ on a diet of *A. anophagefferens* (Fig. 5a).

Lipid indices for *C. virginica* larvae were significantly different between $CO_2$ concentrations, $F(1)$, and food sources, $F(2)$, ($F(1) = 142.873$, $df = 2, 18$ $F(2) = 107.486$, $df = 1, 18$, $p<0.001$; two-way ANOVA), and there was a significant interaction, $F(3)$, between $CO_2$ concentrations and food source suggesting there was an antagonistic effect of increased $CO_2$ and *A. anophagefferens* among the treatments (Fig. 5b; $F(3) = 37.574$, $df = 2, 18$, $p<0.001$; two-way ANOVA). Lipid indices for *C. virginica* larvae decreased from 0.24 ± 0.03 and 0.08 ± 0.001 at 390 and 850 ppm $CO_2$ when fed *I. galbana* to 0.09 ± 0.03 and 0.04 ± 0.004 at 390 and 850 ppm $CO_2$ when fed *A. anophagefferens* (Fig. 5b). Although $CO_2$ did not have a significant impact on clearance rates of *C. virginica* larvae ($F(1) = 0.694$, $df = 2, 18$, $p = 0.429$; two-way ANOVA), food source did ($F(2) = 907.230$, $df = 1, 18$, $p<0.001$; two-way ANOVA, Fig. 5c). Larvae fed *A. anophagefferens* had significantly higher clearance rates (1.55 ± 0.03 µl $min^{-1}$ $larvae^{-1}$) at both $CO_2$ concentrations of ~390 and ~850 ppm respectively when compared to larvae being fed the bioequivalent of *I. galbana* (0.39 ± 0.09 µl $min^{-1}$ $larvae^{-1}$) (Fig. 5c).

***Crassostrea virginica*** larvae exposed to high levels of ***Aureococcus anophagefferens***

With an increase to high concentrations of *A. anophagefferens* ($10^9$ cells $L^{-1}$), *C. virginica* larval performance worsened compared to the lower density of

*A. anophagefferens*. After 20 days of exposure, $CO_2$ concentrations, $F(1)$, and food source, $F(2)$, both had a significant effect on larval metamorphosis ($F(1) = 9.086$, $F(2)= 142.415$, $F(3)= 8.876$, $df =15$, $p<0.05$; two-way ANOVA), survival ($F(1) = 10.254$, $df= 2, 18$, $F(2)= 113.978$, $df= 1, 18$, $F(3)= 9.918$, $df =2, 18$, $p<0.01$; two-way ANOVA), and lipid content ($F(1) = 103.727$, $df= 2, 18$, $F(2)= 55.425$, $df= 1, 18$, $F(3)= 35.653$, $df =2, 18$, $p<0.001$; two-way ANOVA). There was also a significant interaction, $F(3)$, between treatments for metamorphosis ($F(3) = 8.876$, $df =2, 18$, $p<0.05$; two-way ANOVA), survival ($F(3) = 9.918$, $df =2, 18$, $p<0.01$; two-way ANOVA, and lipids ($F(3) = 35.653$, $df =2, 18$, $p<0.001$; two-way ANOVA). While 24 ± 3.8 and 15 ± 5.2% of larvae had metamorphosed at day 20 at 390 and 850 ppm $CO_2$ when fed *I. galbana* treatments, almost none of the individuals fed *A. anophagefferens* metamorphosed (<0.2%; Fig. 2). At day 20, survival of *C. virginica* larvae at 390 ppm fed *I. galbana* and *A. anophagefferens* was 30 ± 5.6 and 4.6 ± 0.6 % respectively whereas at 850 ppm, survival to 19 ± 4.8 and 4.3 ± 0.5 %, respectively (Fig. 2). At $CO_2$ concentrations of 390 and 750 ppm with *I. galbana* as a food source, *C. virginica* lipid indices were 0.25 ± 0.03 and 0.07 ± 0.002 whereas individuals fed *A. anophagefferens* and exposed to the same $CO_2$ concentrations (390 and 750 ppm) has lipid indices of 0.1 ± 0.03 and 0.06 ± 0.02, respectively (Fig. 6b). $CO_2$ concentrations, $F(1)$, and *A. anophagefferens* as a food source, $F(2)$ also significantly affected larval diameters ($F(1)= 53.206$, $df= 2, 18$, $F(2)= 51.215$, $df =1, 18$, $p<0.001$; two-way ANOVA), although there was not a significant interaction between these treatments. Diameters of *C. virginica* larvae fed *I. galbana* were 374.7 ± 46.8 and 259.5 ± 22.2 µm at 390 and 850 ppm $CO_2$, respectively, but decreased to 261.6 ± 25.7 and 163.6 ± 8.3 µm at 390 and 850 ppm $CO_2$ when fed *A.*

*anophagefferens* (Fig. 6a). There was no significant effect of $CO_2$ concentrations on *C. virginica* larvae clearance rates, but there was a significant effect of food source ($F(2)$= 788.673, $df$ =1, 18, $p<0.001$; two-way ANOVA) and an interactive effect between the treatments ($F(3)$ = 8.167, $df$ =2, 18, $p<0.05$; two-way ANOVA). Clearance rates for *C. virginica* larvae fed *I. galbana* at ~390 and ~850 ppm $CO_2$ were 1.06 ± 0.02 and 0.83 ± 0.08 µl $min^{-1}$ $larvae^{-1}$, respectively, but increased to 2.34 ± 0.10 and 2.40 ± 0.12 µl $min^{-1}$ $larvae^{-1}$, respectively, when fed the high density of *A. anophagefferens* (Fig.6c).

***Argopecten irradians*** larvae exposed to low levels of ***Aureococcus anophagefferens***

Compared to *C. virginica*, *A. irradians* larvae were more sensitive to both elevated $CO_2$ and *A. anophagefferens*. Both $CO_2$ concentrations, $F(1)$, and food source, $F(2)$, yielded significant responses and had a significant interactive effect, $F(3)$, on metamorphosis ($F(1)$ = 4,076.740, $df$=2, 18, $F(2)$= 170,133,378, $df$= 1, 18, $F(3)$= 3,001.852, $df$ =2, 18, $p<0.001$; two-way ANOVA), survival ($F(1)$ = 34.816, $df$= 2, 18, $F(2)$=528.348, $df$=1, 18, $F(3)$=16.495, $df$ =2, 18, $p<0.001$; two-way ANOVA), diameter ($F(1)$ = 2118.9, $df$=2, 18, $F(2)$=292.649, $df$= 1, 18, $F(3)$=44.657, $df$ =2, 18, $p<0.001$; two-way ANOVA), and lipid content ($F(1)$ = 75.791, $df$= 2, 18, $F(2)$=75.818, $df$= 1, 18, $F(3)$=14.624, $df$ =2, 18, $p<0.001$; two-way ANOVA). While 84.2 ± 0.24, 70.2 ± 0.69, and 51.5 ± 0.50% of larvae fed *I. galbana* metamorphosed after 20 days at 240, 390, and 850 ppm $CO_2$, these percentages declined precipitously to 3.2 ± 0.24, 0.62 ± 0.14, and 0.50 ± 0.35% when their diet was comprised of *A. anophagefferens* (Fig. 3). Similarly, survival of larvae fed *I. galbana* and exposed to 240, 390, and 850 ppm $CO_2$ was 88.0 ± 0.20, 72.9 ± 0.72, and 44.8 ± 15.5% but dropped to 14.5 ± 0.54, 7.2 ± 0.35, and 5.5 ±

0.24% when fed *A. anophagefferens* and exposed to 240, 390, and 850 ppm $CO_2$ (Fig. 3.). Diameters of individuals fed *I. galbana* decreased from 492.9 ± 4.7, 389.3 ± 8.2, 192.0 ± 3.7 µm with increasing $CO_2$ concentrations (~240, 390, and 850 ppm), and declined further when fed *A. anophagefferens* to 387.6 ± 4.5, 369.9 ± 18.2, and 126.1 ± 6.4 µm for ~240, 390, and 850 ppm, respectively (Fig. 7a). Lipid indices of larvae fed *I. galbana* decreased with increasing $CO_2$ concentrations (~240, 390, and 850 ppm) and were 0.30 ± 0.006, 0.17 ± 0.007, and 0.08 ± 0.001, respectively, but were only 0.13 ± 0.06, 0.10 ± 0.009, 0.04 ± 0.001, respectively, when fed *A. anophagefferens* (Fig. 7b). There was a significant effect of food source on larval clearance rates by *A. irradians* larvae ($F(2)$ = 196.36573, $df$ =1, 18, $p$<0.001; two-way ANOVA, Fig. 7c) as well as an interactive effect between food source and $CO_2$ concentrations on larval clearance rates ($F(3)$ = 6.798, $df$ =2, 18, $p$<0.05; two-way ANOVA, Fig. 7c). Clearance rates for *A. irradians* larvae fed *I. galbana* were 0.29 ± 0.04, 0.18 ± 0.07, and 0.25 ± 0.05 µl $min^{-1}$ $larvae^{-1}$ at ~240, 390, and 850 ppm $CO_2$ respectively, compared to clearance rates of 1.12 ± 0.04, 1.70 ± 0.33, and 1.26 ± 0.23 µl $min^{-1}$ $larvae^{-1}$ for larvae fed *A. anophagefferens* at the same $CO_2$ concentrations (Fig. 7c).

***Argopecten irradians*** larvae exposed to high levels of ***Aureococcus anophagefferens***

The strongest negative effects on bivalve larvae were observed during exposures of *A. irradians* larvae to high concentrations of *A. anophagefferens* ($10^9$ cells $L^{-1}$) and high $CO_2$ concentrations. $CO_2$ concentrations, food source, and the interaction of these factors significantly affected larval metamorphosis ($F(1)$ = 1,739.969, $df$= 2, 18, $F(2)$=92,085.520, $df$=1, 18, $F(3)$=1,739.969, $df$ =2, 18, $p$<0.001; two-way ANOVA),

survival ($F(1) = 22.275$, $df = 2, 18$, $F(2)=663.336$, $df = 1, 18$, $F(3)=22.275$, $df = 2, 18$, $p<0.001$; two-way ANOVA), and lipid content ($F(1) = 1052.96$, $df = 2, 18$, $F(2)=1595.614$, $df = 1, 18$, $F(3)=228.344$, $df = 2, 18$, $p<0.001$; two-way ANOVA). None of the *A. irradians* larvae exposed to the high concentrations of *A. anophagefferens* metamorphosed or survived the 20 day experiment (Fig. 4). Maximal survival times for individuals exposed to high densities of *A. anophagefferens* and ~240, 390, and 850 ppm $CO_2$ were 17, 14, and 11 days, respectively (Fig 4). On day 11 larvae exposed to high concentrations of *A. anophagefferens* and $CO_2$ concentrations of ~240, 390, and 850 ppm has lipid indices of 0.12 ± 0.004, 0.07 ± 0.008, and 0.03 ± 0.001 while larvae fed *I. galbana* has lipid indices of 0.30 ± 0.01, 0.18 ± 0.007, and 0.07 ± 0.002 respectively (Fig. 8b). Food source and $CO_2$ levels both significantly affected larval diameters ($F(1) = 502.438$, $df = 2, 18$, $F(2)=199.156$, $df = 1, 18$, $p<0.001$; two-way ANOVA), but did not have an interactive effect. When fed *I. galbana* and reared under $CO_2$ concentrations of ~240, 390, and 850 ppm larval diameters were 494.33 ± 13.1, 404.10 ± 38.9, and 193.4 ± 14.6 µm whereas individuals fed *A. anophagefferens* were 364.9 ± 9.8, 309.0 ± 10.4, and 93.8 ± 3.1 µm, respectively (Fig. 8a). There was a significant effect of food source only on *A. irradians* clearance rates ($F(1) = 121.304$, $df = 2, 18$, $p<0.001$; two-way ANOVA). Mean clearance rates for all levels of $CO_2$ were higher when larvae were fed bloom concentrations of *A. anophagefferens* (2.11 ± 0.37 µl $min^{-1}$ $larvae^{-1}$) compared to clearance rates in the presence of *I. galbana* (0.62 ± 0.03 µl $min^{-1}$ $larvae^{-1}$; Fig. 8c).

