INTERACTIVE EFFECTS OF OCEAN ACIDIFICATION AND MULTIPLE STRESSORS ON PHYSIOLOGY OF MARINE BIVALVES

by

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ABSTRACT

OMERA BASHIR MATOO.Interactive effects of ocean acidification and multiple stressors on the physiology of marine bivalves (Under direction of Dr. INNA M. SOKOLOVA)

The continuing increase of carbon dioxide (CO_2) levels in the atmosphere leads to increase in sea-surface temperature and causes ocean acidification altering seawater carbonate chemistry. Estuarine and shallow coastal areas, which are hotspots for biological productivity, are especially prone to these changes, because of low buffering capacity of brackish waters, biological CO_2 production, and large fluctuations of temperature and salinity in these habitats. These additional stressors may exacerbate the acidification trend and significantly affect the physiology of marine calcifiers. Bivalves are a key group of marine calcifiers that serve as ecosystem engineers and key foundation species in estuarine and coastal environments. However, the interactive effects of elevated CO_2 and other stressors, including elevated temperature and reduced salinity, are not yet fully understood in bivalves and require further investigation. This study focused on the physiological responses in two ecologically and economically important bivalve species - the eastern oyster (*Crassostrea virginica*) and hard shell clam (*Mercenaria mercenaria*). Juveniles and adults were exposed to environmentally relevant salinities at either ~32 or ~16 with different P_{CO2} levels (~400, 800 and 1500 µatm) and temperature (22°C and 27°C), as predicted by future global climate change scenarios, for 11-21 weeks (in juveniles) and 15 weeks (in adults). Survival, metabolism and calcification were

assessed. Elevated P_{CO2} alone and in combination with either reduced salinity or elevated temperature led to reduced survival and growth, and altered the shell mechanical properties (microhardness and fracture resistance) in juvenile and adult bivalves. Tissue energy reserves (lipid and glycogen) were reduced in juveniles under elevated P_{CO2} and low salinity. Standard energy metabolism (SMR) increased under the conditions of elevated P_{CO2} and temperature indicating higher costs of basal maintenance. In adult bivalves, elevated temperature led to the depletion of tissue energy reserves in both species indicating energy deficiency, and resulted in higher mortality in oysters but not in clams. In adult clams and oysters, elevated P_{CO2} alone had a small effect on the metabolism. No persistent oxidative stress (measured by total antioxidant capacity as well as oxidative markers of proteins and lipids) was seen in the bivalves after prolonged exposure to elevated temperature and P_{CO2}. The results of this study indicate that the interactions between common abiotic stressors (salinity and temperature) and elevated P_{CO2} are complex, non-linear, and species-specific. Our study shows that elevated temperature and reduced salinity are predominant stressors that affect survival, metabolism, and biomineralization response of the studied bivalves. Furthermore, these stressors modulate the responses of these bivalves to ocean acidification while elevated CO_2 levels have a modest impact in the absence of these stressors.

DEDICATION

To the courage of all those who survive in the land without post offices.

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LIST OF ABBREVIATIONS

ABT	arrenius break point
ADP	adenosine diphosphate
AEC	adenylate energy charge
AMP	adenosine monophosphate
ANOVA	analysis of variance
ASW	artificial seawater
ATP	adenosine triphosphate
AZM	acetazolamide
BSA	bovine serum albumin
CA	carbonic anhydrase
DI	deionized water
DIC	dissolved inorganic carbon
DNPH	2, 4-dinitrophenyl hydrazine
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
E_a	apparent activation energy
GLM	generalised Linear Method
HNE	4-hydroxynonenal
IPCC	intergovernmental panel for climate change
LDH	lactate dehydrogenase
MK	myokinase

MDA	malondialdehyde
NADH	nicotinamide adenine dinucleotide hydrogen
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NBS	national bureau of standards
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
PCA	principal component assay
PCA	perchloroic acid
P_{CO2}	patial pressure of carbon dioxide
PEP	phosphoenolpyruvate
PK	pyruvate kinase
PMSF	phenylmethanesulfonylfluoride
p-NPA	p-nitrophenyl acetate
ppmv	parts per million by volume
PSU	Practical salinity units
qRT-PCR	quantative reverse transcription polymerase chain reaction
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SMR	standard metabolic rate
SEM	standard error of means
TA	total alkilinity
TAOC	total antioxidant capacity
TCA	trichloroacetic acid

TSP	trimethylsilyl propionate
µatm	microatmosphere

CHAPTER 1: INTRODUCTION

1.1.Anthropogenic CO_2 Release and the Global Climate Change

The global concentration of carbon dioxide (CO_2) in the atmosphere has steadily increased from a pre-industrial value of ~280 ppmv (parts per million by volume) to ~400 ppmv in 2012 (Intergovernmental Panel for Climate Change (IPCC), 2007). This increase is largely due to anthropogenic emissions from burning of fossil fuels, deforestation and other human activities such as cement manufacture, iron and steel production and mining (IPCC, 2007). This is a rapid increase compared to the 80-ppmv increase over 5000 years spanning the most recent ice age to the present time (IPCC, 2007). The current rate of change in CO_2 concentrations is at least 100 times more rapid than any experienced over the past 100,000 years (Convention on Biological Diversity (CBD), 2009). According to the Special Report on Emissions Scenarios (SRES) by the Intergovernmental Panel on Climate Change (IPCC), atmospheric CO_2 levels are predicted to continue to increase for the next century and beyond (IPCC SRES, 2000). Unless mitigation efforts are put into practice, atmospheric CO_2 concentrations between 730-1020 ppmv are predicted by end of the year 2100 (IPCC SRES, 2000) , which is higher than anything experienced on Earth for several million years (IPCC, 2007). The total anthropogenic CO_2 emissions were about 10 billion tons in 2008 and a cumulative of 560 billion tons was released since the beginning of the industrial era (Doney et al., 2009). CO_2 is the most important anthropogenic greenhouse gas (GHG) contributing a staggering 77% of the total GHG

emissions in 2004 (IPCC, 2007) (Fig 1.1).

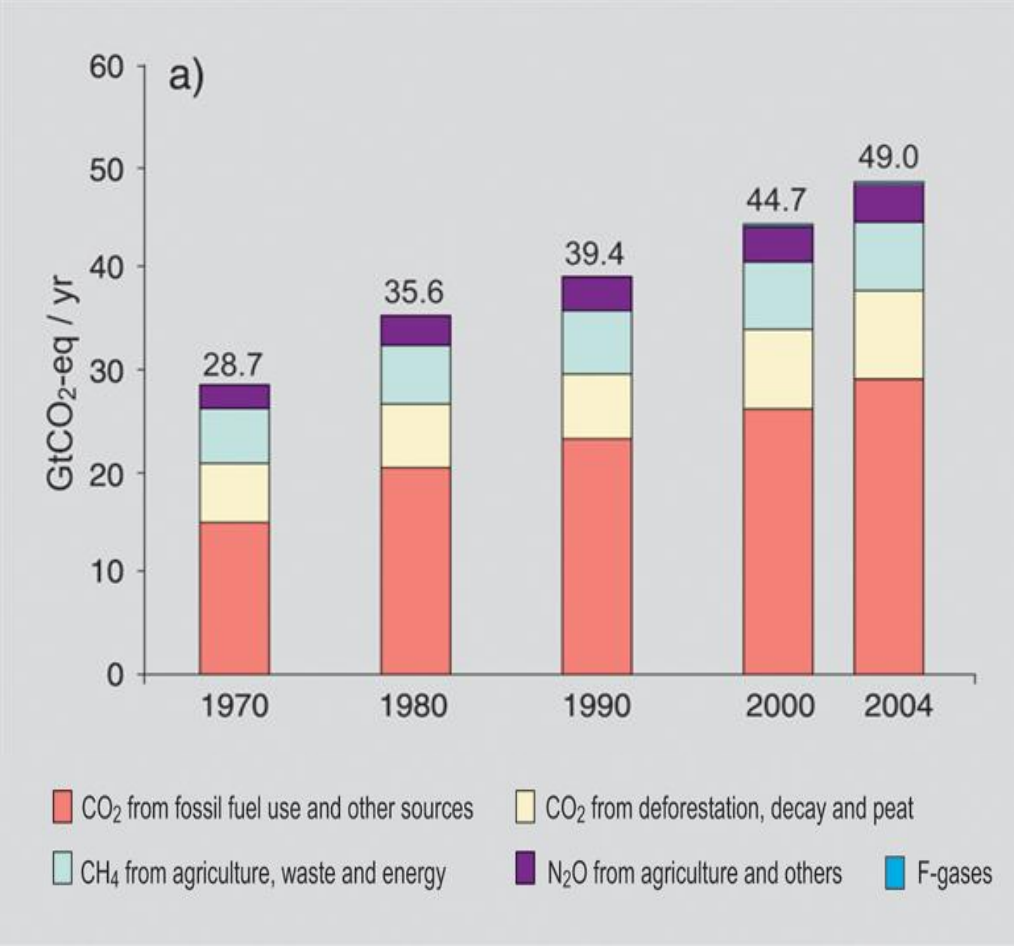

Figure 1.1: Global annual emissions of anthropogenic CO_2 and other greenhouse gases in 1970-2004 (IPCC, 2007).

A quarter of CO_2 emissions are removed by land vegetation, a quarter by the world's oceans and the remaining half remains in the atmosphere (Doney et al., 2009). Accumulation of CO_2 in the atmosphere and oceans has serious consequences for the global climate and the world's oceans. CO_2 is a major greenhouse gas in the atmosphere, raising the surface temperature of the planet with an average linear trend of about 0.2°C per decade (IPCC, 2007; IPCC SRES, 2000). The decade of 2000-2010 was the hottest

decade since the modern record keeping began 130 years ago, with 2009 being the second warmest year on the record (IPCC 2007; IPCC SRES 2000). Recent climate models forecast a further increase of the mean global temperature by approximately 1.8 to 4°C by the year 2100 (Fig 1.2) (IPCC, 2007; Mann et al., 2008).

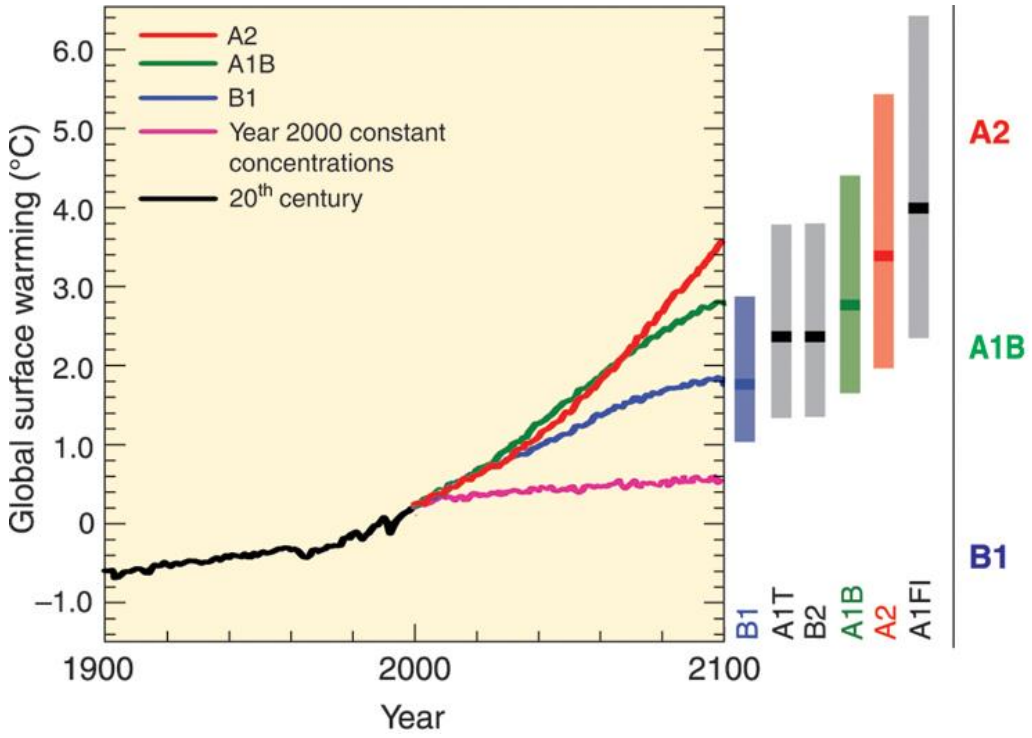

Figure 1.2: Global averages of surface warming for SRES scenarios using Atmosphere-Ocean General Circulation Models (AOGCMs). Bars on the right indicate likely range for marker scenarios (denoted by letter-number combinations) and solid line within each bar represents the best estimate (IPCC, 2007).

Global climate change has potential impacts on ecosystem structure and function. Temperature is one of the most important abiotic factors for species. Deviation of the environmental temperature from the optimum results in thermal stress. This may result in altered species' interactions with their environment, local extinctions, increased invasion of non-native species, changes in population abundances and geographical redistribution

of species (Fig 1.3) (IPCC, 2007; Walther et al., 2002). These impacts of global climate change will have negative consequences for biodiversity and ecosystem services (IPCC, 2007).

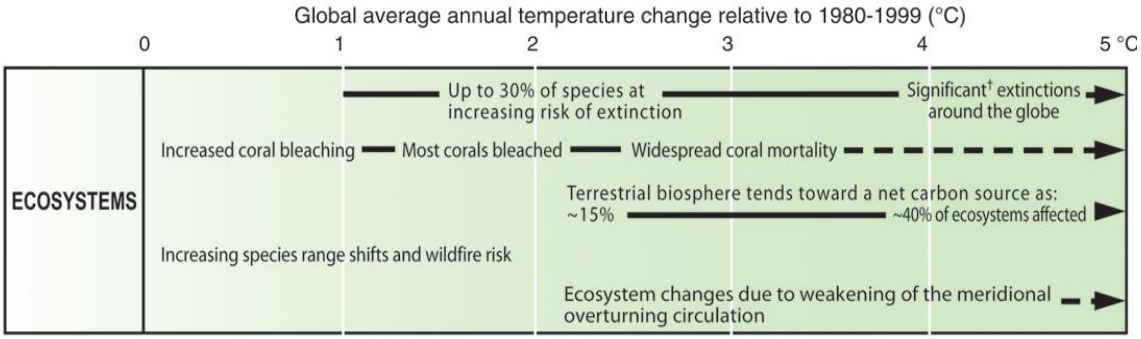

Figure 1.3: Ecosystem impacts associated with global average temperature change. Black lines link impacts; broken line arrows indicate impacts continuing with increasing temperature. Adaptation potential to climate change is not included in estimation (IPCC, 2007).

1.2.Ocean Acidification

The oceans covering about 70% of the Earth's surface are a substantial carbon reservoir (Royal Society, 2005). On the time scales of hundreds of years, oceans have the highest exchanges of carbon with atmosphere (Royal Society, 2005). By virtue of large volume and buffering capacity of the seawater, they play a fundamental role of a natural CO_2 sink and thus temper rising atmospheric CO_2 (IPCC, 2007). Oceans have already absorbed about 112 (± 17) petagrams of Carbon (Pg C) corresponding to about 29% of the total anthropogenic emissions during the past 250 years (Lee et al., 2003; Sabine and Feely 2007; Sabine et al. 2004). Oceanic CO_2 uptake, however, comes at a cost. CO_2 reacts with seawater and forms carbonic acid (H_2CO_3). Carbonic acid dissociates and increases the concentration of H^+ ions. This in turn reduces the pH and alters fundamental

ocean chemistry of the oceans - a process known as ocean acidification (Caldeira & Wickett, 2003, 2005; Feely et al., 2004, 2008; Orr et al., 2005; Solomon et al., 2007). Ocean acidification has already decreased the pH of surface waters by 0.1pH units since the industrial revolution resulting in a 30% increase in acidity (IPCC, 2007). Atmospheric CO_2 concentrations could exceed 1000 ppmv by 2100 according to business-as usual scenario- I92Sa (IPCC, 2007; IPCC SRES, 2000) leading to a decrease of 0.3-0.4 pH units or about 100-150% more acidity since the beginning of industrial era (IPCC, 2007; Orr et al., 2005).

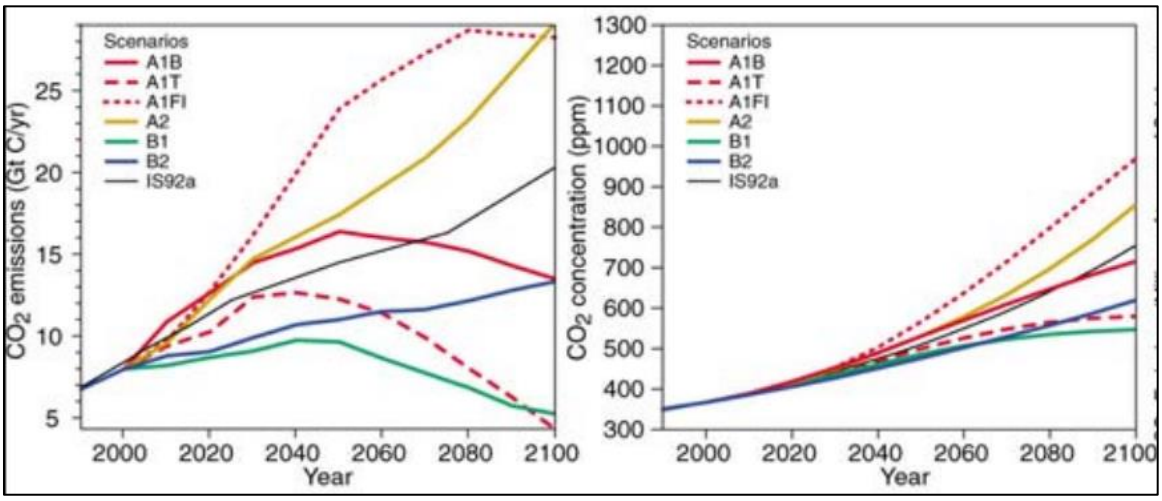

Figure.1.4: CO_2 emissions and concentration for a range of SRES (Special Report on Emission Scenarios) scenarios (IPCC SRES, 2000).

The uptake and solubility of CO_2 in the oceans is modulated by various chemical (CO_2 buffering capacity), physical (temperature, water column stratification and mixing) and biological factors (e.g. biological productivity). Partial pressure difference of CO_2 (ΔP_{CO2}) between the surface seawater and overlying atmospheric column is the thermodynamic force for CO_2 transfer across the sea surface. Gas exchange at air –sea

interface equilibrates surface water CO_2 to atmospheric levels on the timescales of approximately one year (Doney et al., 2009; Royal Society, 2005). Once dissolved in seawater, CO_2 reacts with water to form carbonic acid (H_2CO_3) which dissociates producing bicarbonate (HCO_3^-) and hydrogen ions (H^+).

$$CO_2\,(atm) \leftrightarrow CO_2\,(aq.) + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

The seawater reactions are reversible and near equilibrium (Millero et al. 2002). Surface seawater at a pH of ~8.1, has approximately 90% of the inorganic carbon as bicarbonate ion, 9% as carbonate ion, and only 1% as dissolved CO_2, collectively referred to as dissolved inorganic carbon (DIC). The relative proportions of these three DIC forms reflect the pH of the seawater. DIC acts as a natural carbonate buffer and maintain the pH of seawater within narrow limits. It is the capacity of this buffer system that decreases as increasing amounts of atmospheric CO_2 are being absorbed by the oceans and in turn lead to relative acidification of oceans. The additional hydrogen ions produced are buffered by carbonate (CO_3^{2-}). The net result can be written as follows:

$$H_2O + CO_2 + CO_3^{2-} \leftrightarrow 2HCO_3^{2-}$$

The neutralization, however, is not complete and the extra hydrogen ions remain increasing $[H^+]$ and lowering the pH. Overall, CO_2 added to the sea water increases $[H^+]$ and bicarbonate ion concentration $[HCO_3^-]$ and decreases pH and carbonate ion concentration $[CO_3^{2-}]$. The projected 0.3-0.4 pH unit drop of oceans at the end of 21st century will lead to an approximately 50% decrease in carbonate ion concentrations (Orr et al 2005). Model predictions also suggest that a change in surface ocean DIC per unit change in atmospheric CO_2 (μmol kg^{-1} per ppmv) will be about 60% lower in the year

2100 under IS92a scenario indicating a reduction in the capacity of the ocean to buffer changes in atmospheric CO_2 (Orr et al., 2005).