**Discussion**

Coastal ocean acidification combined with the increasing frequency and intensity of harmful algal blooms such as brown tide will have detrimental effects on larval bivalves. Mankind's footprint on coastal ecosystems has grown progressively larger during the past century. Although issues such as hypoxia and overfishing have received much attention, the loading of anthropogenically derived carbon has acidified coastal seawater (Caldeira and Wickett 2003; Sabine et al. 2004; Feely et al. 2008; Salisbury et al. 2008; Fussel 2009) while anthropogenic nutrient loading has promoted more frequent harmful algal blooms (Anderson et al. 2008; Heisler et al. 2008). Concurrently, populations of marine organisms such as filter feeding bivalves have been depleted (Jackson 2001; Lotze et al. 2006; Beck et al. 2011). This study revealed the strong negative impacts that ocean acidification and HABs can have on coastal bivalves and further demonstrated that the effects of such stressors are additive. This study additionally demonstrates that organisms filling similar ecological niches (e.g. filter feeding, resource bivalves) can vary substantially in their susceptibility to these stressors. Together, this research has important implications for both the management and future trajectories of estuarine ecosystems.

During this study, the harmful alga, *Aureococcus anophagefferens*, had a more dramatic impact on larval performance than elevated $CO_2$. For example, while survival for *C. virginica* declined 10% when $CO_2$ concentrations increased from 250 to 850 ppm, they decreased by 20% when their diet was switched from *I. galbana* to a high level ($10^9$ cells $L^{-1}$) of *A. anophagefferens*. Similarly, for *A. irradians* the higher level of $CO_2$ depressed survival by 40% while bloom densities of *A. anophagefferens* decreased

survival by 90%. Similar declines in survival (60%) were observed in *Mercenaria mercenaria* (hard clam) larvae when fed bloom densities of *A. anophagefferens* (Bricelj and MacQuarrie 2007), although another study found *A. anophagefferens* did not alter the survival of hard clam larvae (Padilla et al. 2006).

The negative effect of *A. anophagefferens* on bivalve larvae may be partly related to the higher clearance rates veliger larvae displayed consuming this alga compared to *I. galbana* (Fig. 5, 6, 7, 8). Notably, these clearance rates were measured during the first two days of larval development, a period corresponding to nearly 100% survival of individuals. *A. anophagefferens* is known to be a nutritionally poor food source that does not allow lipid accumulation in larval bivalves (Padilla et al. 2006; Bricelj and MacQuarrie 2007). The elevated clearance rates of *A. anophagefferens* may be indicative of the larvae not obtaining enough nutrition, forcing greater consumption of toxic *A. anophagefferens*, which promoted high mortality several days later. For *M. mercenaria* larvae fed *I. galbana* for 8 days and then fed *A. anophagefferens*, clearance rates were lower than compared to larvae being fed solely *I. galbana* (Bricelj and MacQuarrie 2007). Similarly, *A. irradians* larvae reared on *I. galbana* for two days and then switched to *A. anophagefferens* displayed a 7% reduction in clearance rates (Gallager et al. 1989). In both cases individuals that had built up lipid reserves on an ideal food source may have had the luxury to decrease their feeding rates on a poor food source. For the current study, the introduction of the harmful alga occurred within hours of fertilization with the larvae never experiencing another food source. Individuals that fed rapidly on *A. anophagefferens* early in development experienced delayed metamorphosis, reduced sizes, and lower lipid indices likely because *A. anophagefferens* is not a nutritionally

complete algae for the bivalve larvae; (Bricelj et al. 1989; Bricelj and Lonsdale 1997). All of the negative impacts on the performance of individuals during larval stages (reduced sizes and lipid content and delayed metamorphosis) would be likely to translate into elevated post-set mortality in an ecosystem setting as declines in larval recruitment is projected to have serious implications for adult populations (Caley et al. 1996; Munday et al. 2010).

Although the impacts of *A. anophagefferens* on oyster larvae have never been investigated, the negative responses of bay scallop larvae to *A. anophagefferens* shown here were stronger than those found by (Gallager et al. 1989). This may be related to the strain of *A. anophagefferens* used in each study. *A. anophagefferens* strain CCMP1850 used in this study is generally more harmful than strains CCMP 1784/1984, CCMP 1708, and CCMP 1794 (Bricelj et al. 2001; Smith et al. 2008; Harke et al. *In press*) used in prior studies of *A. anophagefferens* and bivalve larvae (Gallager et al. 1989; Padilla et al. 2006; Bricelj and MacQuarrie 2007). Strain CCMP1850 was isolated more recently (1998) than these strains and thus may be a more representative example of wild populations of *A. anophagefferens* compared to strains CCMP 1708 and 1794 isolated in 1995, or CCMP 1784/1984 isolated in 1986 (Martins et al. 2004).

As coastal marine ecosystems adapt to anthropogenic stressors such as HABs and coastal ocean acidification, there will be some species that outperform others in these rapidly changing environments. In this study, while *A. irradians* displayed greater survival than *C. virginica* under ideal conditions (ambient $CO_2$, ideal food), the opposite was true in the presence of HABs and coastal ocean acidification. At the end of 20 days, *C. virginica* larvae had twice the percent survival of *A. irradians* larvae at low density

brown tide blooms despite increases in $CO_2$ concentrations (Fig. 1 and 3). At the highest densities of brown tide, some *C. virginica* survived and fully metamorphosed while all *A. irradians* individuals perished (Fig. 2 and 4). This suggests that *C. virginica* larvae are more likely to persist and expand in the face of ecosystem challenges such as HABs and coastal ocean acidification than *A. irradians*. Bivalve restoration efforts are commonly implemented to enhance the densities of depleted wild populations (Arnold et al. 2002; Doall et al. 2008; Tettelbach and Smith 2008). Going forward, such efforts will need to consider the differential vulnerabilities of these species to stressors such as acidification and HABs. Our results specifically demonstrate that, at least at the larval stage, *C. virginica* is a hardier bivalve and perhaps a more appropriate target for bivalve restoration in ecosystems which experience blooms of *A. anophagefferens* and reduced pH.

Harmful algal blooms have become an increasingly common phenomenon across coastal oceans and estuaries where many bivalves reside. Carbon dioxide fluxes have simultaneously acidified these habitats (Miller et al. 2009) via the introduction of acidic river water (Salisbury et al. 2008) and heterotrophic processes stimulated by loadings of organic carbon (Gattuso et al. 1998; Paerl et al. 1998; Thomas et al. 2004; Koch and Gobler 2009). Furthermore, there is synergy between these stressors as the stimulation of HABs by anthropogenic nutrient loading (Anderson et al. 2008; Heisler et al. 2008) may promote coastal ocean acidification since many HAB species rely on heterotrophic nutrition (Smayda 1997); and the degradation of bloom-derived organic matter may result in decreases in pH and increases in $CO_2$. Globally, declines in bivalve populations during the past century have been attributed to the loss of habitat, hypoxia, and overfishing

(Jackson 2001; Lotze et al. 2006; Beck et al. 2011). However, HABs have eliminated important shellfisheries such as the loss of *A. irradians* populations in NY due to *A. anophagefferens* blooms (Gobler et al. 2005). Furthermore, the increase in $CO_2$ which has occurred since the industrial revolution is capable of significantly reducing the survival of some bivalve larvae (Talmage and Gobler, 2010) including *A. irradians*. Therefore, HABs and coastal ocean acidification have likely contributed to the 'functional extinction' (Beck et al. 2011) of some bivalve populations and will continue to do so in this century.

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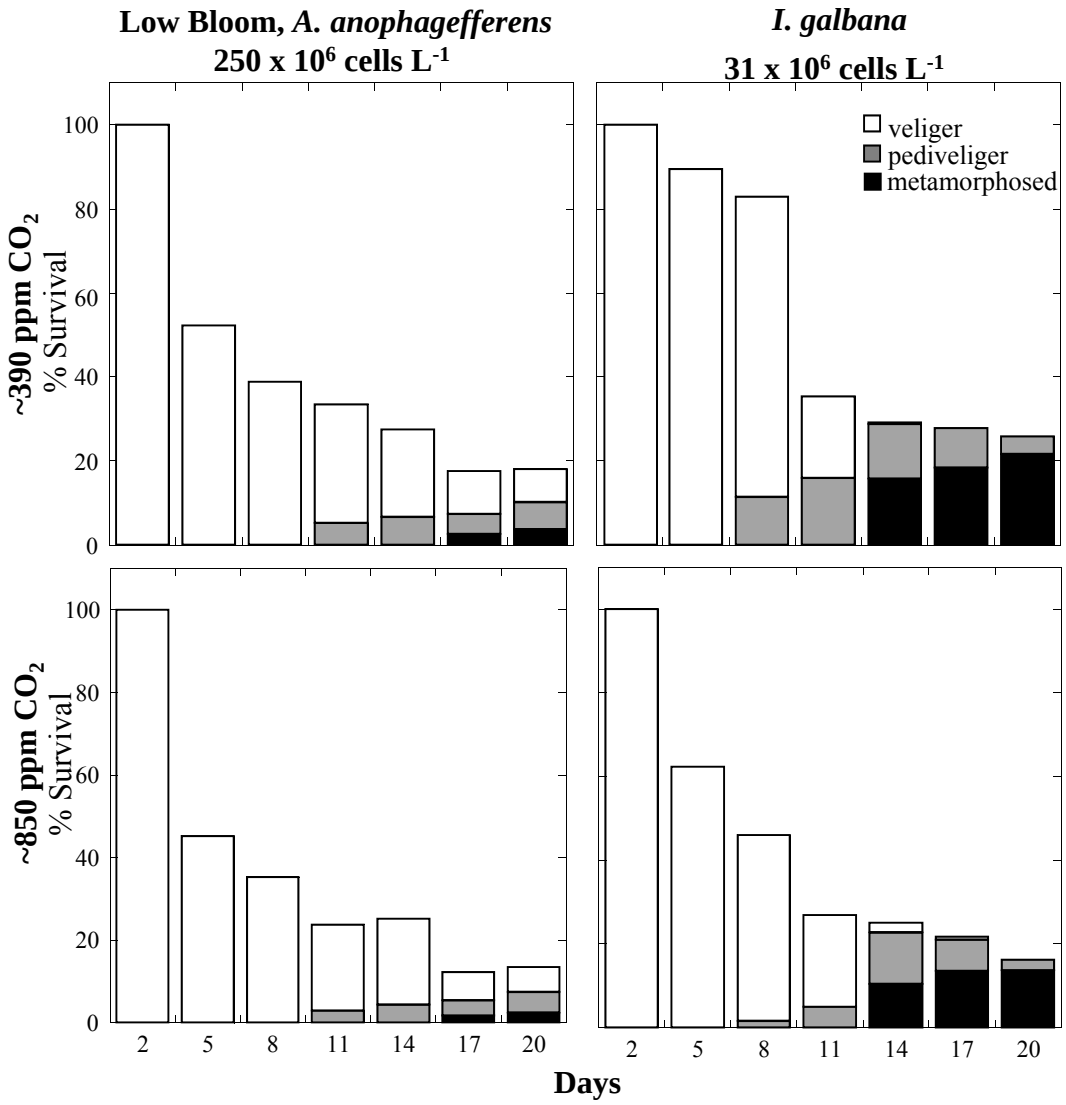


Figure 1: Survival and development of *Crassostrea virginica* grown under two levels of $CO_2$, approximately 390 and 850 ppm (Table 1) and fed either a low bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *I. galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated ($n$ =4 per treatment).

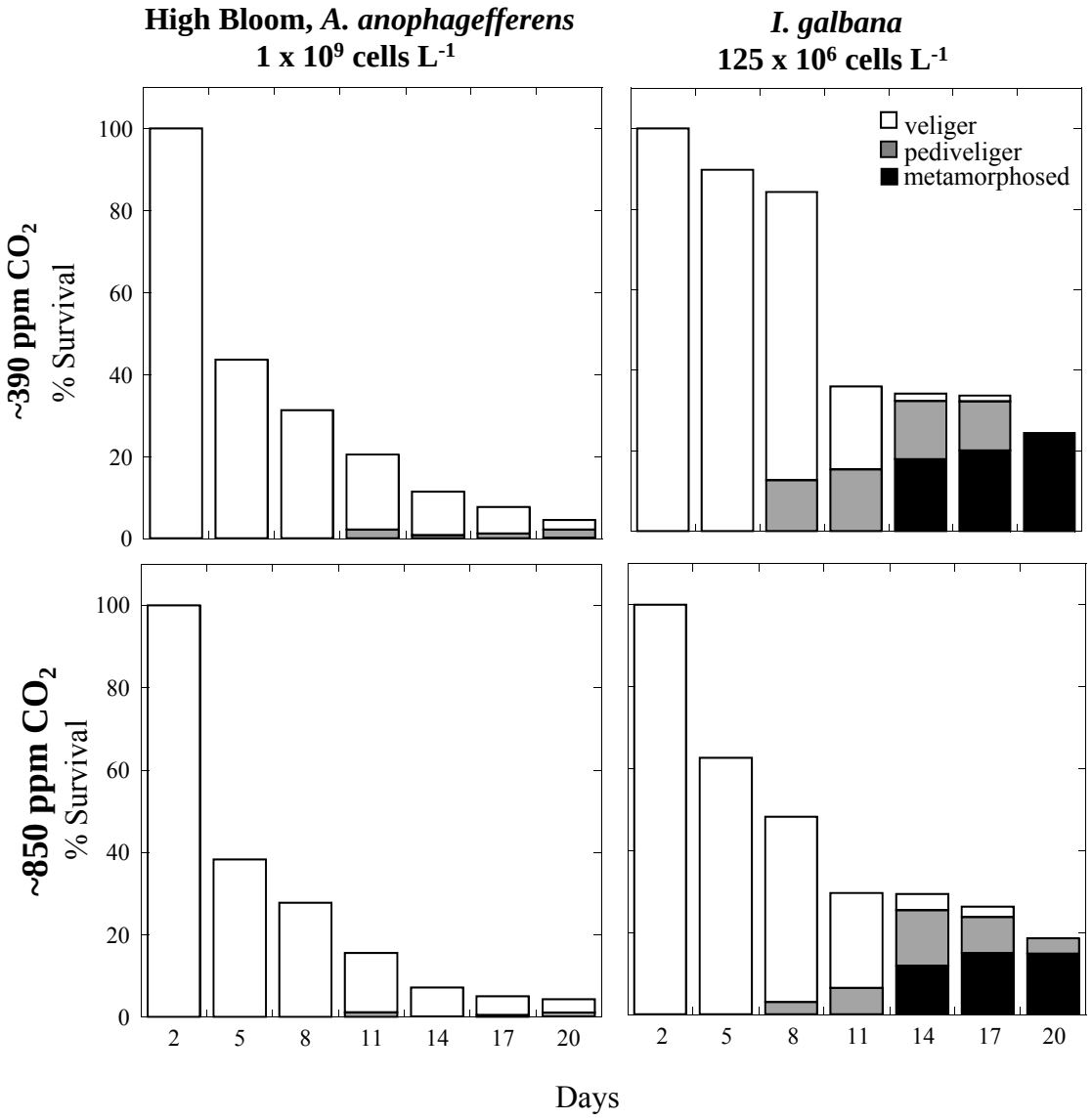


Figure 2: Survival and development of *Crassostrea virginica* grown under two levels of $CO_2$, approximately 390 and 850 ppm (Table 2) and fed either a high bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *I. galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated ($n$ =4 per treatment).

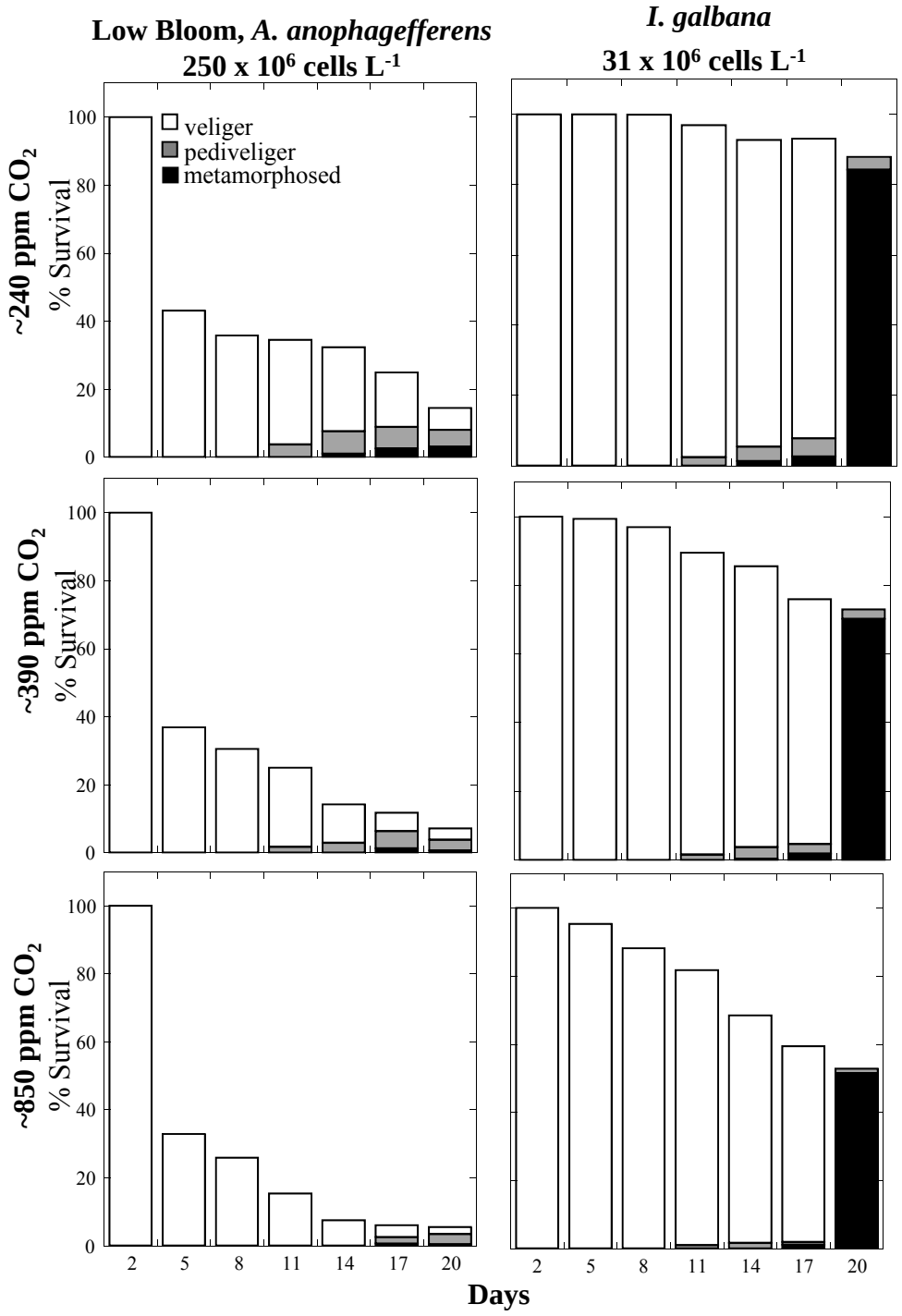


Figure 3: Survival and development of *Argopecten irradians* under three levels of $CO_2$, approximately 240, 390, and 850 ppm (Table 3) fed either a low bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *I. galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated ($n$ =4 per treatment).