On the time scales of hundreds of years and longer, oceanic uptake of atmospheric CO_2 is influenced by $CaCO_3$ counter pump (IPCC, 2007). This pump describes the effects of production and dissolution of calcium carbonate structures on pH of seawater.

$$CaCO_3 \leftrightarrow Ca^{2+} + CO_3^{2-}$$

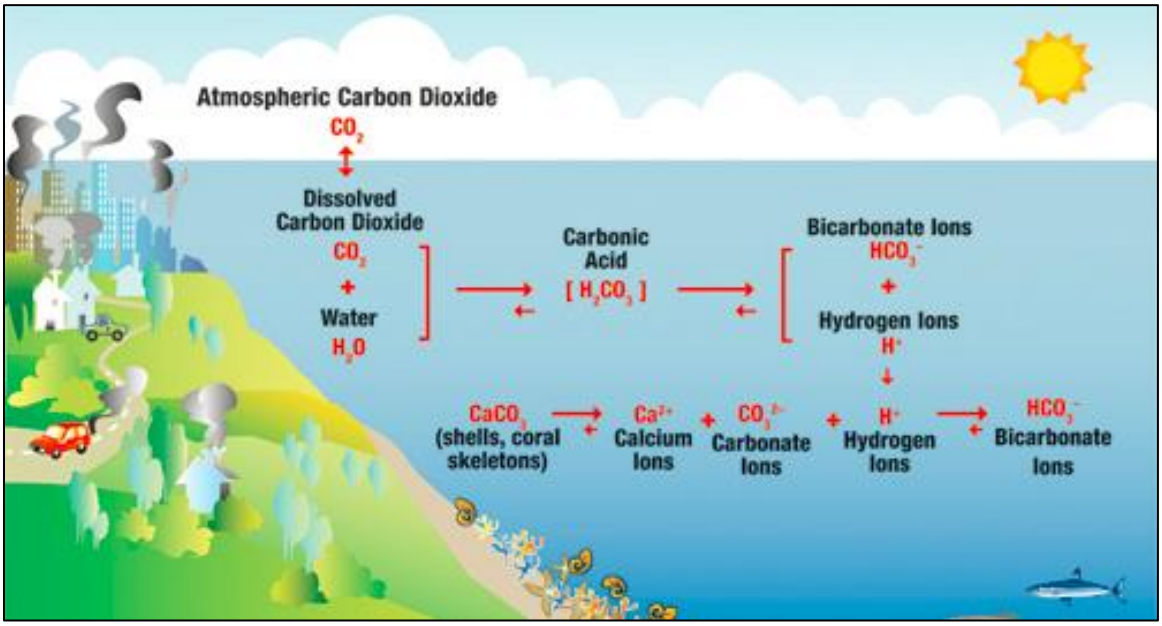

Figure.1.5: Schematic representation of ocean acidification and $CaCO_3$ counter pump (National Research Council of National Academy of Sciences)

Total DIC and the ability of seawater to neutralize acids (total alkalinity, A_T) depend on calcium carbonate dissolution. Calcium carbonate minerals are derived from the skeletons of marine organisms (coralline algae, corals, pteropods, foraminiferans, coccolithophores and other invertebrates). Once the organisms die, the carbonates dissolve (~ 60-80% of exported $CaCO_3$ from the surface ocean may dissolve in the above

1000 m depth) or fall through the water column to be deposited in deep-sea sediments (Feely et al., 2004). The stability of carbonate minerals, and hence calcification and dissolution, is determined by the carbonate saturation (Ω), which is defined by the following equation:

$$\Omega = \frac{[Ca^{2+}]\,[CO_3^{2-}]}{K^*_{sp.}}$$

where $[Ca^{2+}]$ and $[CO_3^{2-}]$ are the effective Ca^{2+} and CO_3^{2-} concentrations in seawater and $K_{*sp.}$ is the in situ solubility product of calcium carbonate. $\Omega > 1$ signifies super saturation (that favors precipitation of carbonate minerals) and $\Omega < 1$ signifies under saturation (that favors dissolution of carbonate minerals as the seawater becomes corrosive). Solubility ($K_{*sp.}$) depends on temperature, salinity, pressure and calcium carbonate polymorph. Calcium carbonate occurs in two natural polymorphs –aragonite and calcite. Calcite can also contain different concentration of magnesium ions that affect the solubility of the mineral. The mole % of magnesium deposited in the shells and skeletons of calcifiers is species-specific and range from a few mol % to as high as 30 mol % (Andersson et al., 2008). Furthermore, most marine calcifiers deposit their incipient shells using a transient amorphous calcium carbonate (ACC) precursor. ACC is isotropic with short range crystalline order and without any preferred growth axis. This allows a biological advantage to the organism. Shells and skeletons can be grown into a particular shape and additional elements can be integrated for stabilization. Different polymorphs of calcium carbonate differ in their solubility with the order of increasing solubility being ACC > high magnesium calcite > aragonite > low magnesium calcite (Mucci 1983; Andersson et al., 2008).

Calcium carbonate is also more soluble with decreasing temperature and increasing ocean depth creating a boundary layer called saturation zone (where $\Omega=1$) above which $CaCO_3$ forms and below which it dissolves. Indian Ocean and the high latitude areas of North Pacific have saturation zones for aragonite between 200m of depth whereas for the Atlantic Ocean a deeper saturation zone (~3,500 m) is reported (IPCC 2007). CO_2-driven ocean acidification lowers the carbonate buffering capacity and moves the saturation zone towards the ocean surface - a process called shoaling. Shoaling of aragonite saturation horizons by 30–200 m from the preindustrial period to the present is already seen (IPCC, 2007). It is predicted that surface waters of Southern Ocean will become undersaturated with respect to aragonite by as early as 2050 (IPCC, 2007). Shoaling reduces the available habitat for calcifiers and have negative effects on ecosystem functioning.

Evidence that anthropogenic CO_2 absorption results in associated changes in pH and carbonate chemistry in sea water have been verified by repeated research cruises, modeling programs as well as time series data collected from in situ experimental stations in major ocean basins over the past decades (Caldeira & Wickett 2003, 2005; Feely et al. 2004, 2008; Orr et al. 2005; Solomon et al. 2007) . The trends in ocean variables (pH , DIC, A_T and carbonate ion concentration) paralleling changes in atmospheric CO_2 have been documented in three subtropical time series stations –North Pacific station ALOHA (22.75°N,158°W) of Hawaii Ocean Time –Series (HOT) program, western North Atlantic gyre of Bermuda Atlantic Time-Series Station (BATS; 31.72°N, 64.17°W) and eastern North Atlantic at the European Time Series in the Canary Islands (ESTOC; 24.04°N, 15.50°W). All three stations shows reductions in pH and

carbonate saturation states with increases in atmospheric CO_2 since the past 20-30 years (Fig 1.4). Earlier seawater data from the GEOSECS (Geochemical Sections) and TTO (Transit Tracers in the Ocean) expeditions in the North Atlantic also show similar trends(Craig & Turekian 1976, 1980; Brewer et al., 1985; Doney et al., 2009) . In all cases, the observed increases in acidity agree with the estimates of oceanic uptake of CO_2 that comes from the human activities.

The closest geological analogue to the current rise in CO_2 concentrations is the Paleocene-Eocene thermal maximum (PETM), about 55 million years ago (Kennett and Scott, 1991; Zachos et al., 2003). Sea surface temperature increased over 5-9°C during 1,000-2,000 years and shoaling of calcite zone by 2 km was observed (Kennett and Scott, 1991; Zachos et al., 2003). During PETM, air –sea CO_2 flux led to severe acidification and shifts in marine planktonic communities. Importantly, the five mass extinction events that occurred in the geological past since the end of Ordovician period (434 million years ago) have their causes linked in some ways to perturbation in carbon cycle and changes in atmospheric CO_2 levels (Veron, 2008).

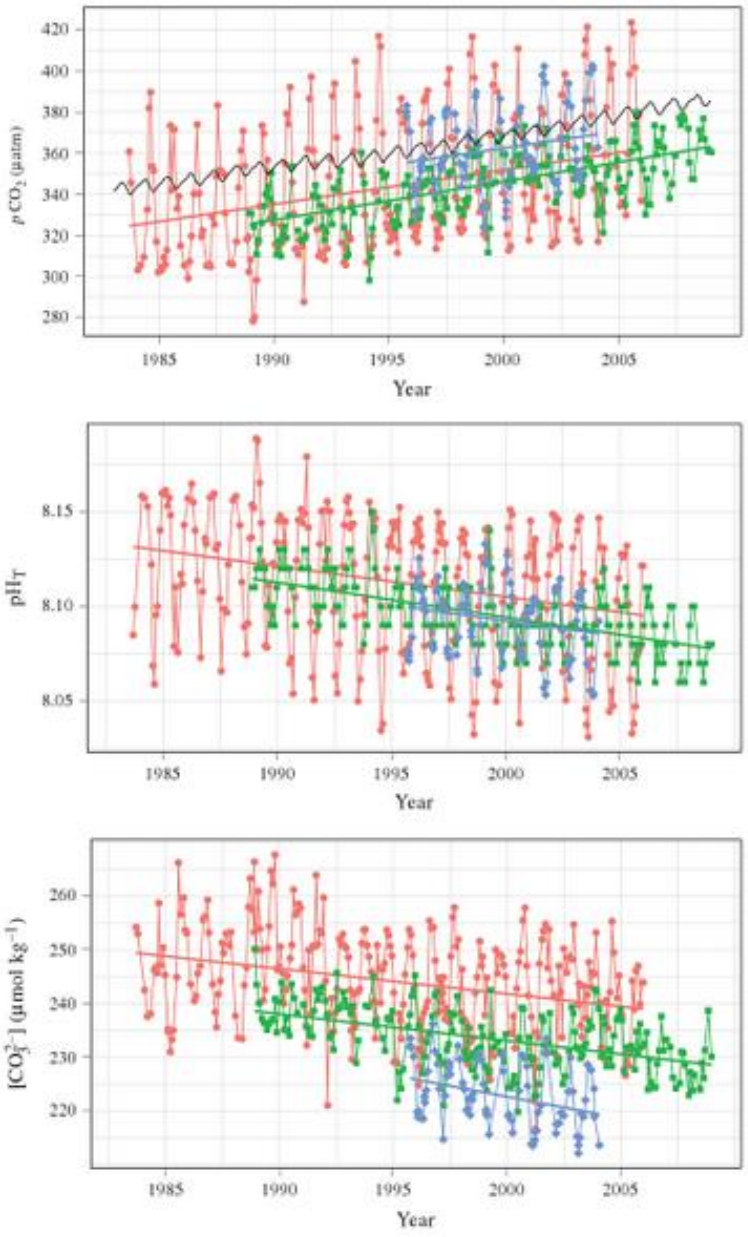

Figure.1.6: Time series of surface-ocean P_{CO2}, pH_T and $[CO_3^{2-}]$, as well as for the atmospheric mole fraction of CO_2 at Mauna Loa (Black), ocean time-series stations ALOHA (green), BATS (red), and ESTOC (blue) (Gattuso and Hansson, 2011).

1.3 Interaction of Ocean Acidification with Other Stressors in Estuaries

Coastal ecosystems including estuaries are estimated to provide over US$14 trillion worth of ecosystem goods (e.g. food and raw materials) and services (e.g. nutrient cycling) per year, or ~ 43% of the global total (Costanza et al., 1997). Estuaries - semi-closed coastal bodies of water, which have free connection with sea, and within which seawater is measurably diluted with fresh water derived from land drainage (Pritchard, 1967) - are among the most productive areas of the ocean. Estuaries have large fluxes of freshwater, sediments, organic matter, nutrients, and pollutants entering from different natural and anthropogenic sources and are especially sensitive to ocean acidification (Royal Society, 2005; Waldbusser et al., 2013). In addition to the elevated carbon from terrestrial inputs, estuaries also experience increase in organic matter (Net Community Production, NCP) due to increase in atmospheric CO_2.This organic matter is eventually used in respiration by organisms (plants, animals and microbes) releasing CO_2 which strongly modulates the carbonate chemistry of coastal and estuarine waters. Many estuaries are dominated by net heterotrophy, leading to increased DIC and reduced pH (Gattuso et al., 1998). These influences make the water chemistry and pH of estuaries temporally and spatially highly variable. In many southeastern US estuaries, pH and P_{CO2} levels can fluctuate from the values close to those of the open ocean (pH ~8.0, ~400 µatm P_{CO2}) during the cold season to pH values as low as 6.0 and P_{CO2} levels as high as 45,000 µatm in summer on a time scale from hours to months (Cochran and Burnett, 1996; Burnett, 1997; Ringwood and Keppler, 2002) (see also long-term seasonal pH monitoring data at http://cdmo.baruch.sc.edu/). This dynamic range, even on a 24-hour period, exceeds the end-of-century predictions for pH decrease. Increasing amount of

atmospheric CO_2 further exacerbates the air-sea exchange flux and shifts the CO_2 and pH baselines in estuaries.

Estuaries are naturally stressful environments characterized by a low diversity of species. For organisms living in estuarine environments, temperature and salinity are the two most important factors limiting their distribution and zonation (Gunter, 1957; Gunter, 1961). Temperature regimes in estuaries and intertidal zones are very variable. Lower capacity of thermal buffering of the shallow waters and the thermal exchanges with the land and fresh waters predispose estuarine and coastal zones to rapid warming and temperature extremes (Helmuth et al., 2002; Gilman et al., 2006). Temperature fluctuations as high as 15-20°C within a few hours can be observed in summer low tides. Temperature affects every physiological process in organisms and determines species' distribution in the intertidal zones. The long-term temperature recordings show that warming of the estuarine and coastal waters have outpaced those observed in the surface waters of the open ocean (Talmage and Gobler, 2011). Temperature also interacts with estuarine acidification due to anthropogenic CO_2. It modulates the solubility of gases as well as thermodynamic equilibrium constants that ultimately influence the carbonate speciation in estuaries. Increases in temperature can also affect pressure gradients and wind circulation patterns in the coastal zones leading to upwelling of CO_2 –rich corrosive acidified waters from deeper depths to the surface. Episodic upwelling can reach estuaries, where this water then becomes a major factor in reducing the pH and lowering the saturation state (Ω) of calcium carbonate (Feeley et al., 2010; Barton et al., 2012). Observations in the California Current System (CCS), one of the four major Eastern Boundary Upwelling Systems, suggest that upwelling is positively related to temperature

and has been increasing since the past 30 years (Pisias et al., 2001; Snyder et al., 2003). Independently, shifting CO_2 baseline due to increases in atmospheric CO_2 has been shown to increase the magnitude, frequency and duration of this upwelling in CSS (Hauri et al., 2013 and Haris et al., 2013).

Estuaries experience fluctuating salinities (between 0.5‰ and 35‰) due to the freshwater inputs. Estuarine species are euryhaline tolerating a wide range of salinities but salinity remains an important factor affecting their survival and distribution. Salinity fluctuations may affects an organism through changes osmotic concentration, proportion of solutes, density and viscosity of seawater. Salinity dilution also changes the concentration of dissolved gases like oxygen. The freshwater inputs into estuaries also results in changes of carbonate chemistry parameters over multiple time scales (Waldbusser et al., 2013). The flushing, tidal cycles, stratification, wind patterns and circulation as well as the basic geometry of the estuary all influence the complexity of dilution, hence changes in seawater chemistry, from riverine inputs (Kennedy, 1996). Salinity influences the dissociation constants (stoichiometric constants) of various chemical species in the carbonate system and the dissociation constant (K_d) of carbonic acid in sea water is greater than in freshwater. River run off also dilutes the total alkalinity (A_T) and calcium concentrations and therefore effectively lowers the saturation state (Ω) of calcium carbonate (Fig. 1.5) (Salisbury, 2008; Cai et al., 2008). Human influences due to land use changes (e.g. drainage and excavation of acid sulfate soils) have also significantly altered the chemical make-up of rivers. Most riverine plumes are acidic and nearly all of the world's large rivers have Ω lower than receiving ocean waters (Salisbury, 2008). Agricultural practices (limestone and nitrogen fertilizer application)

and the subsequent surface riverine flux also significantly change the A_T and DIC in estuarine environments (Waldbusser et al., 2013). These changes in the carbonate chemistry will further be exacerbated by increasing amounts of atmospheric CO_2.

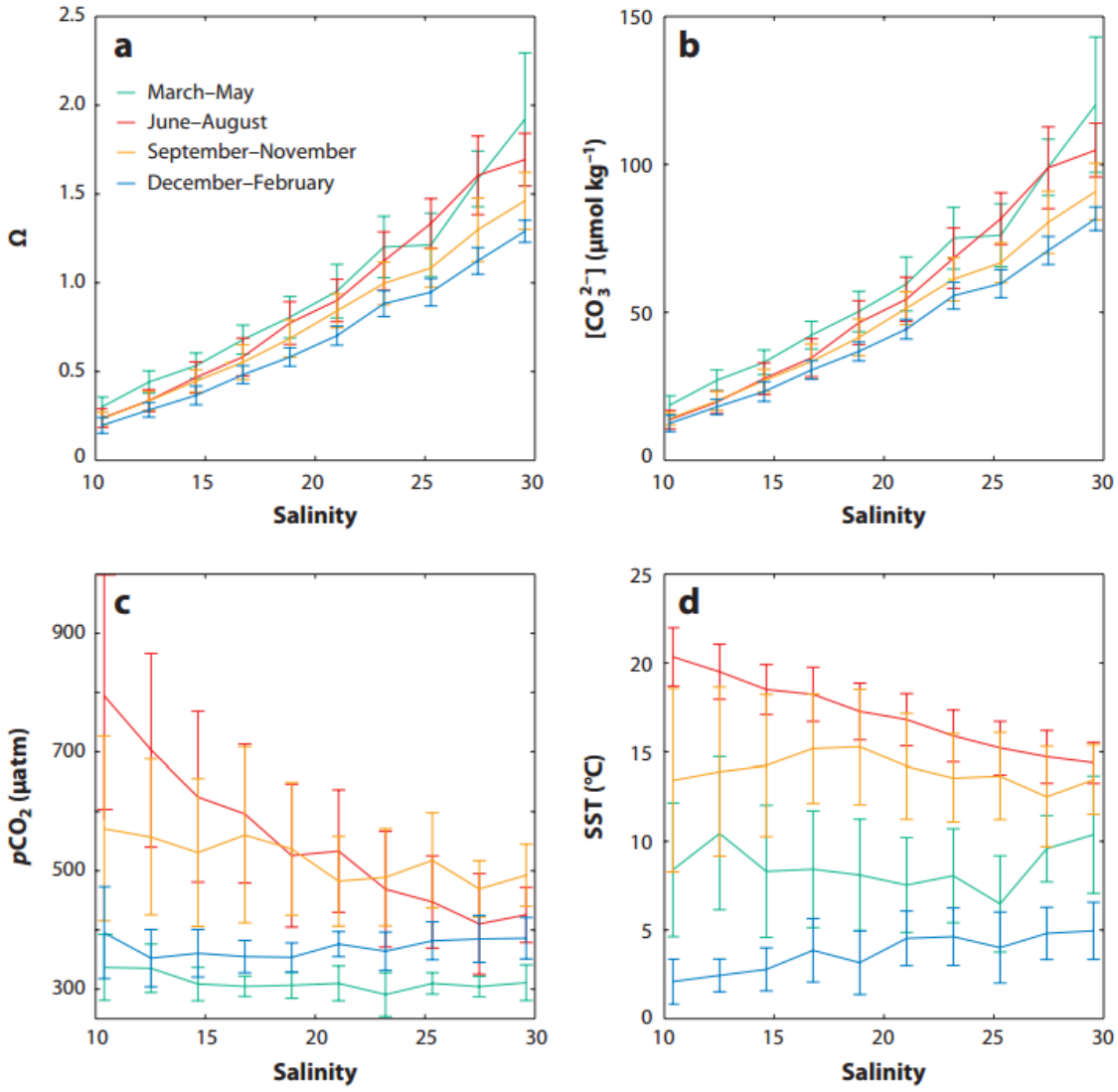

Figure.1.7: Carbonate parameters changes in the Kennebec Estuary, Maine (43.8°N, 68.8°W) with salinity fluctuations : (a) surface saturation state (Ω),(b) CO_3^{2-} concentration, (c) pCO_2, and (d) sea-surface temperature (SST) (Waldbusser et al., 2013; Cai et al., 2008)

1.4. Biological Responses of Marine Animal Calcifiers to Ocean Acidification

Ocean acidification is shown to negatively affect survival, metabolism, calcification, growth, reproduction and immune responses across pelagic (foraminiferans, pteropods, coccolithophores, marine fish and mammals) and benthic (corals, echinoderms, mollusks and crustaceans) taxa of marine organisms (Kroeker et al., 2010, 2013). The magnitude and direction of the response to ocean acidification, however, varies among and within different taxa. Increasing CO_2 concentration is shown to have stimulating effect on primary production in a variety of phytoplankton like coccolithophores, diatoms and dinoflagellates. CO_2 is used as a substrate in the carbon fixation step of photosynthesis. 10-30% increases in photosynthesis have been reported using plankton assemblages and mesocosm ocean acidification experiments (Gattuso and Hanson, 2011). In contrast, calcification is severely affected by ocean acidification. Calcification depends on acidity of sea water, carbonate saturation state, temperature as well as the biogenic polymorph of $CaCO_3$ used by the organism. Ocean acidification reduces the saturation state (Ω) of calcium carbonate making it increasingly difficult to produce and maintain shells and skeletons.