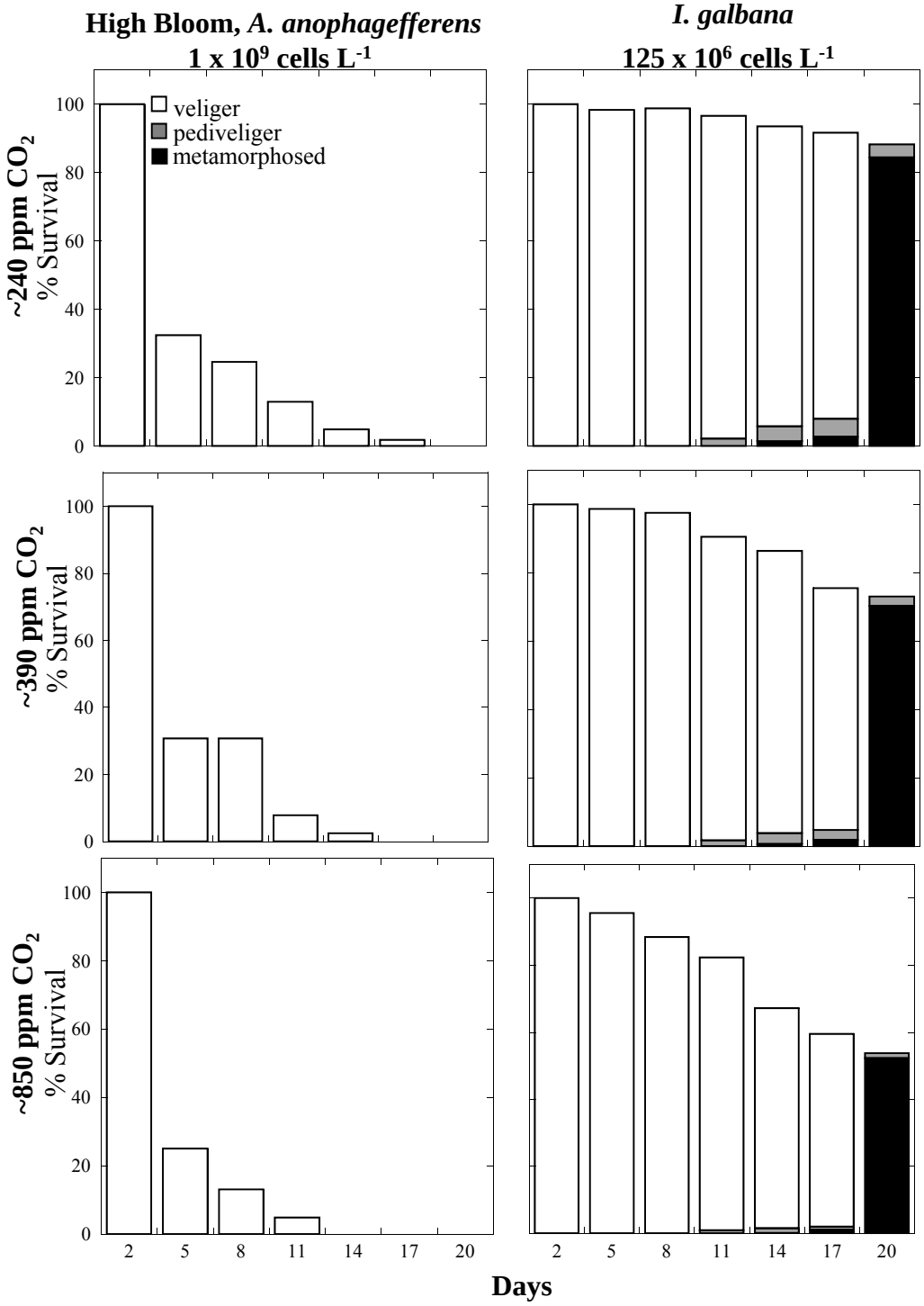


Figure 4: Survival and development of *Argopecten irradians* under three levels of $CO_2$, approximately 240, 390, and 850 ppm (Table 4) fed either a high bloom density of *Aureococcus anophagefferens* or the biovolume equivalent of *I. galbana*. Counts of all larvae at each stage (veliger, pediveliger, and metamorphosed) were made on days indicated ($n$ =4 per treatment).

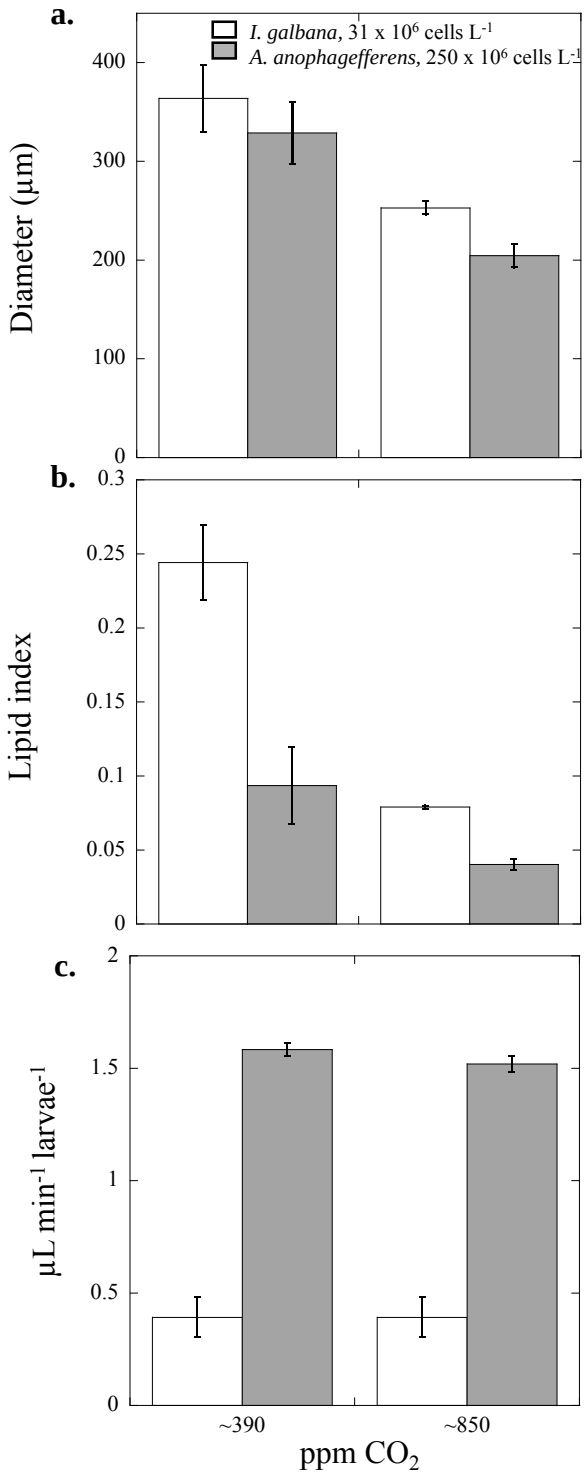


Figure 5: Mean diameter, lipid index, and clearance rates ± standard deviation of *Crassostrea virginica* larvae under two levels of $CO_2$, approximately 390 and 850 ppm (Table 1) fed either a low bloom density of *Aureococcus anophagefferens* (light gray) or the biovolume equivalent of *I. galbana* (white).

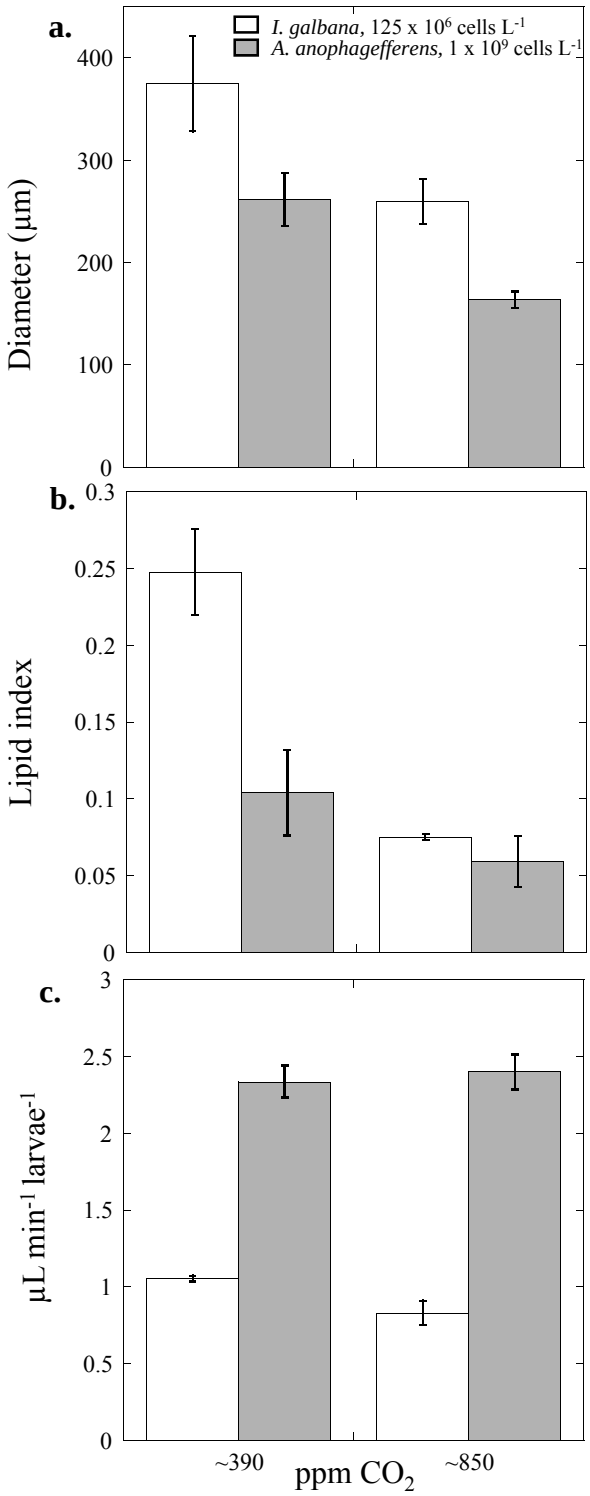


Figure 6: Mean diameter, lipid index, and clearance rates ± standard deviation of *Crassostrea virginica* larvae under two levels of $CO_2$, approximately 390 and 850 ppm (Table 1) fed either a high bloom density of *Aureococcus anophagefferens* (gray) or the biovolume equivalent of *I. galbana* (white).