Calcifiers are ecologically important in all ecosystems, and they range in distribution from high latitude to deep seas. Sensitivity of calcifiers to ocean acidification depends on the habitat; the mineral form used for the formation of the skeleton and varies between the taxa. Marine calcifiers living at high latitude, polar and cold water environments (such as pteropods and cold water corals) that have shells and skeletons made of high solubility $CaCO_3$ (aragonite and high magnesium calcite) are at a greater risk from ocean acidification because these regions are only slightly supersaturated with

regard to these minerals (Andersson et al., 2008). Ocean acidification slows down calcification in corals (Schneider and Erez, 2006; Anthony et al., 2008; de Putron et al., 2011; Crook et al., 2013) and mollusks (Ries et al., 2009; Parker et al., 2012). In crustaceans, calcification is either negligibly affected (Kurihara and Ishimatsu, 2008; Hauton et al., 2009; Small et al., 2010; Carter et al., 2013) or even increased. In echinoderms the negative effects of ocean acidification appear in the gastrulation stage (Byrne et al., 2009; Byrne and Przeslawski, 2013), with calcifying (e.g. echinoplutei) larvae generally more sensitive than non-calcifying ones (Byrne and Przeslawski, 2013). Calcification is a complex process under tight biological control involving many enzymes; ion- and acid base transporters so that some organism can compensate for the unfavorable changes in seawater chemistry (Pörtner, 2008). However, because calcification is an energy demanding process, this compensation comes at a cost and may result in reallocation of energy budget of the organism (Pörtner, 2008).

Ocean acidification also has an impact on cellular and physiological processes beyond calcification. Increased dissolved CO_2 in seawater diffuses across epithelial surfaces and may disturb the acid-base balance in the organism. It has been hypothesized that physiological effects of elevated P_{CO2}, to a large extent, are mediated by changes in the acid-base status of the organism (Pörtner, 2008). Most animal calcifiers have limited capacity to counteract this acidification. The pH regulation mechanisms include passive buffering through $CaCO_3$ dissolution to provide HCO_3^- ions and passive and active transport and exchange of ions (Fabry et al., 2008). The relative role and efficiency of different acid-base regulation mechanisms differs among the species. Benthic bivalves, gastropods and echinoderms that lead hypometabolic sessile life styles are weak ion- and

acid-base regulators with low non-bicarbonate buffering capacity of the body fluids. In contrast, crustaceans possess a well-developed ability to regulate the pH of their extra- and intracellular fluids. Species evolved in naturally rich CO_2 environments like hydrothermal vents or those present in intertidal pools that experience large fluctuations of CO_2 also have a greater capacity for buffering and ion transport (Royal Society, 2005). It remains to be established whether the physiological differences in pH regulation capacity can result in different tolerance of marine species to chronic ocean acidification expected under future climate change.

Shifts in the acid-base balance and difficulty to produce and maintain exo- and endoskeletons in the face of changing CO_2 levels can affect metabolism and increase the energy costs to maintain homeostasis in organisms. Metabolic responses to elevated CO_2 vary both between and within species depending on physiological status, developmental stage and other factors. An increase in standard metabolic rate (SMR) during exposure to elevated CO_2 has been reported in several species of marine bivalves (Beniash et al., 2010; Lannig et al., 2010; Thomsen and Melzner, 2010; Cummings et al., 2011). In contrast, in many species no change in the basal metabolism in response to ocean acidification was found (Marchant et al., 2010; Fernández-Reiriz et al., 2012; Liu and He, 2012). This has been attributed to increased absorption efficiency of food (Fernández-Reiriz et al., 2012) and/or near-complete compensation of extracellular acidosis by HCO_3^- (Marchant et al., 2010). In many species, uncompensated acid base status can depress the metabolism and induce “trade-off” in energy budget. This adaptive strategy is employed to match ATP supply and ATP demand and thus prolong survival (Melatunan et al 2013). Reallocation of energy resources towards maintenance of acid-base

homeostasis can effectively diminish the scope for growth (SFG) of an organism and can negatively affect growth and reproductive output ultimately reducing the persistence of the species on longer timescales.

1.5. Mollusks as Model Species for Ocean Acidification

The phylum Mollusca is a large and diverse group of animals with about 50 000 species of which 30 000 live in the sea (Gosling, 2003). Shelled mollusks occupy a variety of ecological niches from deep sea habitats to intertidal zones and from poles to tropical areas. Mollusks act as ecosystem engineers by introducing heterogeneity and complexity and changing resource availabity to other organisms (Gutiérrez et al., 2003). Mollusks are suspension-feeders and regulate water quality in their environments by clearing particles greater than ~ 1-7µm (Winter, 1978). This also allows for better light penetration, which aids algae and benthic plants. Mollusks also play a key role in the ocean's carbonate cycle and $CaCO_3$ counter pump by depositing massive amounts of $CaCO_3$ in their shells (Gazeau et al., 2007). For example, pteropods (Arctic pelagic mollusks with aragonitic shells) alone are estimated to contribute 50% to the total global $CaCO_3$ flux (Berger, 1978; Fabry and Deuser, 1991). Changes in population densities of shelled mollusks associated with ocean acidification can thus result in habitat restructuring and changing food webs. The phylum Mollusca also includes many economically important species. The global shellfish aquaculture is estimated at a value of $13.1 billion US, representing 12-16% of total seafood consumption (FAO, 2008). Marine sources of protein are increasingly in demand as terrestrial protein sources become limiting due to the human population growth.

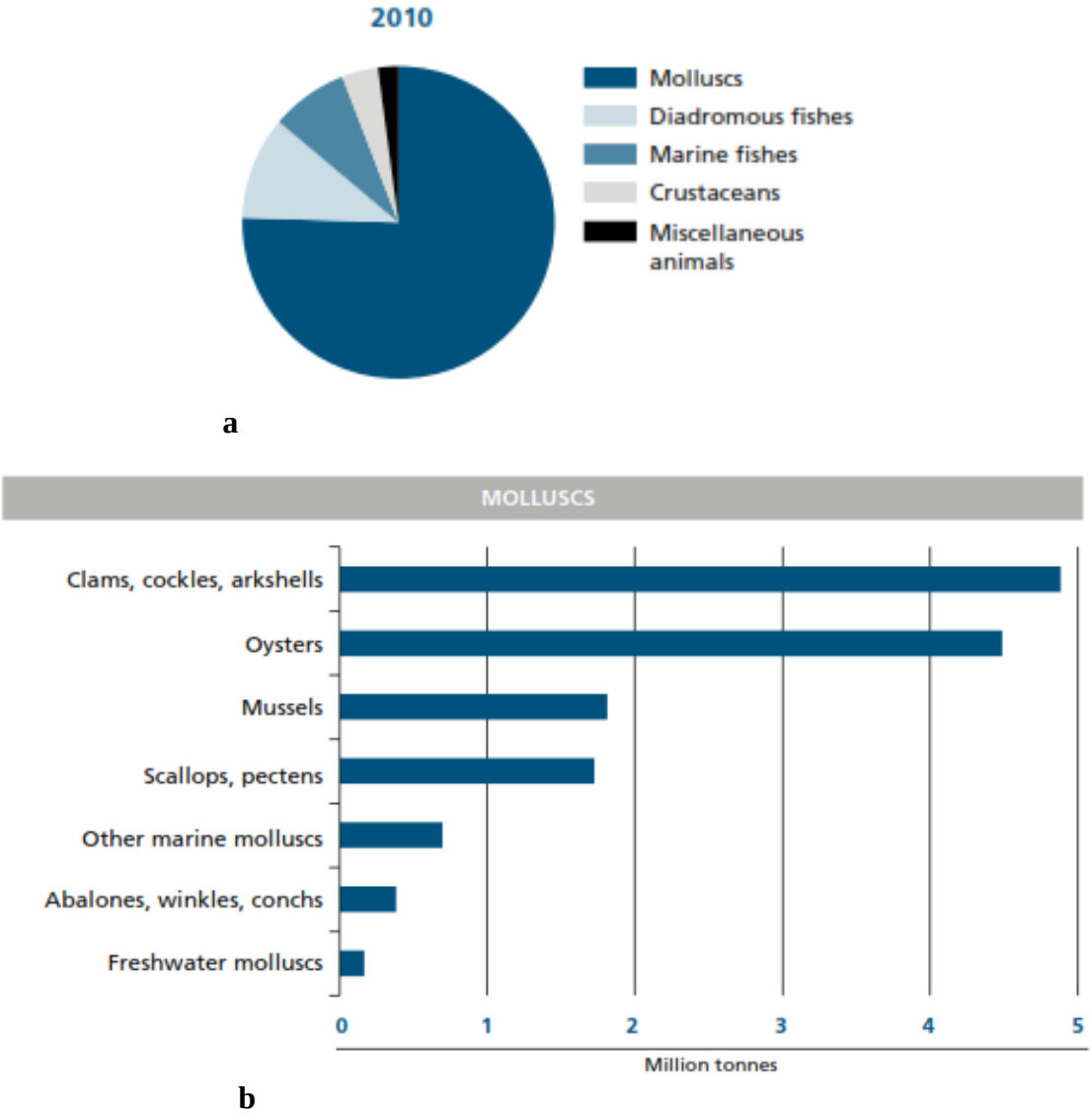

Figure 1.8: (a).World aquaculture production by composition and types of different farmed species. (b).Production of major mollusk species.from.aquaculture in 2010.(FAO, 2012)

Mollusks are a major group of calcifying organisms in shallow coastal areas (Gazeau et al., 2007). Most marine mollusks produce 50-1000 g $CaCO_3$ m^{-2} yr^{-1} and estimated production of $CaCO_3$ by oysters is 90 000 g m^{-2} yr^{-1} (Beukema, 1982; Powell et al., 1989). Based on these rates, mollusks may deposit 0.02 mm to 0.035 mm of $CaCO_3$ yr^{-1}(Gutiérrez et al., 2003), a surprisingly high value given that sedimentation rates in

estuaries are estimated around 1-3 mm yr^{-1}(Nichols, 1989). Shell production is a complex process and depends on environmental factors like water chemistry, pH, temperature, salinity and other trace mineral composition. Shell formation, mineralogy, micro- and macro-morphology is under the genetic control and tightly physiologically regulated (Weiner, 1984; Jackson et al, 2009). Shell deposition primarily occurs in an enclosed compartment (extrapallial cavity) lined by mantle tissue cells with membrane –bound ionic pumps and an extracellular organic matrix (Fig. 1.6) (Marin et al., 2007). Blood cells (hemoctyes) are also involved in intracellular calcium carbonate nucleation and transport of the crystals to the mineralization front (Mount et al., 2004).

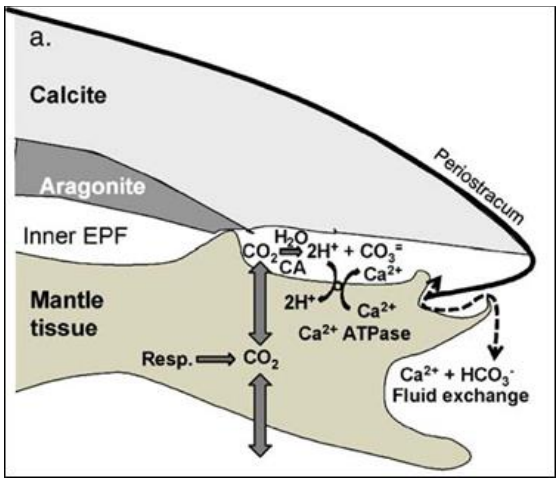

Figure.1.9: Schematic of the shell formation (excluding the hemocyte-related calcification mechanisms) of a mollusk (McConnaughey and Gillikin, 2008)

The shell is a composite layered structure made up of 95-99% $CaCO_3$ and 1-5% organic matrix of proteins and polysaccharides (Palmer, 1983). The mineral composition and organization varies among different mollusk species. The structural organization of the shell can have two or more of seven microstructures (aragonitic prisms, nacre,

crossed lamellar, complex crossed lamellar and homogenous structure, and calcitic prisms and foliae) (Harper et al., 1997). Primitive molluscan shells are believed to be made of aragonite, which is mechanically stronger in terms of fracture resistance, and crack propagation (Harper et al., 1997). Other shell mineralogies have repeatedly evolved among the mollusks suggesting selection pressures for other properties than strength (Harper et al., 1997). In bivalves and gastropods, there is a repeated trend of the loss of nacre and prismatic aragonitic microstructure through evolution possibly due to the high energy costs of these relatively protein-rich structures (Harper et al., 1997). Protection against adverse environmental water conditions has resulted in the presence of calcite in the outer shell layers, sometimes to the extent of a total switch as seen in oysters and scallops whose shells are entirely calcitic (Harper et al., 1997). Bivalve mollusks with calcitic outer shells are mainly subtidal, although some intertidal members evolved from a subtidal lineage (Harper et al., 1997). For infaunal members like clams, carbonate chemistry in the sediments can be significantly different from the seawater and there is no general rule for the selection of a particular mineral polymorph. These species isolate their shell surface by a thick periostracum (outermost proteinous layer of shell) which isolates the mineral phase from the surrounding water (Harper et al., 1997). Shell structure differences are thus likely to play a role in determining the calcification and physiological response of mollusks to ocean acidification and thus they serve as an excellent model species.

Intertidal and estuarine mollusks are subject to multiple stressors in their habitats that may modulate their responses to ocean acidification. Temperature and salinity affects most aspect of bivalve physiology including feeding, respiration, survival, gonad

development, spawning, immunity and host-parasite interactions (Shumway, 1996 as cited in Kennedy et al., 1996). Mollusks are poikilothermic ectotherms and temperature strongly changes the rates of all their physiological and biochemical reactions and therefore, affects growth, survival and reproduction (Hochachka and Somero, 2002). Physiological effects of elevated P_{CO2} may be also modulated by the environmental temperature because of the temperature-dependent changes in solubility of gases, pH and inorganic carbon speciation. Moreover, ocean acidification may reduce thermal tolerance of marine organisms by shifting energy balance and reducing their aerobic scope (Pörtner, 2012; Sokolova et al., 2012). Salinity fluctuations in estuaries and intertidal zones have a strong direct impact on physiology of mollusks, leading to changes in cell volume, extracellular and intracellular osmotic pressure, rates of energy metabolism and enzyme activities, and affecting the rates of protein synthesis and turnover (Kinne, 1971; Deaton et al., 1984; Berger and Kharazova, 1997; Sokolova et al., 2012). A common strategy in mollusks to rapid and large salinity fluctuations is breakdown of free amino acids in cytosol during hyper saline periods to prevent cell shrinking (Kinne, 1971). Conversely, during low salinity conditions these excess amino acids are excreted to prevent cell swelling (Kinne, 1971). In estuaries, where salinity fluctuates rapidly, the frequent breakdown and release of amino acids can be energetically costly and result in reduced fitness (Koehn, 1978, 1983; Hilbish et al., 1982; Deaton et al., 1984). Extreme salinity may also lead to complete shell closure and cessation of ventilation leading to anaerobiosis and metabolic rate depression (Lockwood, 1976; Sokolova and Berger 2000). Salinity also changes seawater carbonate chemistry and may thus exacerbate the negative effects of ocean acidification on estuarine mollusks. This creates a basis for

interactive effects of temperature, salinity and ocean acidification on molluscan physiology that may in turn affect their survival in estuaries.

In the present study, I used two ecologically and economically important bivalve mollusks firm the western Atlantic – the eastern oyster *Crassostrea virginica* (Glemin, 1971) and the hard shell clam *Mercenaria mercenaria* (Linnaeus, 1758). These species commonly found in intertidal and upper subtidal zone of the western Atlantic Ocean from St. Lawerence River estuary in Canada to Florida Keys and Gulf of Mexico (Kraeuter and Castagna, 2001; Carlton and Mann, 1996). These species are ecosystem engineers and are tolerant to large fluctuations of environmental factors including temperature, salinity, CO_2 and O_2 levels. Oysters and clams are also economically important and extensively farmed, and are among the major groups of mollusks produced in the aquaculture settings and harvested in the wild populations (FAO, 2012; Cooley et al., 2011). Clams and oysters differ in their habitat preferences. Eastern oyster is an epifaunal species living in the dynamic environment of intertidal zones which are less prone to acidification (Carlton and Mann, 1996; Green et al. 2009). Hard shell clams, on the other hand, are infaunal species that live buried in sandy to muddy sediments (Kraeuter and Castagna, 2001). Clams are exposed to extreme acidification in sediments due to accumulation of CO_2 and other metabolic acids (Kraeuter and Castagna, 2001). Importantly, the two selected species have different shell composition and mineralogies. Adult oyster shells are made of the less soluble low-Mg-calcite and their periostracum is thin and poorly developed (Digby, 1968; Kennedy et al., 1996). In contrast, the clam shells are made of more soluble aragonite and their outer surface is protected by a thick proteinous periostracum (Kraeuter and Castagna, 2001). This makes it likely that clams

and oysters will differ in their sensitivity to ocean acidification. Understanding of the impacts of ocean acidification in combination with other environmentally important stressors (such as temperature and salinity) on oysters and clams is important for predicting the future of the ecosystems that depend on these species as well as of the coastal economies that depend on fishing and aquaculture (Cooley et al., 2011; Barton, 2012).

1.6. Goals and Significance of the Research

Investigations of the ecological and economic impacts of ocean acidification have come into the focus of research community as well as economists and resource managers manage in the past decade. However, most of the studies (~ 60%) conducted to date on the ocean acidification focused on a single environmental variable (elevated CO_2 and resulting reduced pH) (Dupont and Pörtner, 2013). Although important, these studies are unrealistic in an environmental context where multiple stress exposures are the rule. The interactive effects of ocean acidification with other stressors including temperature and salinity can be quite complex in dynamic environments like estuaries and are poorly understood in mollusks. More ever, species-specific differences in shell mineralogy and physiology among mollusks can affect their vulnerability and hence responses to elevated CO_2 alone or in combination with other stressors in the estuarine habitats. Therefore, multifactorial experimental designs with different species are important to yield important mechanistic insights and help identify ecologically sensitive species. Further, the sensitivity of mollusks to multiple ocean acidification also depends on the life cycle stage with larval and juveniles being typically more sensitive than adults (Parker et al., 2013). Thus, study of the interactive effects of multiple stressors (including ocean

acidification, temperature and salinity) on different life stages will help identifying the physiological processes that can have consequences for survival and recruitment of molluscan populations facing ocean acidification in estuaries. Therefore, the overarching goal of this research is to explore the interactive effects of multiple stressors (ocean acidification, temperature and salinity stress) in juveniles and adults of two model marine bivalve species, eastern oyster –*Crassostrea virginica* and hard shell clam - *Mercenaria mercenaria*. This study focuses on energy metabolism and biomineralization because these physiological functions have direct effects on survival and fitness of the mollusks

The main objectives of this project are:

- To study the interactive effects of elevated temperature, reduced salinity and elevated CO_2 levels (predicted by future global climate change scenarios) on growth, mortality, energy metabolism and biomineralization in the two selected bivalve species (eastern oysters and hard clams) with different shell mineralogies;
- To determine if shifts in cellular redox balance are involved in the physiological response to elevated CO_2 and temperature in the two studied bivalves;
- To determine the sensitivity of juveniles and adults of clams and oysters to elevated temperature reduced salinity and CO_2-driven acidification.