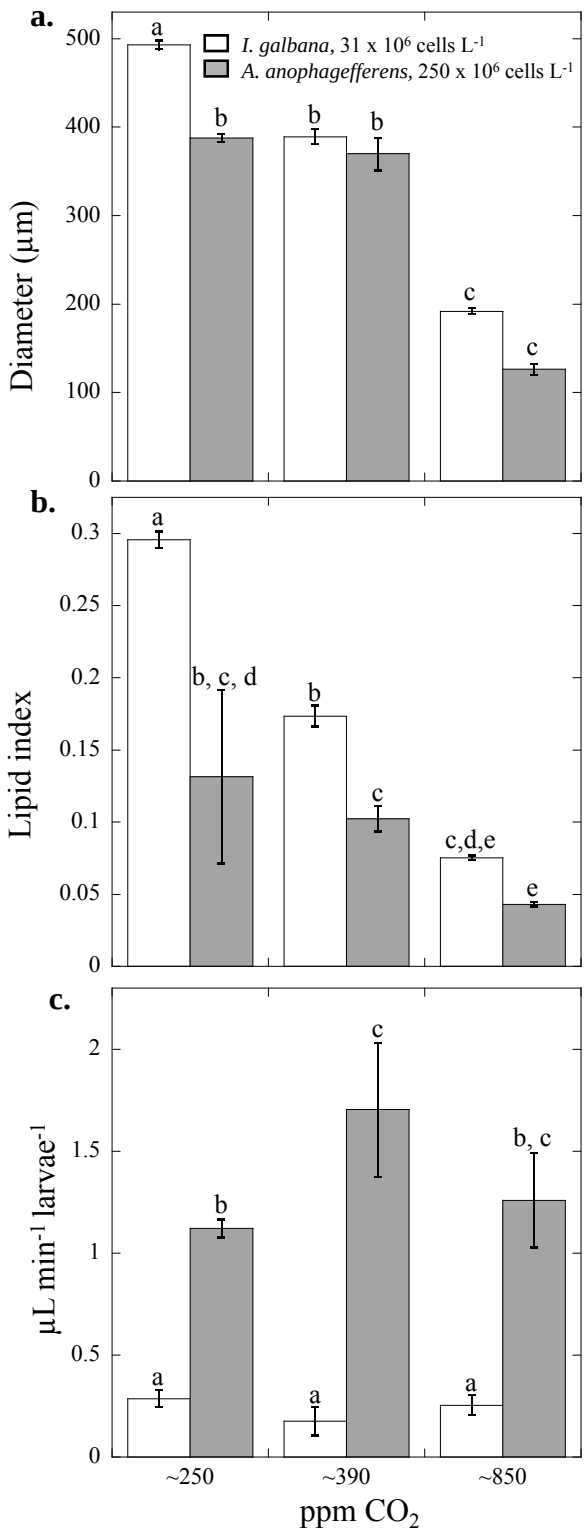


Figure 7: Mean diameter, lipid index, and clearance rates ± standard deviation of *Argopecten irradians* larvae under three levels of $CO_2$, approximately 250, 390, and 850 ppm (Table 2) fed either a low bloom density of *Aureococcus anophagefferens* (light gray) or the biovolume equivalent of *I. galbana* (white), and for Tukey multiple comparisons, $p \leq 0.05$ for all.

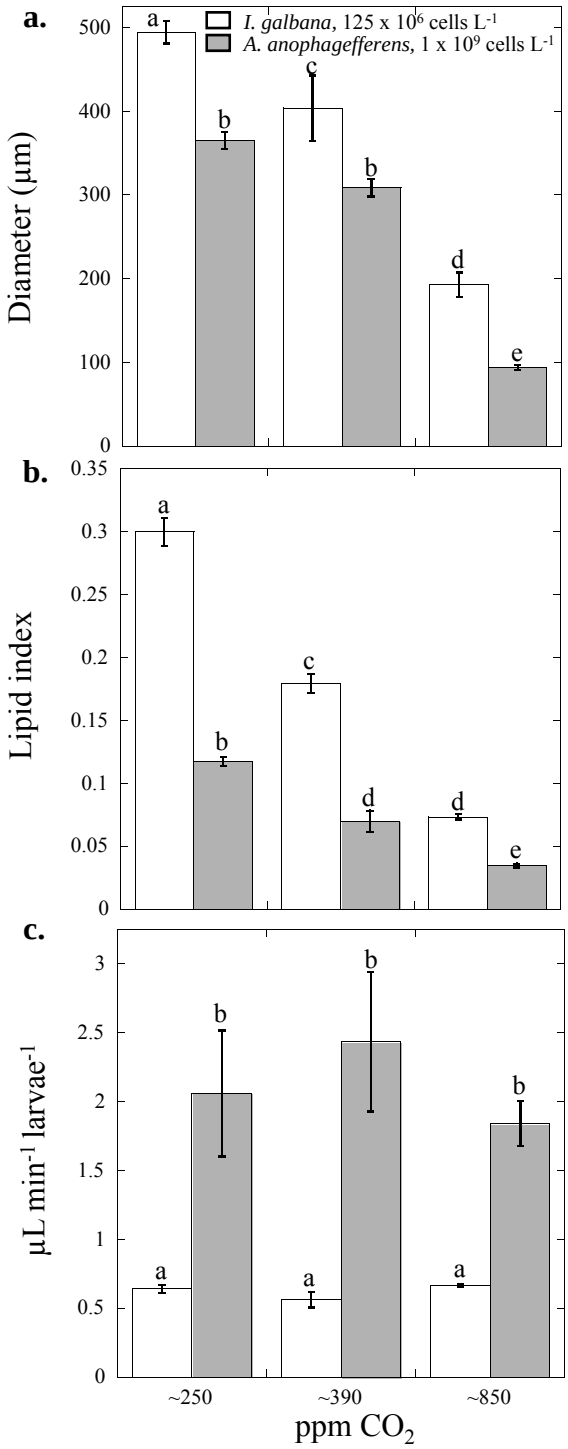


Figure 8: Mean diameter, lipid index, and clearance rates ± standard deviation of *Argopecten irradians* larvae under three levels of $CO_2$, approximately 250, 390 and 850 ppm (Table 2) fed either a high bloom density of *Aureococcus anophagefferens* (gray) or the biovolume equivalent of *I. galbana* (white), and for Tukey multiple comparisons, $p \leq 0.05$ for all.

| **Parameter** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|
| *Crassostrea virginica*<br>Low density brown tide exp | | |
| Temperature (˚C) | 24 ± 0.45 | 24 ± 0.45 |
| pH | 8.100 ± 0.010 | 7.880 ± 0.010 |
| $pCO_2$ (ppm) | 381 ± 14.33 | 833.4 ± 10.95 |
| $\Omega_{calcite}$ | 2.97 ± 0.03 | 2.36 ± 0.07 |
| $\Omega_{aragonite}$ | 1.92 ± 0.02 | 1.52 ± 0.06 |
| Total DIC ($\mu$mol $L^{1}$) | 1466 ± 19.62 | 1880 ± 20.18 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 117.4 ± 1.18 | 93.27 ± 3.10 |
| Alkalinity (TA) | 1632.9± 17.37 | 1987.2 ± 24.35 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | |
| *Crassostrea virginica*<br>High density brown tide exp | | |
| Temperature (˚C) | 28 ± 0.45 | 28 ± 0.45 |
| pH | 8.090 ± 0.010 | 7.873 ± 0.006 |
| $pCO_2$ (ppm) | 398.2 ± 8.97 | 863.5 ± 20.76 |
| $\Omega_{calcite}$ | 2.97 ± 0.08 | 2.37 ± 0.06 |
| $\Omega_{aragonite}$ | 1.91± 0.05 | 1.53 ± 0.03 |
| Total DIC ($\mu$mol $L^{1}$) | 1493 ± 4.07 | 1916 ± 37.40 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 117.2 ± 2.81 | 93.7 ± 2.17 |
| Alkalinity (TA) | 1658.9 ± 7.96 | 2023.4 ± 38.74 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | |
| | | |

Table 1. Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the two-level carbon dioxide and *Aureococcus anophagefferens* experiments with *Crassostrea virginica* larvae.

| **Parameter** | **Near pre-industrial $CO_2$** | **Ambient, present day $CO_2$** | **Elevated $CO_2$** |
|---|---|---|---|
| *Argopecten irradians* Low density brown tide exp | | | |
| Temperature (˚C) | 24 ± 0.6 | 24 ± 0.6 | 24 ± 0.6 |
| pH | 8.208 ± 0.015 | 8.080 ± 0.001 | 7.878 ± 0.005 |
| $pCO_2$ (ppm) | 233.18 ± 14.03 | 368.3 ± 5.327 | 870.1 ± 10.261 |
| $\Omega_{calcite}$ | 2.99 ± 0.04 | 2.63 ± 0.04 | 2.44 ± 0.06 |
| $\Omega_{aragonite}$ | 1.92 ± 0.06 | 1.69 ± 0.02 | 1.58 ± 0.04 |
| Total DIC ($\mu$mol $L^1$) | 1171 ± 37.96 | 1348 ± 19.48 | 1951 ± 31.90 |
| $CO_3^{2-}$ ($\mu$mol $L^{-1}$) | 117.75 ± 5.24 | 103.48 ± 1.49 | 96.25 ± 2.46 |
| Alkalinity (TA) | 1357 ± 38.74 | 1499 ± 20.80 | 2060 ± 34.31 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | |
| *Argopecten irradians* High density brown tide exp | | | |
| Temperature (˚C) | 24 ± 0.5 | 24 ± 0.5 | 24 ± 0.5 |
| pH | 8.200 ± 0.005 | 8.063 ± 0.033 | 7.878 ± 0.010 |
| $pCO_2$ (ppm) | 230.05 ± 9.47 | 377.13 ± 35.525 | 856.68 ± 26.692 |
| $\Omega_{calcite}$ | 2.88 ± 0.08 | 2.47 ± 0.15 | 2.40 ± 0.06 |
| $\Omega_{aragonite}$ | 2.47 ± 0.15 | 1.60 ± 0.09 | 1.55 ± 0.03 |
| Total DIC (umol $L^{-1}$) | 1142 ± 38.15 | 1318 ± 19.29 | 1920 ± 31.72 |
| $CO_3^{2-}$ ($\mu$mol $L^1$) | 113.63 ± 3.32 | 97.58 ± 5.66 | 94.73 ± 2.19 |
| Alkalinity (TA) | 1323 ± 40.78 | 1461± 11.15 | 2028± 32.20 |
| Salinity | 28.0 ± 1.0 | 28.0 ± 1.0 | 28.0 ± 1.0 |
| | | | |

Table 2. Temperature, pH, carbonate chemistry, alkalinity, and salinity (± 1 SD) during the three-level carbon dioxide and *Aureococcus anophagefferens* experiments with *Argopecten irradians* larvae.

# CHAPTER SEVEN:

## Dissertation summary and conclusions

**Dissertation Summary**

The world's oceans have been acidifying since the dawn of the industrial revolution and are expected to do so for at least two centuries (Caldeira and Wickett 2003). Despite the long standing nature of this process, ocean acidification research is in its infancy; and investigations of the effects of ocean acidification on marine organisms had not appeared in the peer reviewed literature until 2003. My dissertation research is among the first to examine the effects of ocean acidification on larvae from bivalves native to the Northwest Atlantic Ocean coast: *Mercenaria mercenaria*, the northern quahog, *Argopecten irradians*, the bay scallop, and *Crassostrea virginica*, the Eastern oyster. For my dissertation I specifically used laboratory experiments to alter the carbonate chemistry of seawater and mimic $CO_2$ concentrations found or estimated in the world's oceans from year ~1600 (pre-industrial; 250 ppm) through $CO_2$ concentration estimates through year 2200 (1,500 ppm). During experiments, I assessed bivalve larval survival and development, and several key physiological processes such as metamorphosis, calcium uptake, lipid synthesis, RNA synthesis, and growth. A sub-set of experiments examined the interactive effects of temperature and harmful algal blooms (HABs) in combination with changes in $CO_2$. Finally, experiments tracking the success of individuals past the larval stages into later juvenile development assessed the longer term effects of larval stage exposure to elevated $CO_2$. Collectively, these approaches provided new insight regarding the manner in which larval stage exposure to $CO_2$ may influence marine bivalve populations.

The results of this dissertation demonstrate that $CO_2$ can play a central role in influencing the survival and physiology of bivalve larvae. In chapter two, I demonstrated

that three species of commercially and ecologically valuable bivalves, *Mercenaria mercenaria*, *Crassostrea virginica*, and *Argopecten irradians* displayed dramatic declines in survivorship and delayed metamorphosis under elevated $CO_2$ concentrations projected for the 21st century and beyond. *Crassostrea virginica* was the most resilient species of the three with lowered growth and survival under elevated $CO_2$, but only significant decreases in survival under the highest $CO_2$ treatments (~1500 ppm). In chapter three, *M. mercenaria* and *A. irradians* larvae both displayed increased growth, survival, and more robust and rapid development when grown under pre-industrial (~250 ppm) $CO_2$ concentrations compared to current levels (~390 ppm). Attempting to elucidate the mechanism by which the survival of bivalve larvae is decreased at high $CO_2$ concentrations, chapter four documented stepwise declines in calcium uptake, RNA:DNA ratios, and growth rates of bivalve larvae exposed to increasing $CO_2$ concentrations. Later in chapter four, experiments varying the elevated $CO_2$ exposure window found that only 4 days of high $CO_2$ can suppress bivalve larval survival but also found that individuals that survived high $CO_2$ concentrations as larvae grew more rapidly as juveniles than individuals exposed to normal $CO_2$ as larvae. However, size deficits established during the larval stages under increased $CO_2$ were still detected, even after eight months of exposure to normal $CO_2$ levels. In chapter five, I examined the combination of higher temperatures and higher concentrations of $CO_2$ and found that simultaneous increases in both of these factors had additive negative impacts on both larval and juvenile stage bivalves. While the precise responses to temperature and $CO_2$ varied by species, larval stages were generally more sensitive to both parameters than juvenile stages. Finally in chapter six, the introduction of a harmful alga as a food source

was combined with increased $CO_2$ and resulted in additive negative effects on the survival and development of *A. irradians* larvae and, to a lesser extent, *C. virginica* larvae, suggesting that multiple environmental stressors have the ability to combine to suppress bivalve larvae but that some species will fare better than others in the face of these challenges.

Many ocean acidification studies to date have used organisms reared under natural and/or ideal conditions that are subsequently introduced into acidified seawater (e.g. Ries et al. 2009). While such a procedure is experimentally straightforward, it does not mimic future ocean acidification and is not entirely characteristic of present day, coastal ocean acidification. Larvae of broadcast spawners, however, may be more likely to experience high $CO_2$ levels in this way. Since the internal pH of adult bivalves is osmotically regulated and relatively static, developing eggs and sperm persist under ideal chemical conditions until spawned; and higher external $CO_2$ is unlikely to drastically affect the acid-base balance of the parent (Kennedy 1996; Kraeuter and Castagna 2001; Cragg 2006). Once spawned, all bivalve larvae enter a new chemical environment that differs from the biochemical stability offered by their parent. Coastal ecosystems already experience elevated levels of $CO_2$ (Salisbury et al. 2008; Talmage and Gobler 2009; Waldbusser et al. 2011), in part due to decomposition of the large amount of organic matter already present in estuaries (Gattuso et al. 1998; Paerl et al. 1998; Thomas et al. 2004), and my dissertation work has demonstrated that bivalve larvae which are spawned into such environments, even for four days, will experience significant reductions in their survival rates. My experiments introduced newly fertilized bivalve larvae into experimental vessels within hours of fertilization; an approach which I believe mimics

what occurs in nature. As our coastal oceans acidify over the next two centuries, there may be selection pressure for bivalves to become more resistant to high $CO_2$ (*see discussion of this topic below*). It seems unlikely, however, that there will be selection pressure that alters the internal chemistry of adult bivalves due to internal mechanisms evolved to maintain homeostasis and regulate internal pH. As such, in the future, bivalve larvae may also experience elevated $CO_2$ in a manner similar to my experiment design: Persisting under ideal conditions as gametes and then thrust into a new, high $CO_2$ environment as larvae. Therefore, while my research had many limitations (*see discussion below*), my approach of introducing hours-old larvae into a new environment may be a more realistic experimental approach than many ocean acidification studies published to date.

Although this dissertation did not study the settlement of larval bivalves onto sediments as they metamorphose, this process may also be strongly influenced by $CO_2$. Bivalve larvae identify an environment to be habitable at the end of their larval life stage and then will settle to the marine benthos to continue their early development as juveniles (Woodin et al. 1998). Newly metamorophosed bivalves are known to search for favorable habitat when settled into an unfavorable location (Marinelli and Woodin 2002; Marinelli and Woodin 2004). This dissertation has documented the negative effects of high $CO_2$ on larval stages, as well as on early developmental stages of bivalves, suggesting that increased $CO_2$ and decreased carbonate saturation could affect the settlement of transitioning bivalve larvae. Shell on the marine benthos has previously been shown to be an important refuge from predators for larvae and can serve as habitat structure (Powell and Klinck 2007). Estuarine sediments supersaturated with respect to

calcium carbonate due to increased shell material have demonstrated the importance of increased $CaCO_3$ availability as a behavioral cue for larval settlement in *Mya arenaria* (Green et al 2009).

While this dissertation provided important insight regarding the effects of $CO_2$ on bivalve larvae, it also had its limitations. The experiments presented throughout my dissertation were designed to assess the response of larval bivalves to changes in $CO_2$ concentrations. These experiments were mostly microcosm (~1L) experiments in a controlled, laboratory setting that allowed for precise estimates of $CO_2$ within each treatment, but also left several questions unanswered. For example, responses would likely have differed if larger volumes were considered. While experiments were conducted with larvae at the densities they would experience in nature, they did not incorporate several key environmental variables that can change simultaneously in an estuarine setting. Chapter 5 and 6 of my dissertation examined other stressors that these organisms are likely to experience in an ecosystem setting in conjunction with increasing $CO_2$ concentrations. Other factors that have strong influences on larval survival such as predation and salinity fluctuations (Kennedy 1996; Kraeuter and Castagna 2001; Cragg 2006) were not considered, however. Along these lines, investigating organismal responses to ocean acidification in natural ecosystem settings would be most likely to provide a realistic estimate of anticipated responses to this phenomenon.

Few ocean acidification studies have been performed in an ecosystem setting, but several locations would be prime destinations for future studies of the effects of $CO_2$ on bivalves. For example, in the Italian, coastal Mediterranean Sea, naturally occurring $CO_2$ vents have been used for in-situ experiments examining ocean acidification (Cigliano et

al. 2010; Dias et al. 2010). Also, there are many locations along the Northwestern coast of the United States that have locally increased concentrations of $CO_2$ due to both upwelling (Feely et al. 2008) and elevated increased respiration that have left many natural shellfish beds in a poor state and on the brink of functional extinction (Beck et al. 2011). These locations certainly warrant the attention of future ocean acidification research. Many other coastal and estuarine settings are already experiencing increased levels of $CO_2$ and decreased pH and thus are an ideal setting to observe the present day effects of ocean acidification on marine organisms (Salisbury et al. 2008; Waldbusser et al. 2011). Going forward, laboratory-based experiments coupled with ecosystem level experiments will provide insight of how marine organisms will respond to rising $CO_2$ concentrations in an ecosystem setting.

To date, there have been a wide array of responses to ocean acidification, both positive and negative, depending on the species studied (Doney et al. 2009). Therefore, it is important to investigate multiple species within clades of organisms to gain a more holistic understanding of responses to ocean acidification. During my dissertation work, I investigated three species of bivalve larvae and found a diversity of responses to elevated $CO_2$ concentrations (Chapter 1, Chapter 6). Future studies should certainly examine additional species of bivalve larvae to better understand whether the strong negative responses of hard clams and bay scallop larvae to elevated $CO_2$ concentrations or the more muted responses of Eastern oysters are typical for bivalve larvae.

Using the data obtained throughout my dissertation, I have begun to elucidate mechanisms by which enhanced $CO_2$ levels influence bivalve larvae survival. If compromised nutrition was the main mechanism causing declines in shellfish larvae,

there would have been no differences in calcification and shell morphology with increasing $CO_2$ levels, which was not the case (Chapters 2 and 3). Since there were declines in calcium uptake rates for bivalve larvae under increased concentrations of $CO_2$ (Chapter 2-4), reduced calcification was probably the primary mechanism by which high $CO_2$ negatively impacted the early development of larval bivalves. The altered morphology and shell structure and smaller shell size as viewed under scanning electron microscopy (Chapter 3) were likely the direct effect of reduced calcification. Recent studies investigating the effects of hypercapnia, and acid-base regulation by ionic transport in marine organisms suggest that internal pH and intracellular conditions are not affected by increased $CO_2$ for both non-calcifying and calcifying marine species (Dissanayake et al. 2010; Thomsen and Melzner 2010). For example, palaemonid prawns (*Palaemon elegans* and *Palaemon serratus*) have been shown to be efficient hypo-ionic/osmo-regulators in seawater under conditions of elevated $CO_2$ (Dissanayake et al. 2010). For the blue mussel (*Mytilus edulis*), enhanced protein metabolism under increased $CO_2$ levels facilitated maintenance of intracellular pH (Thomsen and Melzner 2010). These examples indicate that increased $CO_2$ does not necessarily alter the internal biochemistry of marine organisms and further suggests that a primary impact of elevated $CO_2$ on bivalve mortality during my dissertation research was the reduction of calcification. Since most other parameters I measured declined with increasing $CO_2$, this suggests that reductions in calcification had an overarching, systematic impact on bivalve larval physiology with secondary effects including reduced growth rates, reduced RNA synthesis, lowered lipid accumulation, and delayed metamorphosis that may have

contributed toward the decreases in survival of larval bivalve shellfish with increasing levels of $CO_2$.