Based on previous published literature, the following hypotheses were tested:

- Elevated partial pressure of CO_2 (P_{CO2}) will lead to elevated energy costs of basal maintenance and impaired biomineralization in clams and oysters;
- Elevated temperature and reduced salinity will exacerbate the negative effects of elevated P_{CO2} due to increased energy demand and reduced aerobic scope;

- Elevated temperature can induce oxidative stress in bivalves, and elevated P_{CO2} can potentiate the pro-oxidant effects of the elevated temperatures;
- Effects of elevated P_{CO2} will be more pronounced in oysters than in clams reflecting adaptation of latter species to extreme environmental acidosis in the sediments

Progress in the understanding of the effects of ocean acidification on marine systems, at present, is limited by the scarcity of data and information on reponses of marine life. In, addition, the co-occurrence of other stressors (temperature, salinity, hypoxia) in the natural environment can amplify or dampen the sensitivity of responses of organisms to ocean acidification. The integrative assessment approach to assess the effects of ocean acidification, as in this study, provides the objective and critical data necessay for taking specific acions against the threat of ocean acidification in marine ecosystems. Currently no international policies are in place to stop ocean acidification. Govering bodies and policy makers before taking any specific actions need to see what current studies and research show. Studies like the one addressed here, on a broader scale, aims at bringing the collective concern to the managers and stakeholders to help them take informated decisions about the urgency and threat of ocean acidification in marine ecosystems including marine protected areas (MPAs).

CHAPTER 2: ENVIRONMENTAL SALINITY MODULATES THE EFFECTS OF ELEVATED CO_2 LEVELS ON JUVENILE HARD-SHELL CLAMS, *MERCENARIA MERCENARIA*

The following chapter was published in the scientific journal The Journal of Experimental Biology in 2013. Gary H.Dickinson, Omera B. Matoo, Robert T Tourek, Inna M. Sokolova and Elia Beniash (2013). Environmental salinity modulates the effects of elevated CO_2 levels on juvenile hard-shell clams, *Mercenaria mercenaria*. The Journal of Experimental Biology. 216, 2607-18.

Abstract

Ocean acidification due to increasing atmospheric CO_2 concentrations results in a decrease in seawater pH and shifts in the carbonate chemistry that can negatively affect marine organisms. Marine bivalves such as the hard-shell clam, *Mercenaria mercenaria*, serve as ecosystem engineers in estuaries and coastal zones of the western Atlantic and, as for many marine calcifiers, are sensitive to the impacts of ocean acidification. In estuaries, the effects of ocean acidification can be exacerbated by low buffering capacity of brackish waters, acidic inputs from freshwaters and land, and/or the negative effects of salinity on the physiology of organisms. We determined the interactive effects of 21 weeks of exposure to different levels of CO_2 (~395, 800 and 1500 μatm corresponding to pH of 8.2, 8.1 and 7.7, respectively) and salinity (32 versus 16) on biomineralization, shell properties and energy metabolism of juvenile hard-shell clams. Low salinity had profound effects on survival, energy metabolism and biomineralization of hard-shell

clams and modulated their responses to elevated P_{CO2}. Negative effects of low salinity in juvenile clams were mostly due to the strongly elevated basal energy demand, indicating energy deficiency, which led to reduced growth, elevated mortality and impaired shell maintenance (evidenced by the extensive damage to the periostracum). The effects of elevated P_{CO2} on physiology and biomineralization of hard-shell clams were more complex. Elevated P_{CO2} (~800–1500 μatm) had no significant effects on standard metabolic rates (indicative of the basal energy demand), but affected growth and shell mechanical properties in juvenile clams. Moderate hypercapnia (~800 μatm P_{CO2}) increased shell and tissue growth and reduced mortality of juvenile clams in high salinity exposures; however, these effects were abolished under the low salinity conditions or at high P_{CO2}(~1500 μatm). Mechanical properties of the shell (measured as microhardness and fracture toughness of the shells) were negatively affected by elevated CO_2 alone or in combination with low salinity, which may have important implications for protection against predators or environmental stressors. Our data indicate that environmental salinity can strongly modulate responses to ocean acidification in hard-shell clams and thus should be taken into account when predicting the effects of ocean acidification on estuarine bivalves.

Introduction

Current models of global change predict a rise in the atmospheric carbon dioxide (CO_2) levels from the current value of ~380–400 μatm to 730–1020 μatm by the year 2100 and 1500–2000 μatm in the next 300 years (Doney et al., 2009; Waldbusser et al., 2011). Approximately 30% of the anthropogenically released CO_2 is absorbed by the ocean, resulting in ocean acidification, which involves a drop in seawater pH, shifts in the

inorganic carbon speciation and a decrease in saturation of calcium carbonate ($CaCO_3$) minerals, and can strongly affect marine organisms (Kleypas et al., 2006; Pörtner, 2008; Przeslawski et al., 2008; Cooley and Doney, 2009). Estuarine and coastal habitats, the ocean's hotspots for biological diversity and productivity, may be especially vulnerable to ocean acidification. They often receive acidic inputs from freshwater and land run-off, upwelling of the CO_2-enriched acidified waters as well as from biological CO_2 production, leading to large fluctuations in pH and carbonate chemistry (Mook and Koene, 1975; Cai and Wang, 1998; Thomsen et al., 2010; Amaral et al., 2011). Moreover, the buffering capacity of estuarine waters is considerably lower than that of the open ocean (Mook and Koene, 1975; Cai and Wang, 1998). An increase in atmospheric CO_2 levels can exacerbate acidification of estuarine habitats, and long term pH data show that some estuaries have become more acidic in the past 50 years, with the rate of acidification closely tracking atmospheric CO_2 (Najjar et al., 2010; Waldbusser et al., 2011). Bivalve mollusks play a prominent ecological role as ecosystem engineers and key foundation species in estuarine and coastal ecosystems around the world (Gutiérrez et al., 2003; Kochmann et al., 2008). Mollusks belong to a broad group of marine organisms called marine calcifiers (i.e. organisms that build their skeleton from $CaCO_3$) that are among the most sensitive groups of organisms to ocean acidification (Kleypas et al., 2006; Dupont et al., 2010; Kroeker et al., 2010). Ocean acidification strongly affects biomineralization of calcifiers due to the decrease in pH and saturation of $CaCO_3$ minerals, which slows deposition rates and increases solubility of $CaCO_3$ (Gazeau et al., 2007; Byrne et al., 2011). The effects of elevated CO_2 on molluscan biomineralization

can be very complex depending on the shell structure, mineralogy and biological factors involved in the control of shell formation (Addadi et al., 2006; Stanley, 2006; Ries et al., 2009; Kroeker et al., 2010). Elevated CO_2 levels can have systemic effects on the physiology of marine mollusks that extend beyond calcification, affecting their extracellular and intracellular pH, enzyme activity, protein stability and rates of energy metabolism (Pörtner, 2008; Lannig et al., 2010; Tomanek et al., 2011; Pörtner, 2012). These changes may directly affect the organism's performance and fitness as well as indirectly influence biomineralization via impacts on physiological functions such as activity of biomineralization enzymes and energy metabolism.

In estuaries, ocean acidification can be compounded by other environmental parameters (such as temperature, salinity and anthropogenic pollution) that can modulate the effects of elevated CO_2 (Lannig et al., 2010; Dickinson et al., 2012; Nikinmaa, 2013). Among these parameters, salinity is likely to play a key role due to its direct effect on seawater chemistry and buffering capacity as well as on the physiology of estuarine inhabitants. In osmoconforming animals, such as marine mollusks, reduced salinity has a strong impact on physiology, leading to changes in cell volume and extracellular and intracellular osmotic pressure, altering energy metabolism and enzyme activities, and affecting the rates of protein synthesis and turnover (Berger, 1986; Prosser, 1991; Berger and Kharazova, 1997). Earlier studies also showed that low salinity can negative effects of elevated CO_2 levels on growth, energy balance and biomineralization of a common estuarine bivalve, the eastern oyster, *Crassostrea virginica* (Dickinson et al., 2012). However, the interactive effects of salinity and elevated CO_2 on marine organisms are not yet fully understood and require further investigation.

The aim of the present study was to characterize the interactive effects of two common environmental factors – elevated CO_2 and low salinity – on biomineralization, shell properties and energy metabolism of a common estuarine bivalve, the hard-shell clam *Mercenaria mercenaria* (Linnaeus 1758). *Mercenaria mercenaria* is an ecosystem engineer in temperate estuaries and coastal zones of the Atlantic affecting sediment structure and playing an important role in trophic interactions. Hard-shell clams are also economically important, with annual worldwide harvests ranging from 30,000 to 70,000 tons (Food and Agriculture Organization of the United Nations, Aquaculture and Fishery Statistics available at http://www.fao.org). Shells of hard-shell clams are made of aragonite, a more soluble polymorph of $CaCO_3$ than calcite, and consist of an outer thick prismatic layer and an inner cross-lamellar layer (Kraeuter and Castagna, 2001). This relatively simple shell mineralogy makes this clam a useful model species for studying the effects of ocean acidification and salinity on biomineralization and shell properties. We tested the hypothesis that low salinity (such as commonly occurs in the estuarine habitats of hard-shell clams) will exacerbate the effects of ocean acidification, resulting in reduced growth and biomineralization and elevated basal energy metabolism, which may decrease the amount of energy invested into growth and shell formation.

Materials and methods

Chemicals and enzymes were purchased from Sigma-Aldrich (St Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) unless otherwise stated, and were of analytical grade or higher.

2.1 Experimental Design

The effects of two factors, partial pressure of CO_2 (P_{CO2}) and salinity, were tested in a full factorial design. Three P_{CO2} levels, ~395 μatm (normocapnia, pH_{NBS} 8.25–8.26), ~800 μatm (moderate hypercapnia, pH_{NBS} 8.15–8.16) and ~1500 μatm (extreme hypercapnia, pH_{NBS} 7.74–7.77), were assessed at a salinity of 32 (high salinity) or 16 (low salinity), yielding six treatment groups. P_{CO2} levels were chosen to be representative of the present-day P_{CO2} (~395 μatm), atmospheric P_{CO2} predicted by moderate IPCC scenarios for the year 2100 (~800 μatm CO_2) and a P_{CO2} projection for the year 2250 (~1500 μatm). The two selected salinity conditions were within the environmentally relevant range for *M. mercenaria*. Clams were randomly assigned to one of the six treatment groups and exposed for a total of 21weeks. The group exposed to a salinity of 32 and ~395 μatm P_{CO2} was considered the control. Non-reproductive juveniles were used in this study in order to avoid complications due to the varying energy demands of reproducing organisms in different stages of their reproductive cycle. The salinity and CO_2 levels used in this study are within the range currently found in the estuaries of the southeastern USA where the clams were collected (Burnett, 1997; Ringwood and Keppler, 2002; McElhany and Busch, 2012). It is worth noting that clams can periodically experience much stronger acidification in their present-day habitats (with pH dropping below 7.0) than those used in the present study (Ringwood and Keppler, 2002); however, such extreme events usually only last from a few hours to a few days. The long-term exposures such as those used in the present study are more representative of the future ocean acidification scenarios.

2.2. Animal Collection and Maintenance

Juvenile *M. mercenaria* (8weeks post metamorphosis) were purchased from a commercial supplier (Grant's Oyster House, Sneads Ferry, NC, USA) and shipped overnight to the University of North Carolina at Charlotte. Clams were acclimated for 7 days in plastic trays (28×18×12 cm), each containing 5l of artificial seawater (ASW; Instant Ocean, Kent Marine, Acworth, GA, USA) at 20±1°C and salinity of 32±1 bubbled with ambient air (normocapnia, P_{CO2} ~395 μatm). Animals were then randomly assigned to a high or low salinity treatment. Salinity was maintained at 32 for the high salinity group and was reduced gradually by three units per day until a salinity of 16 was reached.

Once the target salinity was reached, clams were further divided into groups assigned to different P_{CO2} treatments. Target P_{CO2} values were reached by bubbling seawater with gas mixtures containing different CO_2 concentrations. For normocapnic treatment, the tanks were bubbled with the ambient air. For moderate and extreme hypercapnia, ambient air was mixed with 100% CO_2 (Roberts Oxygen, Charlotte, NC, USA) in a fixed proportion using precision mass flow controllers (Cole-Parmer, Vernon Hills, IL, USA) and bubbled into the trays. The air-CO_2 mixture flow rate was set up to maintain the respective systems at a steady-state pH during the exposures. Two replicate trays were used for each species and treatment condition with ~400–500 animals per tray. Water temperature was maintained at 20±1°C throughout the duration of the experiment. Salinity and temperature was measured with an YSI30 salinity, temperature and conductivity meter (YSI, Yellow Springs, OH, USA). Salinity was determined on the practical salinity scale and is reported in practical salinity units (PSU). Water was changed every other day using ASW pre-equilibrated with the respective gas mixtures.

Animals were fed ad libitumon alternative days with 2ml per tray of commercial algal mixture containing *Isochrysis* spp., *Pavlova* spp., *Thalassoisira weissflogii* and *Tetraselmis* spp. with 5–20 μm cells (Shellfish Diet 1800, Reed Mariculture, Campbell, CA, USA). Mortality was checked weekly.

2.3. Seawater Chemistry

Seawater chemistry parameters were determined in all experimental treatments (Table 2.1.) as described elsewhere (Beniash et al., 2010). Water temperature, salinity and pH in the exposure trays was monitored throughout the course of the experiment, and water samples were collected periodically in air-tight containers, poisoned with mercuric chloride and stored at +4°C until further analysis. pH was measured using a pH electrode (pH meter Model 1671, Jenco Instruments, San Diego, CA, USA) calibrated with National Institute of Standards and Technology standard pH buffer solutions (National Bureau of Standards, NBS standards, Fisher Scientific). Total dissolved inorganic carbon (DIC) was measured by the Nutrient Analytical Services (Chesapeake Biological Laboratory, Solomons, MD, USA). Seawater carbonate chemistry parameters [P_{CO2} , total alkalinity, and the saturation state (Ω) for calcite and aragonite] were calculated using co2SYS software (Lewis and Wallace, 1998) using barometric pressure values, as well as DIC, pH, temperature and salinity values for the respective samples. For calculations, we used the NBS scale for seawater pH, constants from Millero et al. (Millero et al., 2006; from Lewis and Wallace, 1998), the KSO_4 constant from Dickson (Dickson, 1990), and concentrations of silicate and phosphate for Instant Ocean seawater (silicate: 0.17 and 0.085μmol kg^{-1} at salinities of 32 and 16, respectively; phosphate: 0.04 and 0.02 μmol kg^{-1} at salinities of 32 and 16, respectively).

2.4. Shell Mass and Tissue Mass

After 16 and 21 weeks exposure, a subset of clams from each treatment group was stored in 70% ethanol and shipped to the University of Pittsburgh for mass measurements, mechanical testing, and structural and mineralogical analyses. Upon receipt, clams were manually inspected and any clams with visible signs of shell damage were discarded.

For dry mass measurements, 11–40 animals (depending on availability) were randomly selected from each treatment group. Individual clams were dried in a vacuum oven at 45°C, 91 kPa for at least 15 days to achieve constant mass and weighed individually on a microbalance (Metler-Toledo XP 26, Columbus, OH, USA) with precision of 0.01 mg or better. Once the total masses of individual clams were determined, each clam was incubated in 500 µl sodium hypochlorite (NaOCl; commercial Clorox diluted to obtain 2% v/v NaOCl and filtered through a 0.2 µm filter) at room temperature (RT) for 10 days, with three changes of NaOCl solution to ensure complete removal of soft tissues. NaOCl-treated shells of individual clams were rinsed three times in deionized water, dried in air (24h at RT) and a vacuum oven (24h at 45°C), and weighed on a microbalance to obtain the shell mass. Tissue dry mass was determined as the difference between the total dry mass of the clam and the dry mass of the shell.

2.5. Mechanical Properties of the Shells

Mechanical properties, structure and mineralogy of the shells were analyzed in the whole shells of experimental clams (i.e. including new growth and pre-existing shell) because the region of new shell growth was too small for the analyses. Therefore, results of these analyses should be interpreted as encompassing both the newly deposited shell

material and changes in the existing shell due to the differences in the seawater chemistry.

Micromechanical testing was conducted on left valves of the NaOCl-treated shells of clams that had been exposed to experimental conditions for 21 weeks. For each treatment group, seven shells were tested. Clams were selected for analysis that approximated the mean mass of all clams when all treatments were combined (3.06 mg). Left shell valves were mounted in epoxy resin (Epofix, EMS, Hatfield, PA, USA) in a flat silicone embedding mold (EMS) and polymerized for 24h at RT. Embedded shells were cut longitudinally, transecting the anterior apical tip to the most posterior distal edge using a slow-speed water-cooled diamond saw (IsoMet, Buehler, Lake Bluff, IL, USA). A second cut was made parallel to the first one to produce a 3 mm thick section, as described previously (Dickinson et al., 2012). Sections were ground and polished with Metadi diamond suspensions at 6, 1 and 0.25 μm particle size on a grinder-polisher (MiniMet 1000, Buehler). Grinding and polishing was conducted using a saturated $CaCO_3$ solution (pH 8.1) prepared by mixing calcium and carbonate salts at very high concentrations and letting the mineral precipitate over several hours. The mixture was centrifuged and the supernatant was used to polish the samples. No etching of the shell samples was observed during grinding or polishing.

Vickers microhardness tests were conducted using a microindentation hardness tester (IndentaMet 1104, Buehler) on polished shells at 0.245N load and 5s dwelling time. Indents were made within the middle layer of the shell (cross-lamellar homogenous layer) in a region equidistant from the apical anterior tip and the most distal posterior edge. Five to seven indentations per shell were made at least 30 μm away from the shell

edges and from other indents. Vickers hardness numbers (VHN) were averaged for each shell sample. Digital photographs were taken before and immediately after each indentation. This enabled quantification of the longest crack produced by each indent, which was measured using Photoshop (version 4.0, Adobe, San Jose, CA, USA) as the radius of a circle radiating from the center of the indent enclosing all visible cracks. The crack radius (μm) for a shell sample was obtained by averaging the crack radii for all indents on that sample. Hardness and crack radius measurements were used to calculate fracture toughness (K_c) for each sample as described elsewhere (Anstis et al., 1981; Baldassarri et al., 2008):

$$K_c = 0.0154\ (E/H)^{½} \times (P/C^{1.5}),$$

where 0.0154 is a calibration constant, *E* is the elastic modulus [empirically determined for *M. mercenaria* as 66 GPa (Currey and Taylor, 1974)], *H* is hardness in GPa, *P* is applied load in N and *C* is crack radius in μm.

2.6. Shell Structure

Scanning electron microscopy (SEM) imaging was conducted on the exterior and interior of the shells collected after 16 weeks of exposure. Imaging of the exterior was conducted on shells that had not been exposed to NaOCl (to avoid destruction of the periostracum), whereas imaging of the interior surface was conducted on NaOCl-treated shells. Right shell valves were affixed to an SEM stub using a copper tape and conductive paint, and sputter-coated with gold/palladium. Imaging was conducted in the secondary electron imaging mode using a field emission SEM (JSM- 6330F, Jeol, Peabody, MA, USA) at 3 kV and 7–8.2 mm working distance. Micrographs of the shell exteriors were taken at ×35 and ×2500 magnifications (for the whole shell and the peripheral growth

ridge, respectively), and interior images were taken within a central region of the shell interior at ×1500 to ×15,000 magnifications. Six shells (three each for the interior and exterior surfaces) were imaged for each treatment group.

2.7. Shell Mineralogy

The right valves of clam shells collected after 21 weeks of experimental exposures and treated with NaOCl were ground with a mortar and pestle, pressed into a KBr pellet, and analyzed in transmittance mode on a Fourier Transform Infrared (FTIR) spectrometer (Bruker Optics, Vertex 70 FTIR, Billerica, MA, USA). Spectra were acquired at 4 wavenumber resolution, with 32 scans for two shells per experimental group. The 600–2000 cm^{-1} region of the spectra were isolated, baseline corrected and normalized, and the ν2, ν3 and ν4 peak positions and heights were measured using Spectrum 5.1 software (Perkin-Elmer, Santa Clara, CA, USA). Relative crystallinity was determined based on ν2: ν3 and ν2: ν4 ratios (Beniash et al.,1997; Gueta et al., 2007).