While much of my dissertation research suggests that calcification had a systematic impact on bivalve larval physiology, an alternative hypothesis is that larvae exposed to high $CO_2$ were smaller and that smaller individuals were in poorer condition with regard to thickness, lipids, RNA, and calcification. To resolve whether the negative secondary effects observed during my dissertation research were a result of increased $CO_2$ concentrations or size dependent effects, I corrected RNA:DNA, calcification uptake, lipid indices, and shell thickness for overall size of the individuals (Figs. 1-4), and compared differences using one-way ANOVA's with Tukey multiple comparisons to determine significant differences between $CO_2$ treatments. For size-corrected RNA:DNA ratios corrected, there were significant differences among treatments ($p \leq$ 0.001, ANOVA) for both *M. mercenaria* and *A. irradians* suggesting that this proxy for growth was impacted by $CO_2$ levels, independent of size (Fig. 1). For size corrected calcification of *A. irradians*, there was a significant difference ($p \leq 0.001$, ANOVA) between $CO_2$ treatments, again evidencing a size-independent effect of $CO_2$. In contrast, size-corrected calcification by *M. mercenaria* did not decrease as a function of $CO_2$ (Fig. 2b). For lipids, there was a negative effect of $CO_2$ on size-corrected lipid indices *A. irradians*, although this was not significant. In contrast, size-corrected the size corrected lipid index for *M. mercenaria* significantly increased with higher $CO_2$ (ANOVA; $p<0.05$; Fig. 3). The size-corrected shell thickness of *A. irradians* declined significantly as $CO_2$ rose ($p \leq 0.001$, ANOVA; Fig. 4b) whereas this trend was present but much less distinct in *M. mercenaria* ($p \leq 0.001$, ANOVA; Fig. 4a).

In summary, all of physiological attributes of *A. irradians* larvae examined during this dissertation varied as a function of $CO_2$, even when corrected for the size of individuals (Figs 1 – 4). These findings support the hypothesis that the main mechanism causing $CO_2$-induced mortality in this species was reduced calcification that had a cascading set of secondary negative effects on individuals, independent of size. These analyses yielded a more complex outcome for *M. mercenaria*, however. In this species, some secondary negative effects seemed to be a function of the size of indivduals (lipid accumulation, calcification rate) where as others (RNA:DNA ratio, shell thickness) varied as a function of $CO_2$ even after size-correction, indicating that some, but not all, of the secondary negative effects of $CO_2$ were due to individuals being smaller. Collectively, these findings emphasize two important points of my dissertation research: 1. The response of marine organisms to ocean acidification is complex and can differ among species even within the same functional group (i.e. filter feeding bivalve larvae; Talmage and Gobler 2009), 2. More research is needed to fully resolve the mechanism leading to mortality of marine bivalve larvae under high $CO_2$.

There remain many unanswered questions regarding the fate of bivalve populations in the face of an acidifying ocean. My longer term growth experiments that focused on the growth and development of bivalves after the larval stage exposure to specific $CO_2$ concentrations provided some preliminary insight regarding the longer term implication of high $CO_2$. Individuals surviving high $CO_2$ exposure as larvae displayed faster short-term (weeks, *M. mercenaria*) and longer-term (month, *A. irradians*) growth under normal $CO_2$ concentrations as juveniles than individuals reared under normal $CO_2$ levels as larvae. After several months, there was no difference in size between individuals

that developed under high and normal $CO_2$ during their larval stages, although both cohorts lagged behind individuals grown under low $CO_2$ as larvae (250ppm; Chapter 4). Hence, although survival of bivalve larvae significantly decreased under increased $CO_2$ concentrations (Chapters 2 and 3), the small numbers of individuals that survive are capable of rapid, compensatory growth.

One key aspect of ocean acidification research that has yet to be conducted is the potential for rapid evolution and possible adaptation to a high $CO_2$ environment. In the earth's geologic history, 35-60 million year's ago, there were higher concentrations of $CO_2$ in the atmosphere (1000-2000 ppm) and calcifying bivalves not studied in this dissertation were present (Pagani et al. 2005). However, the current rates of atmospheric $CO_2$ increases are 100 times faster than any recorded in the past 25 million years, rapidly changing the ocean chemistry to levels not experienced in 25 millions of years (Sabine et al. 2004), suggesting this evolutionary challenge is without precedent for many extant calcifying species. Although modern day and future $CO_2$ concentrations result in reduced survival rates for bivalve larvae compared to pre-industrial $CO_2$ concentrations (Talmage and Gobler 2010), the ability of future populations to survive modern and elevated levels of $CO_2$ and to metamorphose into juvenile bivalves remains unclear. Populations faced with environmental challenges such as ocean acidification may: 1. Display broad phenotypic and physiological plasticity allowing species to tolerate the new conditions, 2. Adapt to the new conditions through genetic change via the process of evolution, specifically natural selection, or 3. Disperse to more hospitable habitats, although this is generally considered not applicable to benthic organisms such as bivalves in the adult stages, and is only vital during larval stages (Pörtner 2008; Pörtner and

Farrell 2008; Bradshaw and Holzapfel 2010; Hoffman and Todgham 2010). A final response would be extinction. Responses, and the extent to which current populations may evolve to adapt to higher $CO_2$ has yet to be established, but we do know that the bivalve fossil record shows different genera of mollusks did exist during periods of history with much higher $CO_2$ concentrations(Haglund 1998; Harte 1998). Although ecological and evolutionary processes are traditionally assumed to occupy different timescales, a wave of recent studies has demonstrated they can, in some instances, proceed at similar rates and reciprocally interact in many biological contexts (e.g. eco-evolutionary dynamics; (Thompson 1998; Post and Palkovacs 2009). Substantial evolution occurring on ecological timescales affects population persistence, drives speciation or extinction (Hendry et al. 2007), alters community structure, and shapes ecosystem function (Post and Palkovacs 2009). Ecological variables, such as spatial habitat heterogeneity, dispersal (Grant et al. 2007), and phenotypic plasticity (Ghalambor et al. 2007) may all influence the potential for evolution.

Beyond evolution, the physiological plasticity in contemporary bivalve larvae may also strongly influence the ability of some organisms to survive the challenge of rising levels of $CO_2$. While climate change may promote evolutionary change in some animals (Root et al. 2003; Parmesan 2006; Visser 2008; Bradshaw and Holzapfel 2010), most evolutionary evidence to date has been associated with the dynamics of temporal and spatial distributions of various marine populations. In many ocean ecosystems, climate change has been hypothesized to influence animals through physiological tolerances (Pörtner 2008; Pörtner and Farrell 2008; Bradshaw and Holzapfel 2010; Hoffman and Todgham 2010). However, there is little mechanistic understanding of such

physiological responses to changes in ocean carbon chemistry. Further, we know little of the mechanisms that tolerant species might possess as compared to more sensitive taxa (Pörtner 2008; Melzner et al. 2009).

While studies on a variety of contemporary calcifiers suggest their persistence in our oceans is uncertain, it is plausible that future organisms may adapt to an acidifying ocean or that current phenotypic plasticity (Visser 2008) is sufficient to overcome the levels of $CO_2$ and pH that are projected for the near future (Orr et al. 2005; Cao and Caldeira 2008). One of the few studies to examine the potential for aquatic organisms to adapt and evolve to high $CO_2$ examined the freshwater green alga *Chlamydomonas* which was grown at high levels of $CO_2$ for 1000 generations. At the end of the experimental period, the alga's carbon concentrating mechanisms, which were advantageous under lower $CO_2$ levels, were lost within high $CO_2$ cultures (Collins and Bell 2004; Collins et al. 2006), demonstrating a short term adaptation to this climatic change. Multiple studies of marine zooplankton and fish have demonstrated that organisms can evolve and adapt to environmental challenges very rapidly, in as few as three generations (Conover and Munch 2002; Colin and Dam 2004). All of these studies suggest evolution through natural selection by high $CO_2$ in natural marine populations is plausible. The extent to which such adaptation may occur within bivalve larvae exposed to high $CO_2$ is unknown and enforces the need for more research focused on the effect of ocean acidification on multiple generations of bivalves.

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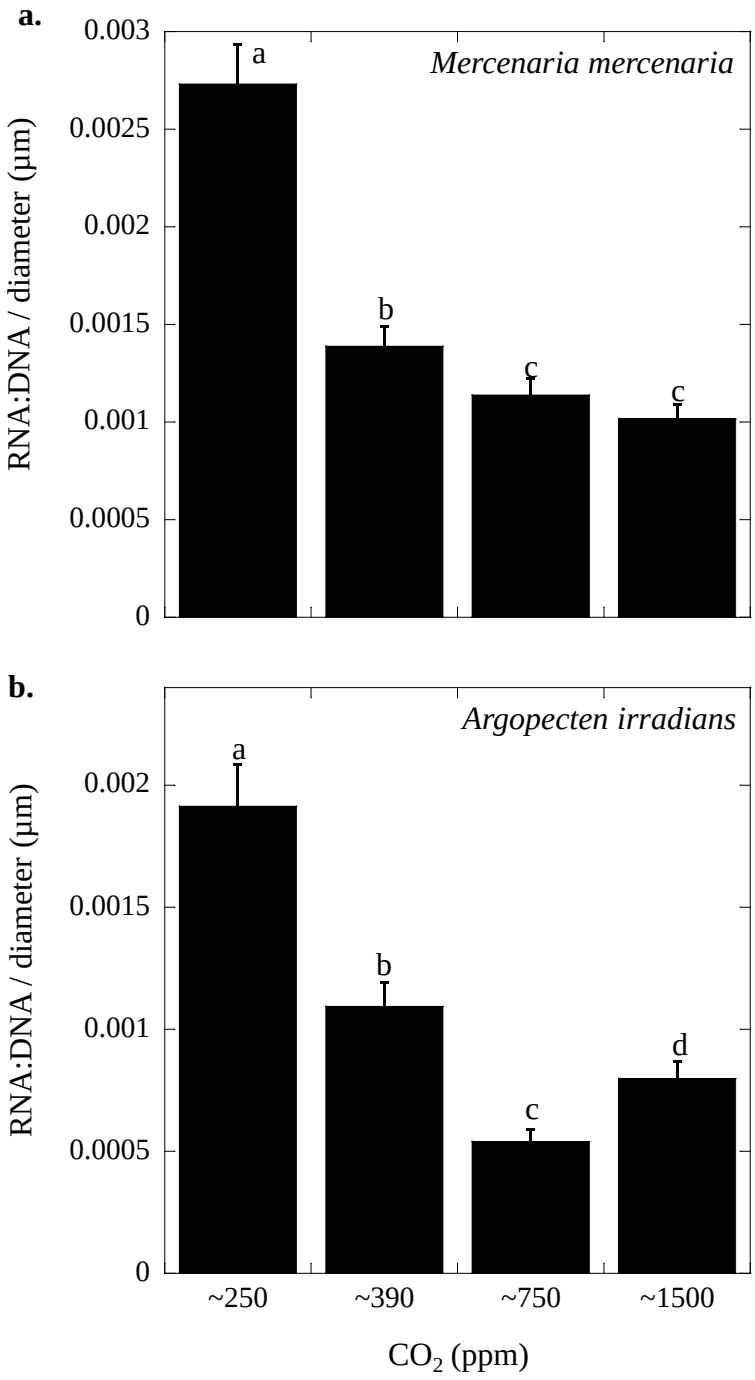