2.8.Standard Metabolic Rate

Standard metabolic rate (SMR) was determined as the resting oxygen consumption rate ($\dot{M}O_2$) in juvenile clams after 2, 8, 11 and 21weeks of experimental exposures. $\dot{M}O_2$ was measured using Clarke-type oxygen electrodes (Qubit Systems, Kingston, ON, Canada) in a water-jacketed respiratory chamber (OX1LP-4ml, Qubit Systems) at 20°C in ASW at the same P_{CO2} and salinity as used in experimental exposures. Two-point calibration with air-saturated seawater and saturated Na_2SO_3 solution was conducted prior to each measurement at the respective salinity and P_{CO2}. The respirometry chamber was equipped with an adjustable air-tight plunger that allowed maintenance of a constant volume of water (2 ml) regardless of the volume of the

experimental animals; therefore, no correction for the volume displacement by clams was needed. To avoid interference with post-prandial metabolism and feces excretion, juveniles were fasted for 24h prior to the start of $\dot{M}O_2$ recordings. For each measurement, three to five similarly sized individuals were selected, placed in the chamber, and allowed to recover from handling stress for 45min. The chambers were closed and $\dot{M}O_2$ was measured as a decrease in O_2 concentration for 30 min. Oxygen levels during the measurement period were never less than 80% of air saturation. Two technical replicates, with 15 min recovery period between the recordings, were obtained for each measurement and these two measurements were averaged. After each experiment, the electrode drift was determined by measuring the oxygen consumption for 15 min in the chamber with 2 ml of seawater without the clams. These values were used to correct the oxygen consumption rates of the experimental clams. A total of 10 biological replicates were obtained for each treatment group, each replicate representing SMR of a separate group of three to five clams. After measurements, total tissue dry mass was determined for all juveniles in the group as described above in 'Shell mass and tissue mass'. SMR was calculated as follows:

$$\mathrm{SMR} = \frac{\Delta P_{O2} \times \beta_{O2} \times V}{M_{tot}} \times \left(\frac{M_{ind}}{M_{av}} \right)^{-0.2}$$

where SMR is the normalized oxygen consumption (μmol O2 g^{-1} dry mass h^{-1}), ΔP_{O2} is the decrease in partial oxygen pressure in the respirometry chamber over time (kPa h^{-1}), V is water volume in the chamber (l), β_{O2} is the oxygen capacity of water (μmol O_2 l^{-1} kPa^{-1}), M_{tot} is the total dry tissue mass of all juveniles in the respirometry chamber (g), M_{ind} is the average individual dry tissue mass of juveniles in the respirometry chamber

(mg), M_{av} is the average individual dry tissue mass of juveniles across all experimental treatments (0.237 mg) and −0.2 is an allometric coefficient (Lannig et al., 2006).

2.9. Statistical Analysis

Experimental data sets were tested for the presence of potential outliers using Grubbs' test (extreme studentized deviate method) as implemented in GraphPad Prism version 5.03 (GraphPad Software, La Jolla, CA, USA). A small number of statistically significant outliers were detected and removed from the analysis; the outlier occurrence was random among the treatment groups. Regression analyses were performed to determine the relationship between shell or tissue mass and aragonite saturation level. Linear, exponential and quadratic curves were fit to the data, and the best-fit curves were chosen based on the significance of the fit and the percentage of variation explained by the regression line estimated by R^2. Average monthly mortalities were estimated from the weekly mortality counts in different treatment groups and compared among treatments using Fisher's exact test. The effects of the factors P_{CO2} and salinity and their interaction on shell mechanical properties and SMR were tested using a generalized linear model (GLM) ANOVA after testing for the normality of data distribution and homogeneity of variances. Both factors were treated as fixed, and P_{CO2} had three levels (~395, 800 and 1500 μatm) while salinity had two levels (32 and 16). Post hoc tests (Fisher's least significant difference) were used to test the differences between the group means; only planned contrasts were used. Pearson correlation (R) and principal component analyses (PCA) were conducted using Origin 8.6 software (OriginLab, Northhampton, MA, USA). Pearson correlation analysis for individual shell mass (M_{sh}) versus tissue mass (M_{ti}) was conducted for each experimental group at 16 and 21 week time points. Furthermore,

Pearson correlation and PCA analyses were conducted across all experimental groups and time points; for this analysis we used average values of SMR, M_{sh}, M_{ti} and mortality for each treatment group at the respective time point with exposure time, salinity, P_{CO2} and the degree of aragonite saturation (Ω_{Arg}) as the potential explanatory variables.

Sample sizes for all experimental groups were 360–505 for mortality estimates and 6–40 for all other traits. Each replicate represents a sample from an individual clam, except SMR, where each biological replicate represents a group of three to five juveniles. Unless otherwise indicated, data are presented as means ± s.e.m. The differences were considered significant if the probability of Type I error was less than 0.05.

Results

3.1. Seawater Chemistry

Aragonite saturation state decreased with increasing P_{CO2} and with decreasing salinity (Table 2.1.). An increase in P_{CO2} from ~395 to ~1500 μatm resulted in a reduction in Ω_{Arg} from Ω=4.83 to 1.46, and a similar decrease of Ω_{Arg} (to 1.54) was seen when salinity was reduced from 32 to 16 under the current P_{CO2} conditions (~395 μatm). At the low salinity (16), two experimental P_{CO2} treatments (~800 and 1500 μatm) resulted in undersaturation of the seawater for aragonite (Ω_{Arg}=0.77–0.42). In all other experimental treatments, Ω_{Arg} values were above the saturation threshold for aragonite (Table 2.1.).

3.2. Mortality

Juvenile clams exhibited significantly higher mortality in low salinities (Fisher's exact test: $P<0.05$ for 8–20 weeks of exposure; Fig.2.1.A). In the high salinity groups, P_{CO2} had no effect on mortality of juvenile clams during the first 12 weeks of exposure. After 16 weeks of exposure at salinity 32, mortality of juveniles was significantly lower

at ~800 μatm P_{CO2} (<2%) than at ~1500 (~3–7%) or ~395 μatm P_{CO2} (13–26%; $P<0.05$ among all P_{CO2} groups). Mortality was also lower in clams exposed for 20 weeks to ~1500 μatm P_{CO2} at salinity 32 compared with their normocapnic counterparts ($P<0.05$); no mortality data are available for this time point for clams kept at ~800 μatm P_{CO2}. At salinity 16, the highest mortality was observed in juveniles exposed to extreme hypercapnia (~1500 μatm P_{CO2}) after 8–20 weeks of exposure ($P<0.05$ for contrasts between extreme hypercapnia and the other two P_{CO2} treatments; Fig.2.1A). Mortality of juveniles exposed to moderate hypercapnia (~800μatm P_{CO2}) and salinity 16 was slightly but significantly lower than in the normocapnic low salinity group after 8–12 weeks of exposure ($P<0.05$) but not after prolonged (16–20 weeks) exposure ($P>0.05$).

3.3. Standard Metabolic Rate

SMR of clams was significantly affected by the interaction of P_{CO2} and salinity, indicating that metabolic response to P_{CO2} was modulated by the acclimation salinity (Table 2.2.). At high salinity, P_{CO2} had no significant effect on SMR (Fig.2.1.B). At all P_{CO2} levels, there was a slight but significant increase in SMR after 8weeks of exposure, which quickly returned to the initial levels in all high salinity treatments. In contrast, in low salinity groups, there was an initial depression of SMR after 2 weeks of exposure followed by a dramatic increase (8- to 10-fold) after 8–11 weeks of exposure (Fig.2.1.B). SMR peaked at different exposure times in juveniles exposed to different P_{CO2}; the maximum SMR was reached earlier in hypercapnic groups (after 8 weeks of exposure) than in the normocapnic group (after 11weeks). After 21weeks at low salinity, SMR had decreased in all P_{CO2} treatment groups but remained significantly elevated above the initial levels measured after 2 weeks of low salinity exposures (Fig.2.1.B).

3.4. Shell and Tissue Mass

In clams exposed to moderately elevated P_{CO2} (800 μatm) at high salinity, the shell masses (M_{sh}) were significantly higher than in the control after 16 and 21weeks of exposure, while no difference was observed between the control (395 μatm) and extreme P_{CO2} (1500 μatm) groups maintained at high salinity (Fig.2.2.). At low salinity, M_{sh} values were significantly lower in all P_{CO2} treatment groups compared to their high salinity counterparts. Soft tissue mass (M_{ti}) determined after 16 weeks of exposure was also significantly lower in clams exposed to low salinity compared with their high salinity counterparts (Fig.2.2.). In high salinity treatments, clams exposed to hypercapnia tended to have higher tissue mass compared with their normocapnic counterparts, but this trend was only significant after 21 weeks of exposure (Fig.2.2.B). At low salinity, the trend was reversed, and hypercapnic groups tended to have lower tissue mass compared with their normocapnic counterparts; this trend was likewise significant only after 21 weeks of exposure (Fig.2.2.B). Analysis of the effects of Ω_{Arg} on shell and soft tissue mass after 16 and 21 weeks of exposure revealed a non-linear biphasic relationship between Ω_{Arg} and M_{ti} or M_{sh} ($P<0.001$, R^2 =0.16–0.42 for all regressions; Fig.2.2.). Shell and tissue mass were the highest at Ω_{Arg}=3.3, which corresponds to the group exposed to moderate hypercapnia (~800 μatm P_{CO2}) at high salinity. Shell and tissue masses were reduced above and below this Ω_{Arg} and were the lowest at $\Omega_{Arg}<1$ (Fig.2.2.). To better understand the relationships between M_{ti} and M_{sh} we conducted Pearson correlation tests within each experimental treatment group. Overall, in high salinity groups, the correlations between M_{ti} and M_{sh} were much stronger and highly significant compared with the low salinity treatments (supplementary material Table S1). The only exception

was the control group (normocapnia at salinity 32), where no correlation between M_{ti} and M_{sh} was found after 21 weeks of exposure (supplementary material Table S1). This may be due to the fact that this group experienced high accidental mortality between 16 and 21weeks of exposure, and the lack of correlation might be related to the survivor effect.

3.5. Mechanical Properties and Mineralogy of the Shells

Vickers microhardness tested on the shells of clams exposed to different P_{CO2} and salinity conditions for 21weeks was significantly affected by P_{CO2} but not by salinity (Table 2.2., Fig.2.3.). At high salinity, shell microhardness was significantly reduced in clams exposed to ~800 and ~1500 μatm P_{CO2}. At low salinity, only the group exposed to ~1500 μatm was significantly different with respect to the shell microhardess from the respective normocapnic counterparts. Microhardness did not differ between high and low salinity treatments when groups exposed to the same P_{CO2} levels were compared ($P>0.05$).

Fracture toughness was significantly affected by the interaction of P_{CO2} and salinity, indicating that the effects of elevated P_{CO2} on this trait are modulated by the exposure salinity (Table 2.2.). At high salinity, fracture toughness of the shells was significantly reduced in clams exposed to ~1500 μatm P_{CO2} (Fig.2.3.). The fracture toughness of the shells of clams exposed to ~800 μatm P_{CO2} at high salinity did not differ from the normocapnic controls, despite a significantly lower hardness in the former group (Fig.2.3.). Shell fracture toughness was generally lower in the clams maintained at low salinity in normocapnia and moderate hypercapnia (~395 and ~800 μatm P_{CO2}) compared with their counterparts from the high salinity treatments, although the decrease was only significant at ~800 μatm P_{CO2} (Fig.2.3.). Interestingly, the fracture toughness values were

higher in the 1500 μatm P_{CO2}, 16 salinity group than in the 800 μatm P_{CO2}, 16 salinity group. We attribute this increase to higher porosity due to shell dissolution. The cracks generated by the indenter tip can be arrested or deflected by pores, although this porosity may or may not add to the materials strength, depending on other factors (Shigegaki et al., 1997; Xu et al., 2001).

FTIR spectra collected from the shells of clams exposed to different P_{CO2} and salinity regimes for 21weeks showed that shells were comprised of aragonite with no other mineral forms present (supplementary material Fig.S1). Analysis of ν2 and ν4 peak position and absorption intensity relative to the ν4 peak revealed no differences among treatment groups, indicating that no changes in crystallinity had occurred in response to experimental treatments (data not shown).

3.6. Shell Structure

SEM imaging of the exterior (Fig.2.4.) and interior (Fig.2.5.) of the shells of clams exposed to different P_{CO2} and salinity conditions revealed distinct differences in shell structure among treatment groups. In high salinity exposures where Ω_{Arg} remained above the saturation level, only minor differences in the structure of shell exterior were observed (Fig.2.4.A–C). Pronounced growth ridges were found in clam shells from high salinity treatments regardless of the exposure P_{CO2} (Fig.2.4.C, inset). At low salinity, distinct flaking of the periostracum was observed at all levels of P_{CO2}, with a nearly complete loss of periostracum at ~1500 μatm P_{CO2} (Fig.2.4.D–F). Major erosion and pitting of the underlying mineral were also observed at low salinity in shells of the clams exposed to ~800 and 1500 μatm P_{CO2}, where Ω_{Arg} was <1. Growth ridges were less pronounced in the shells of clams maintained at low salinity, and at ~1500 μatm P_{CO2} and

salinity 16 they were barely visible (Fig.2.4.D–F, inset). These results demonstrate that the exposed regions of the shells are susceptible to chemical erosion in undersaturated environments. In contrast, changes in structure of the interior of the shells did not vary directly with experimental Ω_{Arg} levels. Under the control conditions (salinity 32, ~395μatm P_{CO2}), the interior of clam shells was composed of closely interlocking aragonite crystals (Fig.2.5.A). In all other experimental treatments, a distinct etching of aragonite was observed, resulting in a porous interior (Fig.2.5.). This etching was the most extreme in shells of the clams exposed to ~1500 μatm P_{CO2} at low salinity (Fig.2.5.F). Clams exposed to ~1500 μatm P_{CO2} at low salinity also showed substantial degradation and etching of the hinge region, which was not observed for other treatment groups (Fig.2.6.).

3.7. Correlation Analysis

Correlation analysis revealed a number of significant associations between the studied parameters ($P<0.05$). As expected, Ω_{Arg} of the seawater was strongly correlated with salinity ($R=0.73$) and P_{CO2} ($R= –0.59$) (supplementary material Table S2). Shell mass (M_{sh}) and soft tissue mass (M_{ti}) were significantly positively correlated with salinity ($R=0.64$ and 0.50, respectively) and with each other ($R=0.62$). Shell mass was also positively correlated with Ω_{Arg} ($R=0.39$). Mortality had a strong positive correlation with exposure time ($R=0.67$) and a negative correlation with salinity ($R= –0.51$), M_{ti} ($R=–0.61$) and Msh ($R= −0.39$). These data indicate that changes in water chemistry had a direct effect on tissue and shell growth as well as mortality in clams, with salinity having the most profound effect. We have tested for the possible associations between the studied parameters separately within high and low salinity groups. In the high salinity

group, the only significant correlation was found between M_{ti} and M_{sh} (R= 0.69; supplementary material Table S3). In contrast, in the low salinity group a strong negative correlation was observed between P_{CO2} and M_{sh} (R= –0.55) and an equally strong positive correlation was observed between Ω_{Arg} and M_{sh} (R= 0.54), while no significant correlations were found between the water chemistry parameters and M_{ti} (supplementary material Table S4). Interestingly, exposure time was negatively correlated with M_{ti} (R= −0.54), indicating selective mortality and/or tissue loss in juvenile clams during prolonged exposure at low salinity.

3.8. Principal Component Analysis

PCA revealed that salinity and Ω_{Arg} had the highest loadings on the first principal component (PC1), responsible for 38% of data variance (supplementary material Table S5). PC2, accounting for another 25% of variance, was dominated by P_{CO2} and pH, while PC3 (15% of the variance) was predominantly associated with the experimental exposure time. Notably, PC4, accounting for 10% of the variance, was dominated by SMR (a loading of 0.92); all other loadings in PC4 were very low except M_{ti} (a loading of 0.31). Together, the first three principal components (PC1, PC2 and PC3) accounted for almost 80% of the data variance. On the PCA plots, mortality and SMR grouped together in the plane formed by PC1 and PC2, consistent with the concomitant increase of SMR and mortality with decreasing salinity. There was also a strong positive relationship between salinity and shell and tissue masses. Interestingly, analysis of PC2 reveals that M_{ti} and M_{sh} are positively influenced by P_{CO2} , probably reflecting the fact that at moderately elevated P_{CO2} conditions, M_{ti} and M_{sh} values are generally higher than in normocapnia.To eliminate possible artifacts due to the fact that pH and Ω_{Arg} strongly depend on salinity

and P_{CO2}, we conducted PCA using only salinity, P_{CO2} and time of exposure as potential explanatory variables (supplementary material Table S6). In PC1, which accounts for 42% of variance, salinity was the dominant factor (loading 0.45). All studied biological variables had high loadings the PC1, indicating that salinity was the major factor affecting clam biology. Exposure time dominated PC2 (a loading of 0.76) and primarily affected mortality and shell mass (supplementary material Table S6). In contrast, P_{CO2} had the highest loading (0.96) on PC3, which accounted for 15% of the variance, and was weakly associated with the tissue mass and SMR (loadings of 0.18 and 0.21, respectively). An overview of the putative relationships between the experimental factors and studied biological traits based on the results of correlation analysis and PCA is shown in fig 2.7.

Discussion

Combined exposure to elevated P_{CO2} and reduced salinity strongly affected growth, bioenergetics and biomineralization of juvenile hard shell clams. Under the conditions of this study, salinity exerted a dominant influence on growth and bioenergetics of hard-shell clams and significantly modified how shell structural and mechanical properties responded to elevated P_{CO2}. In contrast, the direct effects of P_{CO2} on biomineralization and physiology of *M. mercenaria* were small in comparison to those of salinity. This may reflect high tolerance of hard-shell clams to P_{CO2} variations in the range tested in this study, but may also partially reflect the non-linear effects of P_{CO2} on some studied biological traits.

Acclimation of juvenile clams to low salinity resulted in a strong increase of SMR during the mid-term experimental exposures (8–11 weeks), indicating elevated basal

maintenance cost in these organisms. During this period, SMR of clams from the low salinity group were approximately three times higher than in their high salinity counterparts and approximately eight to 10 times higher than during the initial exposure to low salinity. Notably, low salinity exposure also led to an increase in activity of carbonic anhydrase in gills, possibly reflecting an elevated need for gas exchange due to the higher SMR. Elevated costs of basal maintenance typically result in reduced aerobic scope for fitness-related functions including growth (Sokolova et al., 2011; Sokolova et al., 2012), and a negative relationship between SMR and growth rate is commonly found in marine bivalves (Hawkins et al., 1986; Awkins and Day, 1996; Bayne and Hawkins, 1997; Fraser and Rogers, 2007).

The high energy cost of basal maintenance also goes hand-inhand with elevated mortality and reduced shell and tissue mass in clams exposed to low salinity, indicating that energy deficiency may contribute to the reduced growth and survival of this group. A similar increase in mortality associated with the depletion of energy stores has been observed in juvenile oysters during the combined exposure to low salinity and high CO_2 levels (Dickinson et al., 2012). In the present study, SMR was slightly reduced after 21 weeks of exposure at low salinity conditions (even though it still remained significantly above the control levels and above SMR recorded during the initial period of low salinity exposure). This moderate reduction in SMR after the long-term exposure to low salinity may reflect physiological acclimation; however, if true, this would indicate an unusually slow acclimation process in juvenile hard-shell clams. Typically, physiological acclimation to salinity shifts is completed and a new metabolic steady state is achieved within 3 to 6 weeks in marine mollusks (Berger, 1986; Prosser, 1991; Berger and

Kharazova, 1997). Alternatively, a decrease in SMR after 21 weeks in low salinity may be due to the selective mortality of experimental clams, as the individuals with highest SMR are expected to develop the strongest energy deficiency and therefore will be most prone to salinity-induced stress. Indeed, earlier studies in marine bivalves showed that individuals with lower SMR are more resistant to environmental stress and have higher survivorship under the stress conditions (Hawkins et al., 1986; Myrand et al., 2002).

The shell mass of clams was affected by P_{CO2} and Ω_{Arg} in a nonlinear manner. At moderately elevated P_{CO2} (~800 μatm, salinity 32) we observed an increase in shell mass of clams compared with their normocapnic counterparts. However, the average shell mass of clams exposed to extreme P_{CO2} (~1500 μatm, salinity 32) was similar to that of those maintained in normocapnia. These observations indicate that moderate acidification can increase rates of shell deposition in this species, while further increase in P_{CO2} abolishes this effect. This 'bell-shaped' response is even more apparent when the mass data are plotted against aragonite saturation levels corresponding to each experimental condition. The exact physiological mechanisms of this apparent increase in shell deposition rates under the moderately hypercapnic conditions are currently not known. However, a similar bell-shaped response of calcification rates to increasing P_{CO2} and decreasing in Ω_{Arg} has been previously reported for other species of marine calcifiers (Doney et al., 2009; Ries et al., 2009). Interestingly, many other species, including eastern oysters (*Crassostrea virginica*) demonstrate a very different biomineralization response to changes of P_{CO2}, with a linear decrease of shell deposition rates with increasing CO_2 levels (Ries et al., 2009; Beniash et al., 2010). This is especially noteworthy in light of the fact that the shells of eastern oysters are made of calcite, which

is a more thermodynamically stable $CaCO_3$ isoform, with a higher degree of saturation than that of aragonite under any given set of conditions. Hence, these differences in response of clams and oysters to elevated P_{CO2} indicate that biological factors may play a more significant role in the shell deposition of these species than physicochemical properties of seawater, and that biological mechanisms of biomineralization are different in these bivalve species.

Environmental P_{CO2} and salinity conditions also had profound effects on shell structure and mechanical properties of juvenile clams. Exposure to conditions where aragonite saturation was below 1 (i.e. elevated P_{CO2} and low salinity) caused significant etching of the shells' exterior surfaces. In all experimental groups in which $\Omega_{Arg}$was above 1, the etching of the exterior was relatively modest, suggesting that erosion of the shell exterior is primarily affected by hydrochemistry. This chemical dissolution of the shells may have also contributed to the observed decrease in the shell mass of clams from the low salinity treatments compared with their counterparts maintained at high salinity. Indeed, experiments by Nienhuis and colleagues (Nienhuis et al., 2010) demonstrated that a decrease in the rate of shell growth in *Nucella lamellosa* under elevated CO_2 conditions is primarily caused by higher rates of shell dissolution and not a decrease in the shell deposition rates. In the case of *M. mercenaria*, these relationships appeared more complex, as a moderate increase in the CO_2 levels led to an increase in the shell deposition rate. However, it is likely that at low Ω_{Arg} levels dissolution plays a major role in the mass balance of the shells. Shell erosion may become a major factor affecting survival of bivalves in brackish coastal and estuarine waters by weakening the shells and making them more vulnerable to predators (Green et al., 2009; Amaral et al., 2012).

Interestingly, etching of the shell interior was observed in all groups with elevated P_{CO2} even under the supersaturation conditions for aragonite. These observations indicate that etching of the shells' interior is not directly related to the water chemistry. One possible explanation is that the mollusks dissolve the interior of the shells to compensate for the effects of CO_2-induced acidosis in the tissues as described in mollusks (Crenshaw, 1972; Sokolova et al., 2000). SEM analysis of the shells has also revealed that at low Ω_{Arg} values, a significant erosion of the material in the hinge area occurred, leading to weakening of the ligament insertion site and separation of the shell valves. This, in turn, can lead to a major impediment of the shell opening mechanism and affect growth and survival of the mollusks. Our observations are in agreement with an earlier report of compromised hinge structures in juvenile clams maintained low aragonite saturation conditions (Talmage and Gobler, 2010). Salinity and Ω_{Arg} had much less of an effect on microhardness of the shells than P_{CO2}. For example, the shell microhardness of the clams exposed to normocapnia at low salinity were not significantly different from that of the high salinity normocapnic controls, although there was a threefold difference in Ω_{Arg} between these two experimental conditions. At the same time, microhardness of the shells of clams exposed to elevated CO_2 levels were significantly lower than in those from normocapnic treatments. Notably, fracture toughness of the shells of clams exposed to ~800μatm P_{CO2} at high salinity did not differ from that of the normocapnic controls (salinity 32, ~395 μatm P_{CO2}), despite having significantly lower hardness. The fracture toughness in the shells from the ~1500 μatm, low salinity group also did not differ significantly from that of the control group, likely reflecting the effect of increased shell porosity in limiting crack propagation (Shigegaki et al., 1997; Xu et al., 2001). Taken

together, these data indicate that factors other than chemical erosion contributed to the differences in shell hardness and fracture toughness in clams exposed to different salinity and P_{CO2} levels. It is possible that elevated CO_2 affect the structural organizationof the shell mineral and organic components and/or the proportion of the organic matrix to the mineral in the shell, which in turn will affect its mechanical properties. Determination of the precise biological and structural mechanisms underlying these effects of elevated P_{CO2} is outside the scope of this study and requires further investigation.

As a corollary, our study demonstrates complex interactive effects of salinity and P_{CO2} on physiology and biomineralization of hard-shell clams. The major effects of low salinity under the conditions of this study are driven by the elevated basal energy demand that can lead to energy deficiency, reduced growth and elevated mortality of juvenile clams, and possibly to impaired shell maintenance, as evidenced by the extensive damage to the periostracum at low salinity. The effects of elevated P_{CO2} on the physiology and biomineralization of hard-shell clams appear to be more complex and subtle. The metabolic effects of high P_{CO2} in the studied range (~380–1500 μatm) are minimal, while the most pronounced changes are seen with respect to the growth and mechanical properties of the shell. Effects of elevated P_{CO2} on biomineralization of hard-shell clams involve a complex interplay between the chemical effects of corrosive seawater and biological responses to elevated P_{CO2} and/or reduced Ω_{Arg}. Moderate hypercapnia (~800 μatm P_{CO2}) appears to stimulate shell and tissue growth and reduce mortality of juvenile clams; however, exposureto low salinity or extreme hypercapnia (~1500 μatm P_{CO2}) abates these effects. Mechanical properties of the shell (such as microhardness and fracture toughness) are negatively affected by elevated CO_2 alone or in combination with

low salinity, which may have important implications for protection against predators or environmental stressors. Overall, our data indicate that environmental salinity may strongly modulate responses to ocean acidification in hard-shell clams as well as other marine bivalves (Dickinson et al., 2012) and thus should be taken into account when predicting the effects of ocean acidification on estuarine ecosystems.

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Author Contributions

O.B.M. conducted the experimental exposures, measured mortalities, respiration rates of the clams and participated in data analysis and interpretation, and in writing and revising the manuscript.

Figures

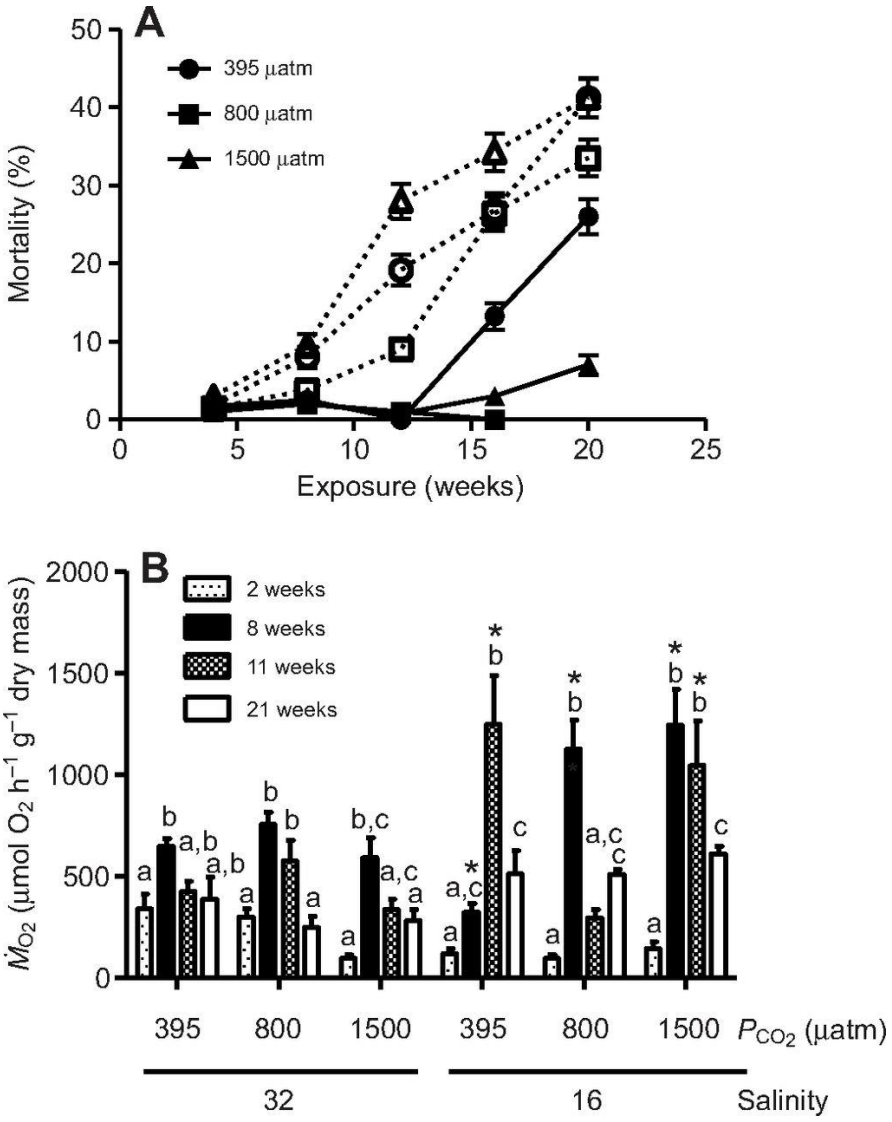


Figure 2.1: Mortality and standard metabolic rate (SMR) in juvenile clams (*Mercenaria mercenaria*) exposed to different P_{CO2} and salinity treatments. (A) Mortality over 20 weeks of exposure period. Solid symbols and solid lines, salinity 32; open symbols and broken lines, salinity 16. Circles, squares and triangles correspond to exposure P_{CO2} of ~395, 800 and 1500 µatm, respectively. There are no mortality estimates for juveniles exposed to ~800 µatm P_{CO2}, salinity 32, due to an accidental loss of experimental animals. *N*=360–505. (B) SMR measured as mass-specific oxygen consumption rates standardized to the average mass of experimental clams (0.237 mg dry tissue mass). Different letters denote significant differences between exposure periods within the same experimental condition (P<0.05). Asterisks denote the values that are significantly different from the respective values for the control clams (maintained at ~395 µatm P_{CO2}

and salinity 32) for the same duration of time (P<0.05). Error bars represent s.e.m. N=9–10.

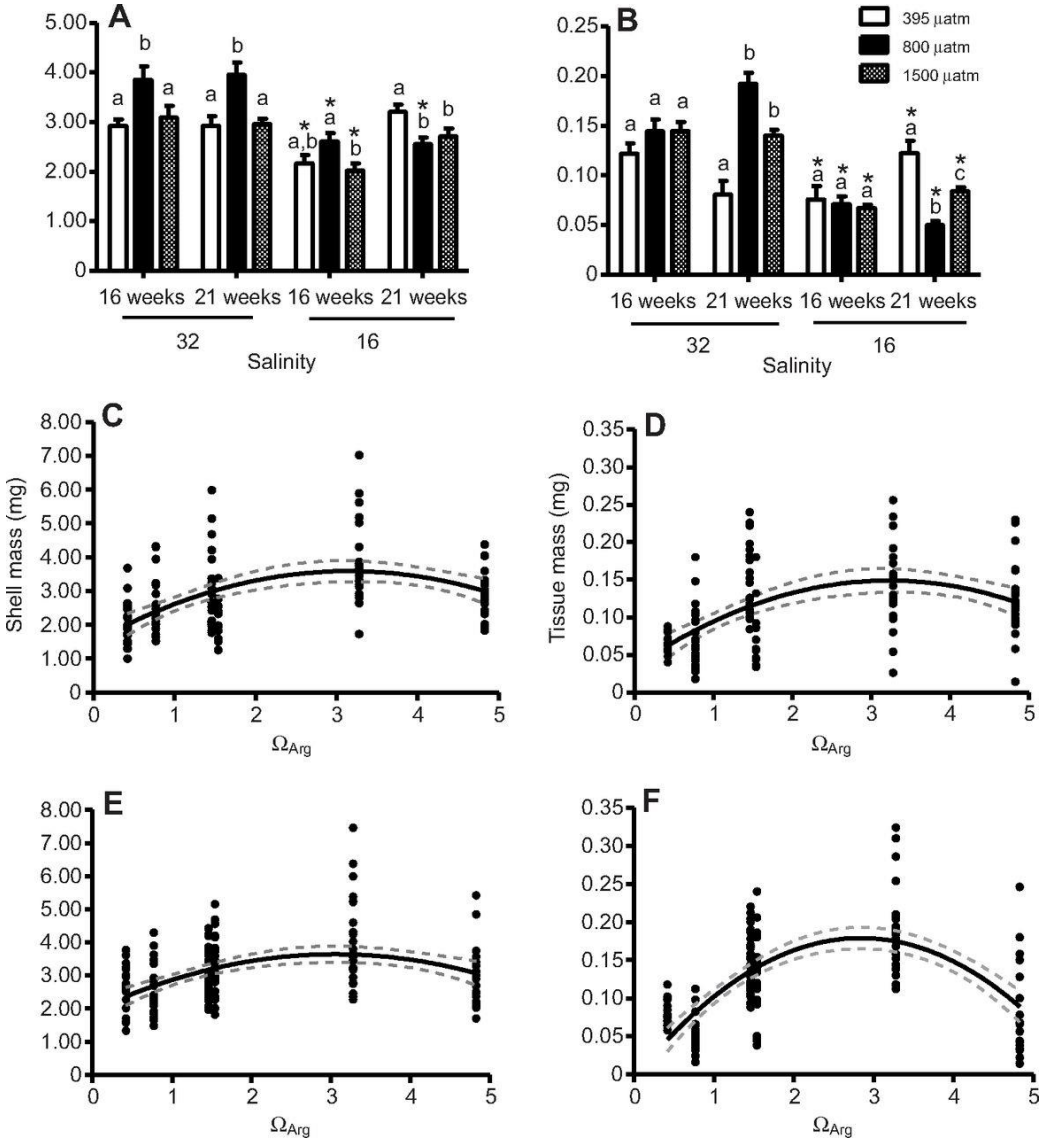


Figure 2.2: Changes in *M. mercenaria* shell and tissue mass in response to salinity, P_{CO2} and aragonite saturation (Ω_{Arg}). (A) Average shell mass after 16 and 21 weeks of exposure; (B) average tissue mass after 16 and 21 weeks of exposure. Different letters denote significant differences between different P_{CO2} levels at the same salinity and exposure period (P<0.05). *Significant differences between high and low salinity at the same P_{CO2} levels and exposure period (P<0.05). (C, E) Regressions of shell mass versus aragonite saturation levels after 16 and 21 weeks of exposure, respectively; (D,F) regressions of tissue mass versus aragonite saturation levels after 16 and 21 weeks of exposure, respectively. Polynomial regressions (solid lines) and 95% confidence intervals (broken lines) are given. N=11–40 in each experimental group.

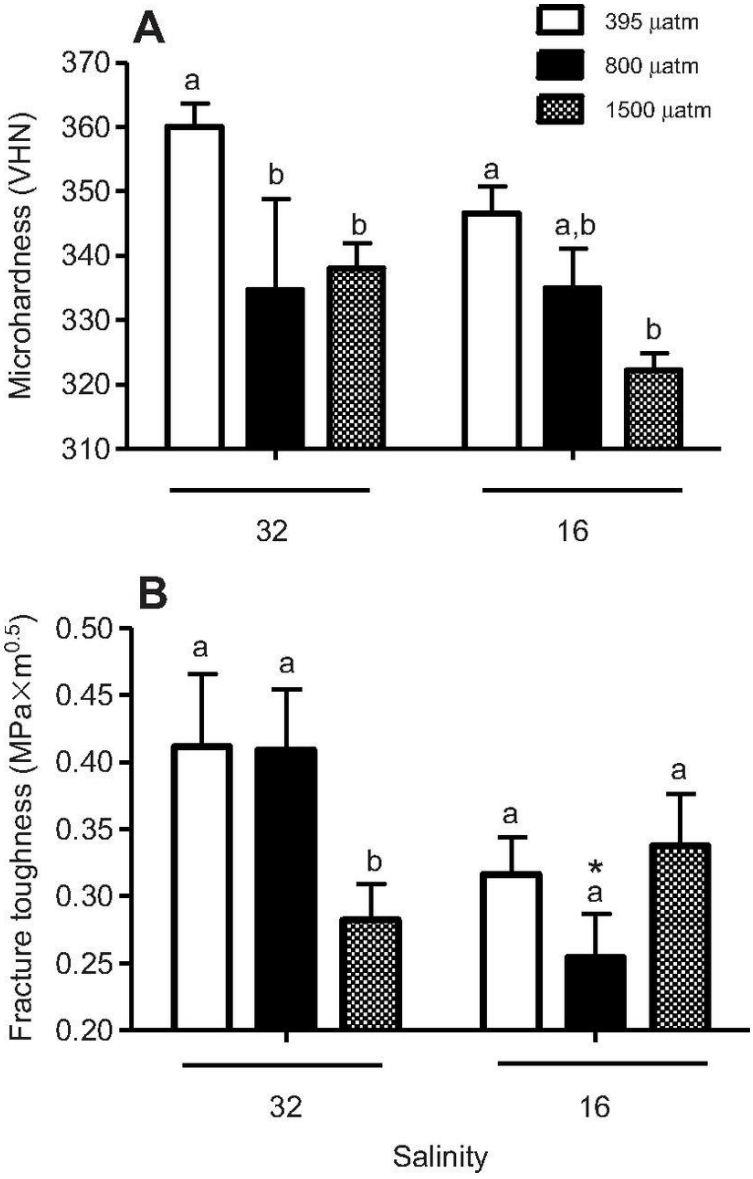


Figure 2.3: Mechanical properties of the shells of *M. mercenaria* exposed to different P_{CO2} and salinity levels. (A) Vickers microhardness of clam shells exposed to experimental conditions for 21 weeks. (B) Fracture toughness of clam shells exposed to experimental conditions for 21 weeks. Within each salinity treatment, groups marked with different letters are significantly different ($P<0.05$). *Significant difference between high and low salinity groups at same P_{CO2}. Data are presented as means ± s.e.m. N=6–7.