Figure 1. Mean RNA:DNA / mean diameter for **a.** *Mercenaria mercenaria* ± relative standard deviation (RSD)(7.3%) and **b.** *Argopecten irradians* individuals ± RSD (8.9%). Data for RNA:DNA was taken from Ch. 4 and diameters from Ch. 3. Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

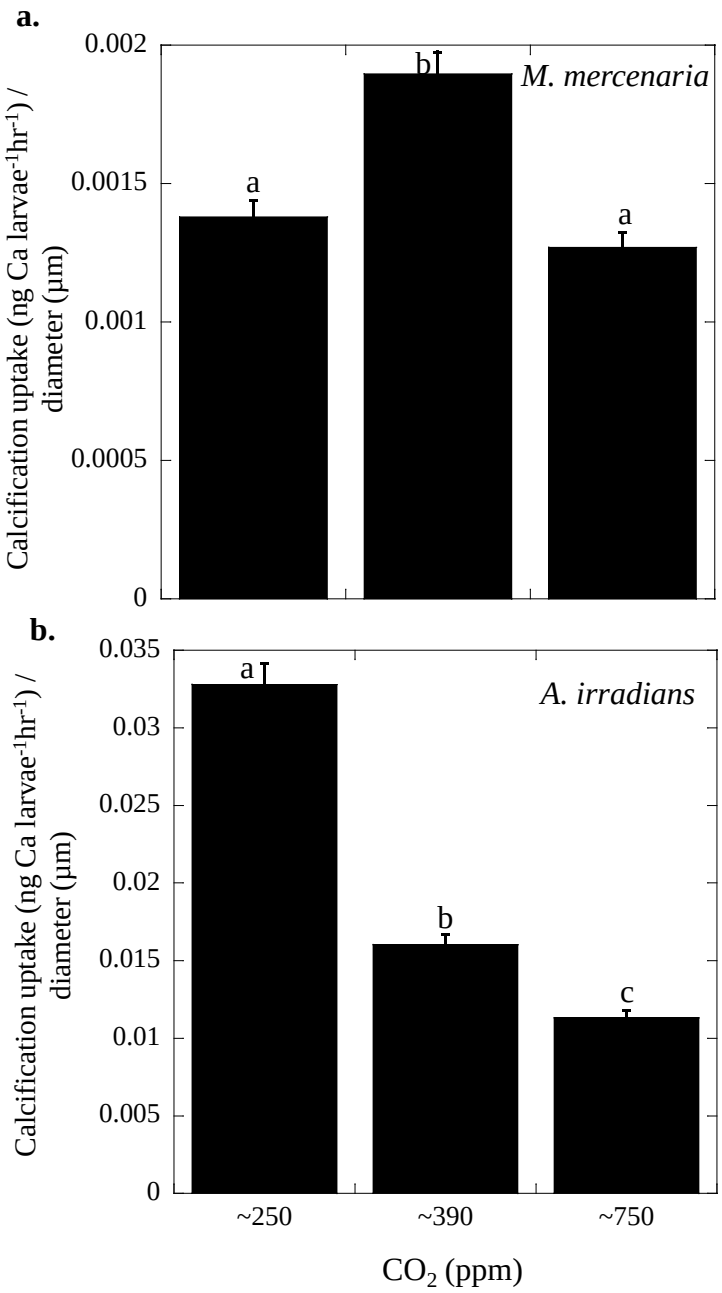


Figure 2. Mean calcification uptake rate (ng Ca larvae$^{-1}$hr$^{-1}$) / mean diameter for **a.** *Mercenaria mercenaria* pediveligers ± relative standard deviation (RSD)(4.2%) and **b.** *Argopecten irradians* pediveligers ± RSD (5.1%). Data for RNA:DNA was taken from Ch. 4 and diameters from Ch. 3. Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

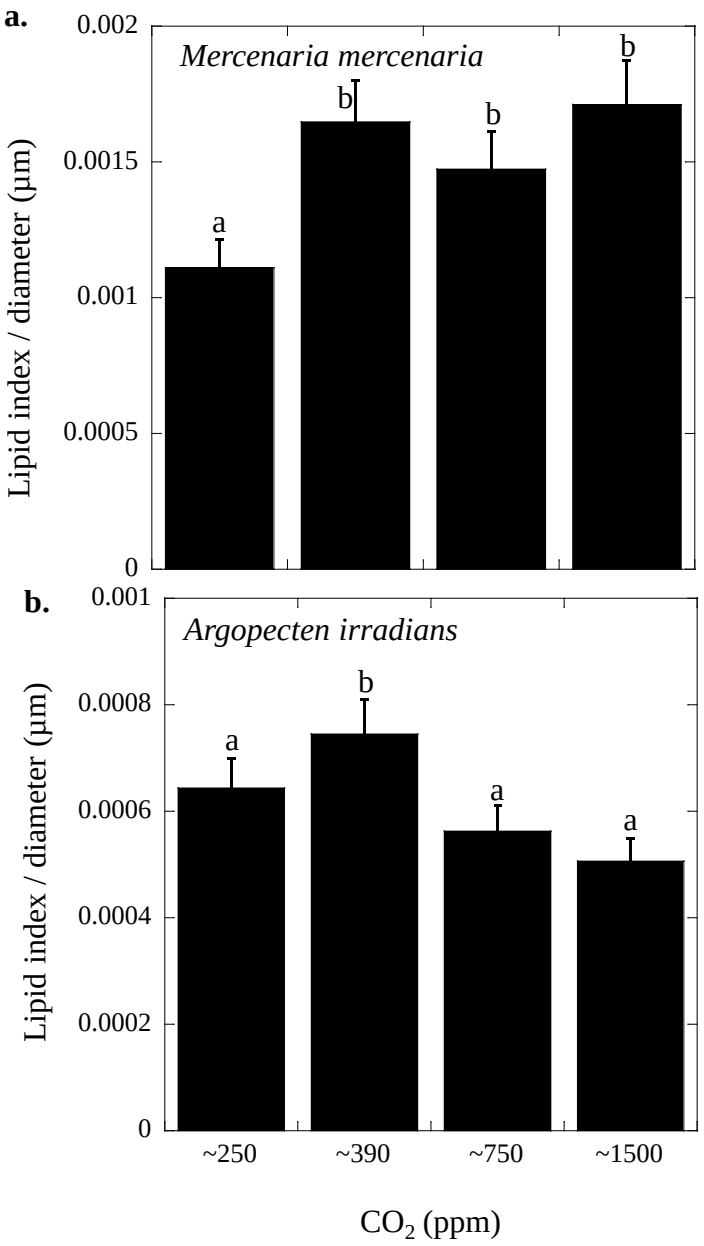


Figure 3. Mean lipid index / mean diameter for **a.** *Mercenaria mercenaria* ± relative standard deviation (RSD)(9.4%) and **b.** *Argopecten irradians* individuals ± RSD (8.5%). Data for lipid indices and diameters was taken from Ch. 3. Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

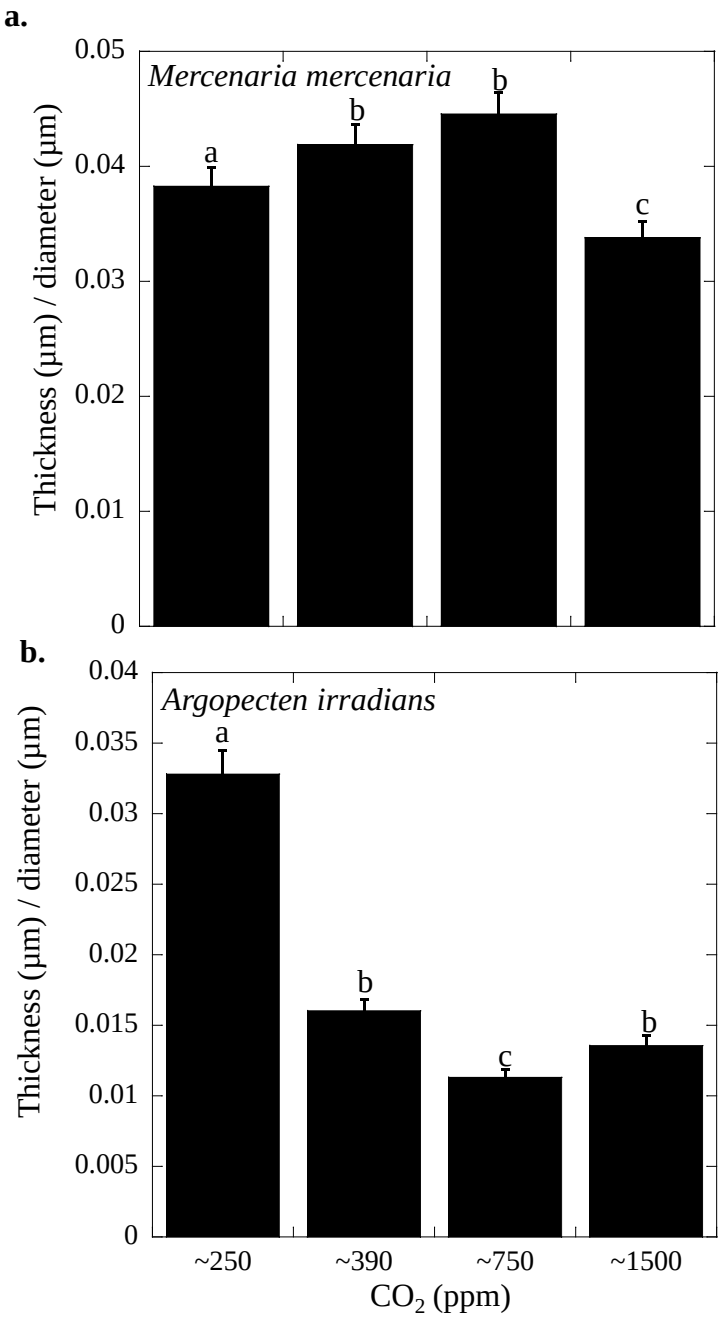


Figure 4. Mean thickness / mean diameter for **a.** *Mercenaria mercenaria* ± relative standard deviation (RSD)(4.2%) and **b.** *Argopecten irradians* individuals ± RSD (5.1%). Data for thickness and diameters was taken from Ch. 3. Letters indicate significant differences revealed from Tukey *post-hoc* multiple comparisons, $p \leq 0.05$ for all.

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