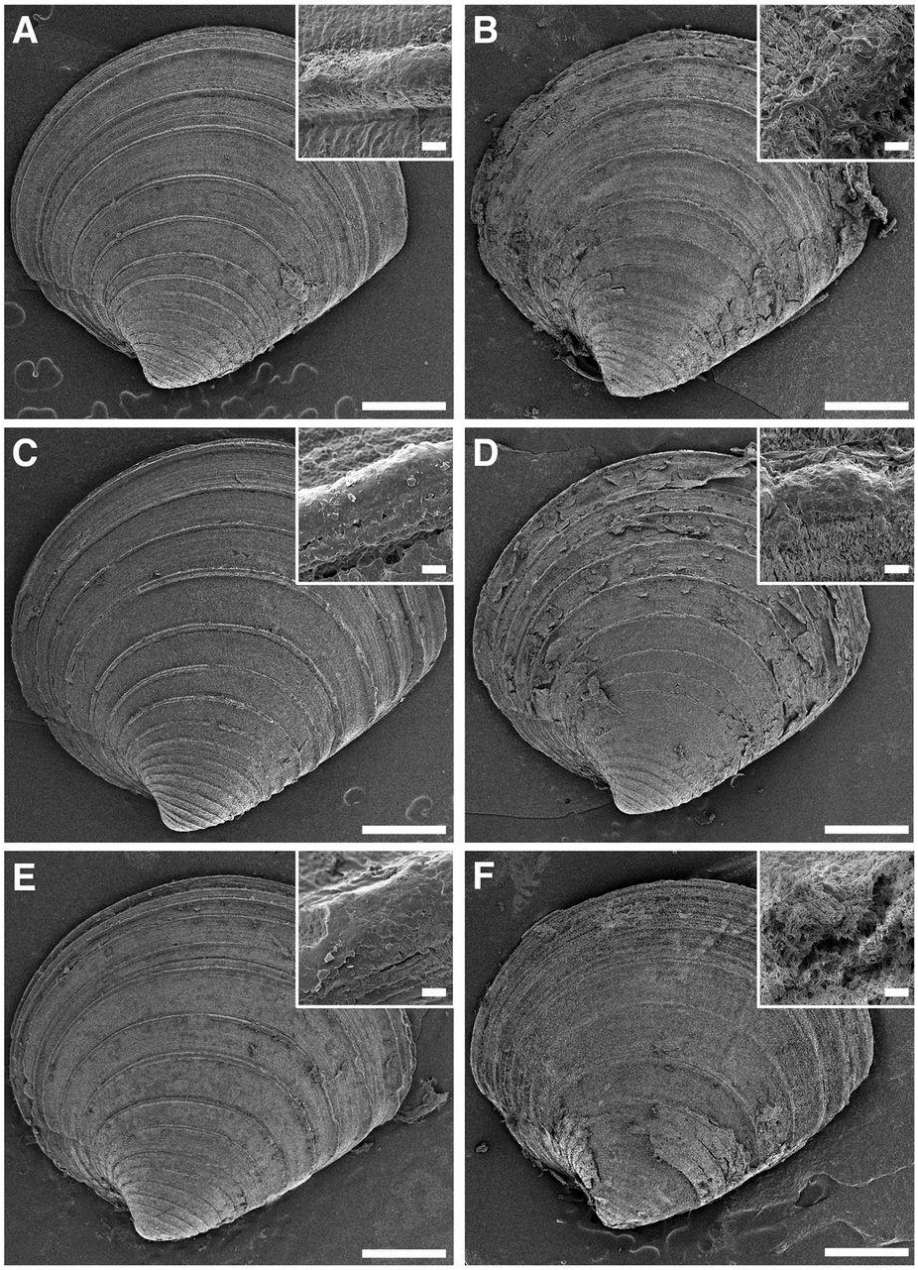


Figure. 2.4: SEM micrographs of the exterior of *M. mercenaria* shells after 16 weeks exposure to experimental conditions. The inset on each panel is a high magnification image of a growth ridge near the periphery of the shell. (A) 395 µatm, salinity 32; (B) 395 µatm, salinity 16; (C) 800 µatm, salinity 32; (D) 800 µatm, salinity 16; (E) 1500 µatm, salinity 32; (F) 1500 µatm, salinity 16. Scale bars, 500 µm main panels; 5 µm insets.

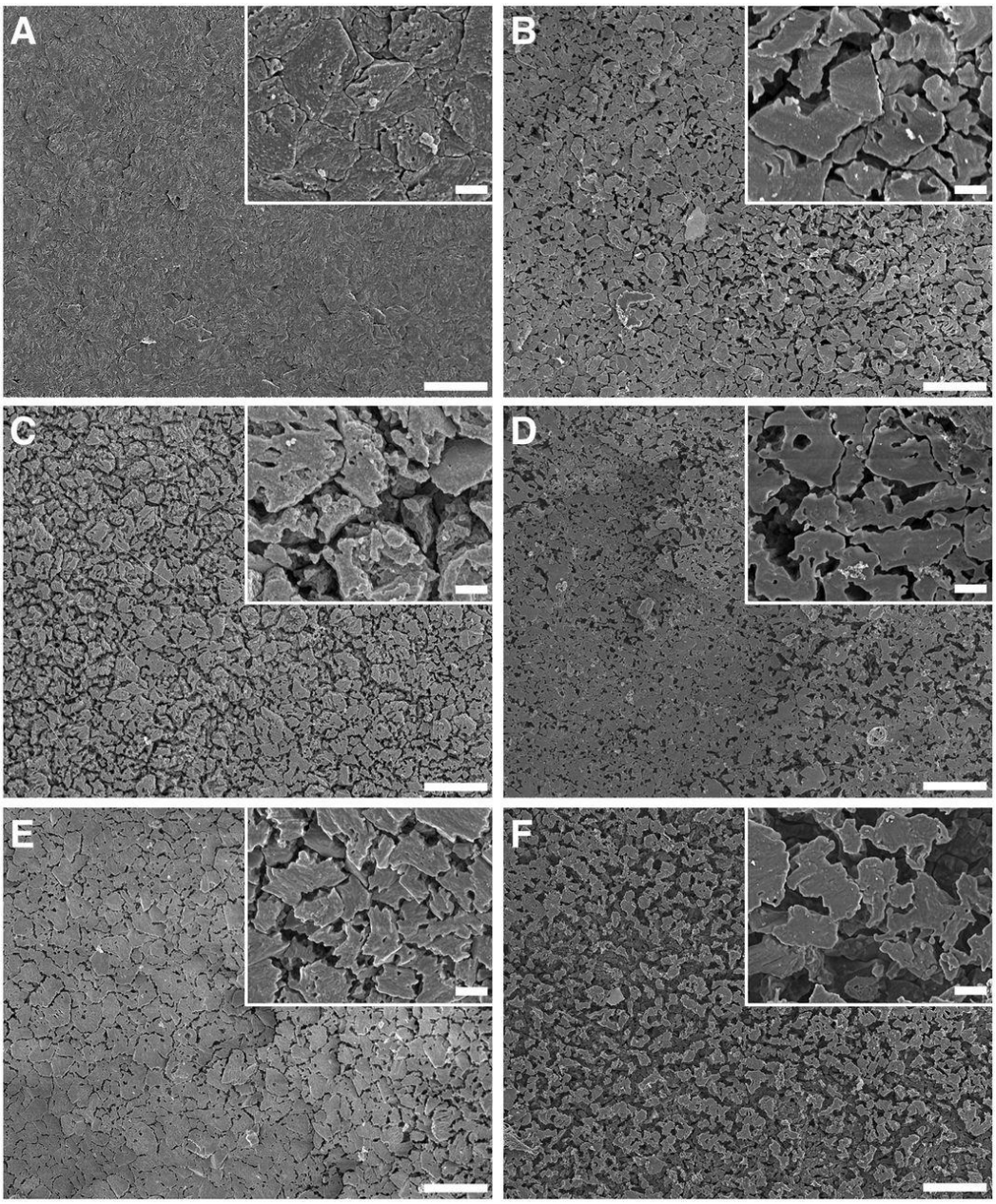


Figure 2.5: SEM micrographs of the interior of *M. mercenaria* shells after 16 weeks exposure to experimental conditions. Images were taken within the center region of the shell interior. Higher (inset) and lower (outer) magnification images were taken from the same region. (A) 395 µatm, salinity 32; (B) 395 µatm, salinity 16; (C) 800 µatm, salinity 32; (D) 800 µatm, salinity 16; (E) 1500 µatm, salinity 32; (F) 1500 µatm, salinity 16. Scale bars, 100 µm main panels; 1 µm insets.

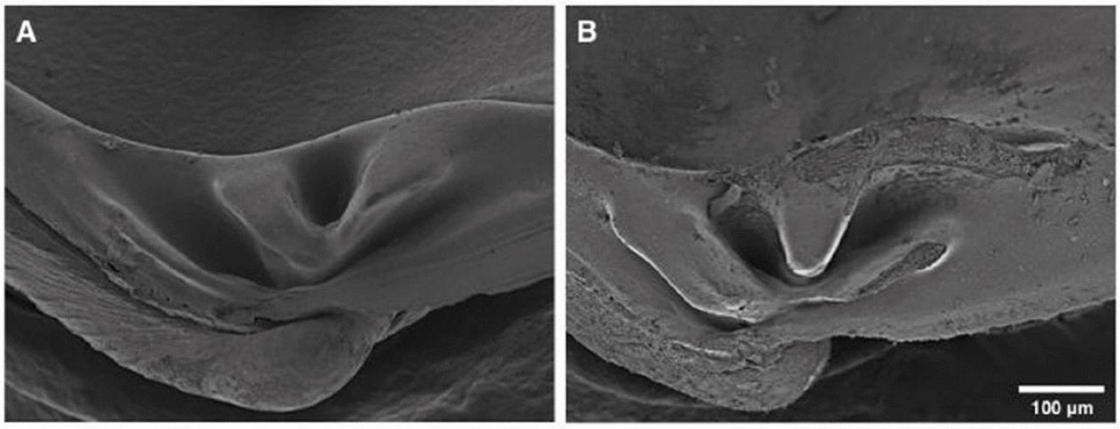


Figure 2.6: SEM micrographs of the interior of the hinge region of M. mercenaria shells after 16 weeks exposure to experimental conditions. (A) 395 µatm, salinity 32; (B) 1500 µatm, 16 salinity.

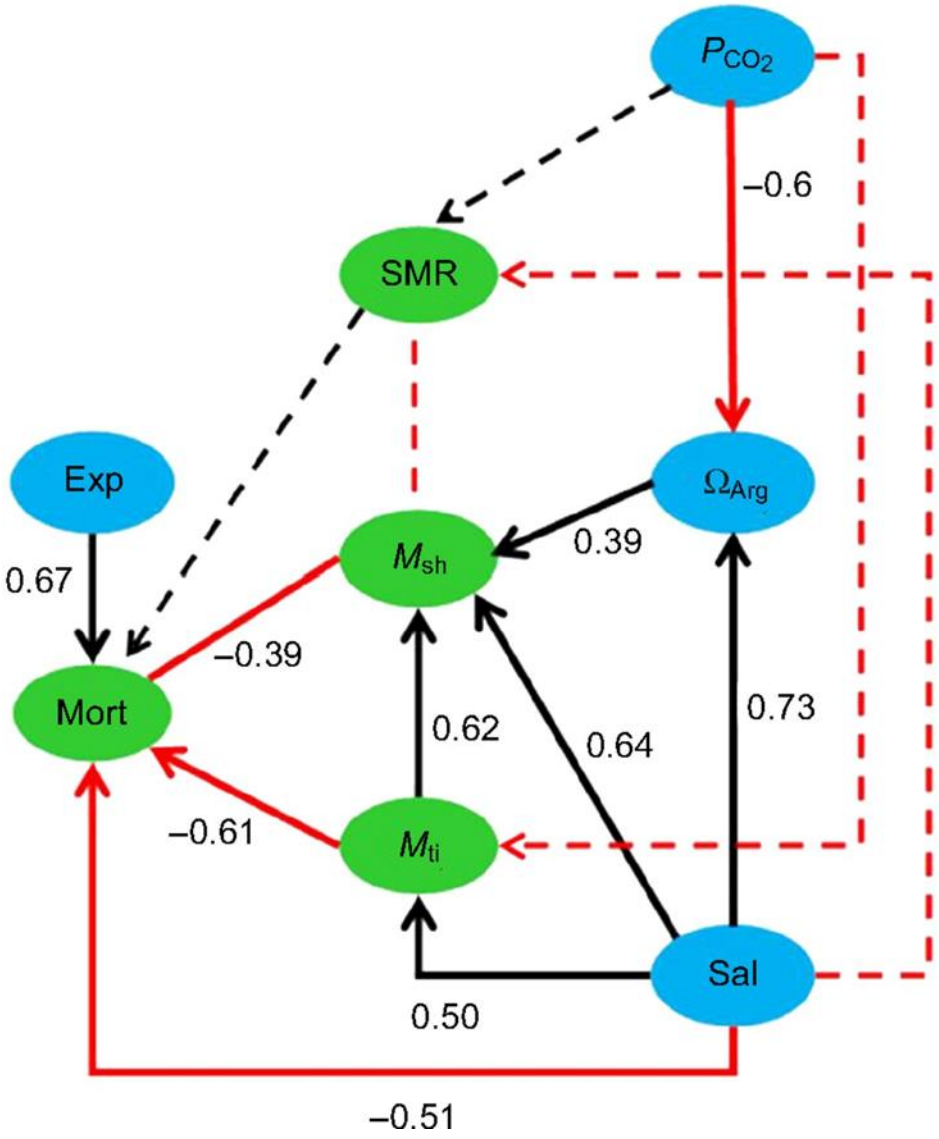


Figure. 2.7: A schematic representation of the relationships between the environmental factors (blue ovals) and the studied biological traits (green ovals). Black and red connector lines represent relationships with positive and negative correlations, respectively. Solid lines represent significant correlations (based on Pearson correlation analysis). Dashed lines represent relationships that did not show significant Pearson correlation coefficients but were identified as important by PCA. Arrows identify putative causality and numbers by the connector lines are Pearson correlation coefficients (R). Abbreviations: P_{CO2}, partial pressure of CO_2; Exp, duration of experimental exposure; Ω_{Arg}, aragonite saturation; Sal, salinity; Mti, tissue mass; Msh, shell mass; SMR, standard metabolic rate; Mort, mortality.

Table 2.1: Summary of water chemistry parameters during experimental exposures. Salinity, temperature, pH and total alkalinity were determined in samples from experimental tanks as described in the Materials and methods. Other parameters were calculated using co2SYS software. Data are presented as means ± s.d. N=45–51 for temperature, salinity and pH, and N=5–8 for all other parameters

	Exposure salinity					
	16			**32**		
	~395 µatm	**~800 µatm**	**~1500 µatm**	**~395 µatm**	**~800 µatm**	**~1500 µatm**
pH	8.25 ±0.26	8.16 ±0.14	7.77 ±0.11	8.26 ±0.08	8.15 ±0.10	7.74 ±0.18
Temperature (°C)	19.3 ±1.6	20.4 ±0.9	19.7 ±0.8	20.1 ±1.0	20.8 ±0.8	20.4 ±0.6
Salinity	17.0 ±1.0	16.3 ±0.9	16.5 ±0.8	32.7 ±1.6	31.6 ±1.1	31.5 ±1.0
P_{CO2} (µatm)	289.1 ±30.2	704.5 ±263.5	1277.2 ±235.2	385.1 ±103.6	656.6 ±212.7	1712.6 ±346.9
TA (µmol kg^{-1} SW)	1564.9 ±86.8	1502.0 ±56.46	1518.4 ±82.69	3025.7 ±178.5	2944.6 ±113.4	2913.9 ±106.38
CO_3^{2-} (µmol kg^{-1} SW)	94.2 ±11.2	47.11 ±19.27	25.9 ±6.0	306.4 ±41.2	206.9 ±37.0	91.9 ±15.3
Ω_{Ca}	2.56 ±0.29	1.29 ±0.52	0.71 ±0.16	7.46 ±1.01	5.07 ±0.90	2.26 ±0.37
Ω_{Arg}	1.54 ±0.18	0.77 ±0.32	0.42 ±0.10	4.83 ±0.65	3.28 ±0.58	1.46 ±0.24

Table.2.2: Effects of exposure P_{CO2}, salinity and their interaction on shell and physiological properties in juvenile *Mercenaria mercenaria*. F-values are provided with degrees of freedom for the factor and the error in subscript. Significant P-values are in bold

Parameters	P_{CO2}	Salinity	P_{CO2} x Salinity
Shell mass, 16 weeks	$\mathbf{F_{2,119} = 37.8}$ **p < 0.001**	$\mathbf{F_{1,119} = 8.00}$ **p < 0.001**	$F_{2,119}$ = 0.68 p = 0.507
Shell mass, 21 weeks	$\mathbf{F_{2,163} = 11.2}$ **p = 0.001**	$\mathbf{F_{1,163} = 3.30}$ **p = 0.039**	$\mathbf{F_{2,163} = 13.2}$ **p < 0.001**
Tissue mass, 16 weeks	$F_{2,117}$ = 0.42 p = 0.653	$\mathbf{F_{1,117} = 63.4}$ **p < 0.001**	$F_{2,117}$ = 1.44 p = 0.242
Tissue mass, 21 weeks	$F_{2,147}$ = 2.41 p = 0.093	$\mathbf{F_{1,147} = 51.8}$ **p < 0.001**	$\mathbf{F_{2,147} = 53.9}$ **p < 0.001**
Vickers microhardness	$\mathbf{F_{2,36} = 6.16}$ **p = 0.005**	$F_{1,36}$ = 2.86 p = 0.099	$F_{2,36}$ = 0.77 p = 0.472
Fracture toughness	$F_{2,33}$ = 0.92 p = 0.408	$F_{1,33}$ = 4.00 p = 0.054	$\mathbf{F_{2,33} = 3.75}$ **p = 0.034**
Standard metabolic rate (SMR)	$F_{2,427}$ =0.36 p =0.701	$\mathbf{F_{1,427} = 37.19}$ **p < 0.0001**	$\mathbf{F_{2,427} = 12.93}$ **p < 0.0001**

Supplementary Materials

Supplementary Table 1: Results of Pearson correlation test of M_{ti} vs. M_{sh} at two latest time points (16 and 21 weeks of exposure) conducted for individual clams within each experimental treatment group. Significant correlations ($P<0.05$) are shown in bold.

Experimental group	Exposure time	R	σ
Sal 32, [CO_2] 395 ppm	16 wk	**0.81**	1.90E-06
Sal 32, [CO_2] 800 ppm	16 wk	**0.81**	4.41E-06
Sal 32, [CO_2] 1500 ppm	16 wk	**0.85**	6.74E-08
Sal 16, [CO_2] 395 ppm	16 wk	0.33	0.29107
Sal 16, [CO_2] 800 ppm	16 wk	**0.44**	0.03608
Sal 16, [CO_2] 1500 ppm	16 wk	0.27	0.28251
Sal 32, [CO_2] 395 ppm	21 wk	-0.04	0.86218
Sal 32, [CO_2] 800 ppm	21 wk	**0.96**	8.44E-14
Sal 32, [CO_2] 1500 ppm	21 wk	**0.90**	4.66E-15
Sal 16, [CO_2] 395 ppm	21 wk	**0.56**	0.00876
Sal 16, [CO_2] 800 ppm	21 wk	**0.63**	4.58E-04
Sal 16, [CO_2] 1500 ppm	21 wk	0.33	0.15065

Supplementary Table 2: Pearson Correlation coefficients and the corresponding σ values for the experimental conditions and biological parameters of the clams. Asterisks identify significant correlations.

		Exposure Time	Salinity	pCO2	Arg Saturation	shellmass	tissuemass	Resp
"Exposure Time"	Pearson Corr.	1	1.83773E-16	-1.35269E-17	4.17204E-17	0.037	-0.17461	0.15595
	Sig.	--	1	1	1	0.84611	0.35609	0.46682
"Salinity"	Pearson Corr.	1.83773E-16	1	2.94432E-17	0.73396*	0.64004*	0.50092*	-0.26491
	Sig.	1	--	1	3.91646E-6	1.39588E-4	0.00481	0.21094
"pCO2"	Pearson Corr.	-1.35269E-17	2.94432E-17	1	-0.57962*	-0.09547	0.05438	0.02041
	Sig.	1	1	--	7.88718E-4	0.61578	0.77532	0.9246
"Arg Saturation"	Pearson Corr.	4.17204E-17	0.73396*	-0.57962*	1	0.38754*	0.24605	-0.14509
	Sig.	1	3.91646E-6	7.88718E-4	--	0.03435	0.18996	0.49876
"shellmass"	Pearson Corr.	0.037	0.64004*	-0.09547	0.38754*	1	0.62391*	-0.37672
	Sig.	0.84611	1.39588E-4	0.61578	0.03435	--	2.29563E-4	0.06959
"tissuemass"	Pearson Corr.	-0.17461	0.50092*	0.05438	0.24605	0.62391*	1	-0.18506
	Sig.	0.35609	0.00481	0.77532	0.18996	2.29563E-4	--	0.38665
"Resp"	Pearson Corr.	0.15595	-0.26491	0.02041	-0.14509	-0.37672	-0.18506	1
	Sig.	0.46682	0.21094	0.9246	0.49876	0.06959	0.38665	--

2-tailed test of significance is used
*:Correlation is significant at the 0.05 level

Supplementary Table 3: Pearson Correlation coefficients and the corresponding σ values for the experimental conditions and biological parameters of the clams grown at salinity 32. Asterisks identify significant correlations.

		Exposure Time	pCO2	shellmass	tissuemass	Resp	Arg Saturation
"Exposure Time"	Pearson Corr.	1	-5.41075E-17	-0.12916	-0.53677*	0.32716	1.39625E-16
	Sig.	–	1	0.64641	0.03911	0.29926	1
"pCO2"	Pearson Corr.	-5.41075E-17	1	-0.54752*	-0.16868	0.17644	-0.91008*
	Sig.	1	–	0.03463	0.54788	0.58331	2.50354E-6
"shellmass"	Pearson Corr.	-0.12916	-0.54752*	1	0.1878	-0.42349	0.53995*
	Sig.	0.64641	0.03463	–	0.5027	0.17012	0.03774
"tissuemass"	Pearson Corr.	-0.53677*	-0.16868	0.1878	1	-0.26921	0.17773
	Sig.	0.03911	0.54788	0.5027	–	0.39749	0.52628
"Resp"	Pearson Corr.	0.32716	0.17644	-0.42349	-0.26921	1	-0.1208
	Sig.	0.29926	0.58331	0.17012	0.39749	–	0.70843
"Arg Saturation"	Pearson Corr.	1.39625E-16	-0.91008*	0.53995*	0.17773	-0.1208	1
	Sig.	1	2.50354E-6	0.03774	0.52628	0.70843	–

2-tailed test of significance is used
*:Correlation is significant at the 0.05 level

Supplementary Table 4: Pearson Correlation coefficients and the corresponding σ values for the experimental conditions and biological parameters of the clams grown at salinity 16. Asterisks identify significant correlations.

		Exposure Time	pCO2	shellmass	tissuemass	Resp	Arg Saturation
"Exposure Time"	Pearson Corr.	1	-5.41075E-17	0.20782	0.12214	-0.20945	1.56696E-16
	Sig.	--	1	0.45734	0.66456	0.51354	1
"pCO2"	Pearson Corr.	-5.41075E-17	1	0.25629	0.2867	-0.37009	-0.972*
	Sig.	1	--	0.35652	0.3002	0.23635	1.48072E-9
"shellmass"	Pearson Corr.	0.20782	0.25629	1	0.69006*	-0.09883	-0.38207
	Sig.	0.45734	0.35652	--	0.00441	0.75993	0.15991
"tissuemass"	Pearson Corr.	0.12214	0.2867	0.69006*	1	0.28327	-0.35902
	Sig.	0.66456	0.3002	0.00441	--	0.37229	0.18878
"Resp"	Pearson Corr.	-0.20945	-0.37009	-0.09883	0.28327	1	0.3196
	Sig.	0.51354	0.23635	0.75993	0.37229	--	0.31122
"Arg Saturation"	Pearson Corr.	1.56696E-16	-0.972*	-0.38207	-0.35902	0.3196	1
	Sig.	1	1.48072E-9	0.15991	0.18878	0.31122	--

2-tailed test of significance is used
*:Correlation is significant at the 0.05 level

Supplementary Table 5: Principal component analysis for all parameters. Loadings of principal components, Eigenvalues and % of variance are given for each principal component.

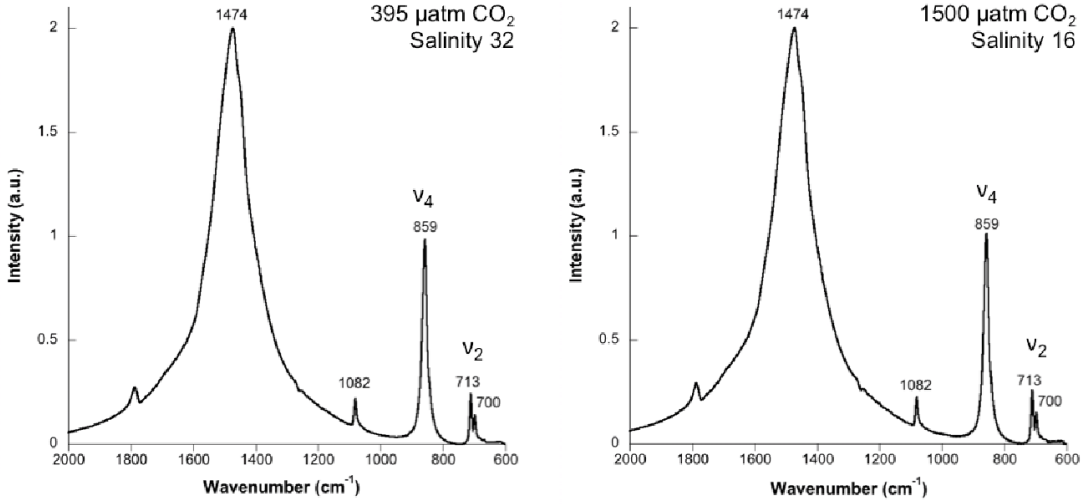
	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3	Coefficients of PC4	Coefficients of PC5
Exposure Time	-0.13196	**0.24594**	**0.73981**	-0.00886	0.09012
Salinity	**0.42971**	-0.12826	**0.30161**	0.16318	**-0.51884**
pCO2	**-0.23407**	**-0.55255**	**0.22775**	0.08182	**-0.27392**
Aragonite Saturation	**0.43541**	**0.28835**	0.07309	0.12724	**-0.4148**
pH	**0.32002**	**0.51596**	-0.14429	-0.02521	0.16471
Mortality	**-0.3627**	**0.33353**	**0.38615**	-0.13791	0.05686
Shell mass	**0.39105**	-0.16556	**0.35215**	-0.10242	**0.36555**
Tissue mass	**0.34569**	**-0.31737**	0.102	**0.26307**	**0.55383**
SMR	**-0.21721**	0.17036	-0.01273	**0.92253**	0.06979

	Eigenvalue	Percentage of Variance	Cumulative
1	3.45974	38.44%	38.44%
2	2.22381	24.71%	63.15%
3	1.34317	14.92%	78.07%
4	0.88109	9.79%	87.86%
5	0.62364	6.93%	94.79%

Supplementary Table 6: Principal component analysis without pH and aragonite saturation. Loadings of principal components, Eigenvalues and % of variance are given for each principal component.

	Coefficients of PC1	Coefficients of PC2	Coefficients of PC3	Coefficients of PC4	Coefficients of PC5
Exposure Time	-0.23	**0.76**	0.08	0.02	0.03
pCO2	0.00	-0.07	**0.96**	-0.26	-0.05
Salinity	**0.45**	**0.30**	0.05	0.12	**-0.74**
Mortality	**-0.49**	**0.40**	0.00	-0.13	0.14
Shell mass	**0.45**	**0.40**	-0.05	0.00	**0.21**
Tissue mass	**0.47**	0.08	0.18	**0.30**	**0.61**
Resp	**-0.29**	-0.04	**0.21**	**0.90**	-0.10

	Eigenvalue	Percentage of Variance	Cumulative
1	2.90999	41.57%	41.57%
2	1.34704	19.24%	60.81%
3	1.02078	14.58%	75.40%
4	0.85644	12.23%	87.63%
5	0.47371	6.77%	94.40%



Supplement Figure 1: Representative transmittance FTIR spectra of *M. mercenaria* shells after 21 weeks exposure to experimental conditions. Since no variation in peak position or intensity was observed among any of the experimental treatments, only spectra for the 395 µatm, salinity 32 (left) and 1500 µatm, salinity 16 (right) are shown.

CHAPTER 3: INTERACTIVE EFFECTS OF SALINITY AND ELEVATED CO_2 ON JUVENILE EASTERN OYSTERS, *CRASSOSTREA VIRGINICA*

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Abstract

Rising levels of atmospheric CO_2 lead to acidification of the ocean and alter seawater carbonate chemistry, which can negatively impact calcifying organisms, including mollusks. In estuaries, exposure to elevated CO_2 levels often co-occurs with other stressors, such as reduced salinity, which enhances the acidification trend, affects ion and acid–base regulation of estuarine calcifiers and modifies their response to ocean acidification. We studied the interactive effects of salinity and partial pressure of CO_2 (P_{CO2}) on biomineralization and energy homeostasis in juveniles of the eastern oyster, *Crassostrea virginica*, a common estuarine bivalve. Juveniles were exposed for 11 weeks to one of two environmentally relevant salinities (30 or 15 PSU) either at current atmospheric P_{CO2} (~400 μatm, normocapnia) or P_{CO2} projected by moderate IPCC scenarios for the year 2100 (~700–800 μatm, hypercapnia). Exposure of the juvenile oysters to elevated P_{CO2} and/or low salinity led to a significant increase in

mortality, reduction of tissue energy stores (glycogen and lipid) and negative soft tissue growth, indicating energy deficiency. Interestingly, tissue ATP levels were not affected by exposure to changing salinity and P_{CO2}, suggesting that juvenile oysters maintain their cellular energy status at the expense of lipid and glycogen stores. At the same time, no compensatory upregulation of carbonic anhydrase activity was found under the conditions of low salinity and high P_{CO2}. Metabolic profiling using magnetic resonance spectroscopy revealed altered metabolite status following low salinity exposure; specifically, acetate levels were lower in hypercapnic than in normocapnic individuals at low salinity. Combined exposure to hypercapnia and low salinity negatively affected mechanical properties of shells of the juveniles, resulting in reduced hardness and fracture resistance. Thus, our data suggest that the combined effects of elevated P_{CO2} and fluctuating salinity may jeopardize the survival of eastern oysters because of weakening of their shells and increased energy consumption.

Introduction

Ocean acidification associated with increasing atmospheric CO_2 levels is an urgent problem in the present and future state of oceans. An increase in dissolved CO_2 reduces seawater pH and alters its carbonate chemistry. These changes affect multiple biological processes that depend on pH and/or the levels and speciation of inorganic carbon in seawater, such as photosynthetic carbon fixation and $CaCO_3$ deposition via biomineralization (Doney et al., 2009). Estuaries and coastal habitats, which are hotspots for biological diversity in the oceans, are likely to be strongly affected by an increase in atmospheric CO_2. Although the chemistry and hydrodynamics of estuarine waters are complex and highly variable, the long-term trend of seawater pH in certain estuarine

systems correlates with the respective trends in the open ocean, suggesting that estuaries will also experience effects of ocean acidification. For example, mean seawater pH in polyhaline sites [>18 practical salinity units (PSU)] of the Chesapeake Bay decreased by 0.012 and 0.006 units $year^{-1}$ (in spring and summer, respectively) over the past 25years (Waldbusser et al., 2011), a rate above the 50-year trend for the surface waters in the open ocean (–0.0019 units $year^{-1}$) (Doney et al., 2009). Moreover, brackish waters can experience large fluctuations in seawater pH and carbonate chemistry because of a lower buffering capacity (compared with open ocean waters with higher salinity), acidic inputs from land-based sources, and biological CO_2 production (Pritchard, 1967; Burnett, 1997; Ringwood and Keppler, 2002). In fact, the seawater dilution in estuaries exacerbates the acidification trend induced by elevated CO_2 (Denman et al., 2011). Because of this natural variability of seawater pH in estuaries, estuarine organisms are often considered to be more tolerant of pH fluctuations and ocean acidification than their open ocean counterparts. However, the effects of high partial pressure of CO_2 (P_{CO2}) and low pH on estuarine organisms and their tolerance limits in the face of ocean acidification are not yet fully understood.

Marine calcifying organisms (such as mollusks, echinoderms and corals) that build calcium carbonate ($CaCO_3$) skeletons are susceptible to changes in seawater carbonate chemistry because both biomineralization and $CaCO_3$ dissolution can be directly affected by reduced pH and the degree of saturation for $CaCO_3$ (Kleypas et al., 2006). Moreover, biomineralization is a complex, biologically regulated process that requires energy (Digby, 1968; Palmer, 1983; Palmer, 1992; Wheeler, 1992; Day et al., 2000; Furuhashi et al., 2009). Susceptibility to ocean acidification varies among marine

calcifiers, although most studied species show reduced biomineralization rates in response to elevated P_{CO2} (Doney et al., 2009). In acidified seawater, an increase in energy consumption required for carbonate sequestration and mineral deposition may incur a significant energy cost to marine calcifiers (Palmer, 1983; Geller, 1990; Palmer, 1992; Day et al., 2000; Wood et al., 2008; Wood et al., 2010). Ocean acidification can also affect energy metabolism of marine organisms either directly, via metabolic effects of changing intracellular pH, and/or indirectly via the elevated energy demands for acid–base and ion homeostasis (Pörtner, 1987; Lannig et al., 2010; Pörtner, 2010). This may result in trade-offs of limited energy resources between different biological processes, including homeostasis, growth, reproduction, development and biomineralization (Sokolova et al., 2011). The metabolic response to ocean acidification is variable and depends on the species, degree of acidification and other environmental factors [see Pörtner and Bock, Beniash et al. and Lannig et al., and references therein (Pörtner and Bock, 2000; Beniash et al., 2010; Lannig et al., 2010)].

In estuarine waters, CO_2-driven acidification commonly co-occurs with other stressors, including temperature, hypoxia and salinity, that can affect both biomineralization and energy metabolism. The potential interactions between hypercapnia and other environmental stressors are not well understood, but recent studies indicate that such interactions may be quite complex (Gazeau et al., 2007; Pörtner, 2008; Gooding et al., 2009; Ries et al., 2009; Byrne et al., 2010; Pörtner, 2010). For example, a moderate increase in temperature partially alleviated negative effects of low pH on biomineralization in the sea urchin *Heliocidaris erythrogramma* and the oyster

Crassostrea virginica (Byrne et al., 2010; Waldbusser et al., 2011), but not in the abalone *Haliotis coccoradiata*, while a more extreme warming led to inhibition of biomineralization in H. erythrogramma (Byrne et al., 2010). These results indicate species-specific and potentially non-linear effects of temperature and temperature–pH interactions. Environmental salinity is another factor that can affect seawater chemistry and modify responses to hypercapnia and low pH in estuarine organisms. Brackish waters have lower alkalinity and less buffering capacity compared with open ocean waters, leading to lower pH of the brackish waters both in normocapnia and under the elevated P_{CO2} conditions (Mook and Koene, 1975; Hofmann et al., 2009). Low salinity also results in major changes in water chemistry, such as reduced Ca^{2+} concentrations and total inorganic carbon (Mook and Koene, 1975; Hofmann et al., 2009), which – in conjunction with changes in alkalinity, buffering capacity and pH – may affect metabolism and biomineralization in marine calcifiers. Both salinity and pH can strongly affect energy metabolism as well as ion and acid–base homeostasis (Kinne, 1971; Ballantyne and Moyes, 1987a; Truchot, 1988; Hawkins and Hilbish, 1992; Lannig et al., 2010), thus creating a physiological basis for the interactive effects of these stressors on estuarine organisms. The combined effects of hypercapnia and salinity on metabolic physiology and biomineralization of estuarine organisms, however, are not well understood and require further investigation.

Eastern oysters, *Crassostrea virginica* Gmelin 1791, are common bivalves in West Atlantic estuaries. They build thick, predominantly calcitic shells used for protection against predators and environmental stressors such as extreme salinity or pollutants (Davenport, 1985; Kennedy et al., 1996; Checa et al., 2007; Checa

et al., 2009). Like other estuarine invertebrates, oysters can experience wide fluctuations of salinity, P_{CO2} and pH in their natural habitats and these natural pH fluctuations may be further compounded by future ocean acidification. Oysters have a low capacity to compensate for disturbances in ion and acid–base status induced by changes in seawater pH and/or salinity, and their metabolism is sensitive to disturbances in extracellular and intracellular pH (Crenshaw, 1972; Pörtner, 2008). Mollusks, including oysters, are also osmoconformers, and therefore changes in environmental salinity directly translate into changes in intracellular osmolarity (Kinne, 1971; Prosser, 1973; Berger, 1986; Berger and Kharazova, 1997). Thus, salinity and pH stress, alone and in combination, can strongly affect metabolism and biomineralization in these organisms.

The goal of this study was to assess the combined effects of salinity (15–30) and P_{CO2} (400–800 µatm) on biomineralization, energy homeostasis and metabolite profile of juvenile *C. virginica*. Survival, body size, biomineralization-related parameters [shell mass and mechanical properties, and activity and mRNA expression of carbonic anhydrase (CA)], parameters of energy status (high-energy phosphates and tissue energy stores) as well as concentrations of anaerobic end products (alanine, acetate and succinate) and free amino acids were determined in oyster juveniles after 11 weeks exposure to different salinity and P_{CO2} levels.

Materials and Methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma Aldrich (St Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Experimental Design

The effects of two factors were assessed in this study: salinity and P_{CO2}. Experiments were carried out at two salinity levels, 30 (high salinity) and 15 (low salinity), and two P_{CO2} levels, ~400 µatm (normocapnia) and ~700–800 µatm (hypercapnia), yielding four treatment groups. The salinity conditions were within the environmentally relevant range for this species, and the two selected P_{CO2} levels were representative of the present-day conditions (~400 µatmCO_2) and atmospheric P_{CO2} predicted by the moderate scenarios of the Intergovernmental Panel for Climate Change (IPCC 2007) for the year 2100 (~700–800 µatm CO_2). Oysters were randomly assigned to one of these four treatment groups. The group exposed to a salinity of 30 and a P_{CO2} of ~400 µatm was considered the control, as these conditions were close to the natural habitat conditions of the studied population. Non-reproductive juveniles were used in this study in order to avoid complications due to the varying energy demands of reproducing organisms in different stages of their reproductive cycle.

2.3. Animal Collection and Maintenance

Juvenile oysters (7weeks post-metamorphosis) were obtained from a local oyster supplier (J & B Aquafood, Jacksonville, NC, USA) and pre-acclimated for 5–7 days at 20°C and a salinity of 30 in recirculating water tanks with artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) prior to experimentation. Salinity was maintained at 30 for high salinity treatments and gradually lowered by approximately 2 PSU day^{-1} to reach a salinity of 15 in the low salinity treatments. Once this was completed, oyster shells were stained with calcein {2,4-bis-[N,N-di(carbomethyl)-

aminomethyl]-fluorescein} to create an artificial growth mark to distinguish new shell growth. Calcein is incorporated into growing $CaCO_3$ structures, creating a growth mark that brightly fluoresces upon excitation (Heilmayer et al., 2005; Riascos et al., 2007; Kaehler and McQuaid, 1999). Animals were incubated for 12h in gently aerated ASW containing 50 mg l^{-1} calcein. Calcein staining was conducted in normocapnia at the two respective salinities, and pH of the calcein solution in ASW was adjusted to 8.3 using Seachem Marine Buffer (Seachem, Madison, GA, USA). After calcein staining, oyster juveniles were rinsed with clean ASW and placed in the experimental incubation tanks.

For hypercapnic treatments, the seawater was bubbled with CO_2- enriched air (certified gas mixtures containing 21% O_2, 0.08% CO_2 and balance N_2; Roberts Oxygen, Charlotte, NC, USA), whereas the normocapnic treatments were bubbled with ambient air. The gas flow rates were adjusted in such a way that further increase in the bubbling rate did not lead to a change in seawater pH, indicating that our systems were in a steady state. Salinity was determined using a YSI30 salinity, temperature and conductivity meter (YSI Inc., Yellow Springs, OH, USA). Water temperature was maintained at 21±1°C in all tanks and salinity either at 30±0.5 or 15±0.5. Water was changed every other day using ASW pre-equilibrated with the respective gas mixtures. Artificial seawater was prepared from the same batch of Instant Ocean® sea salt throughout the experiment to minimize variations in pH, alkalinity and ionic composition. A single batch of seawater was prepared during every water change and used for all experimental treatments; seawater with a salinity of 15 was prepared from seawater at a salinity of 30 by dilution. The experimental incubations of juvenile oysters lasted 11weeks.

During the preliminary acclimation and experimental incubations, oysters were fed ad libitum every other day with a commercial algal blend (5ml per 30l tank) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* sp. with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Algae were added to the tanks following each water change. Experimental tanks were checked for mortality daily, and oysters that gaped and did not respond to a mechanical stimulus were recorded as dead and immediately removed.

2.4. Seawater Chemistry

Carbonate chemistry of seawater was determined as described in an earlier study (Beniash et al., 2010). Briefly, samples were periodically collected from experimental tanks during the 11weeks of exposure, placed in air-tight containers without air space, stabilized by mercuric chloride poisoning (Dickson et al., 2007) and kept at +4°C until further analysis. Water pH was measured at the time of collection using a pH electrode (pH meter Model 1671 equipped with a 600P pH electrode, Jenco Instruments, San Diego, CA, USA) calibrated with National Institute of Standards and Technology standard pH buffer solutions (National Bureau of Standards, NBS standards) (Fisher Scientific). Water temperature and salinity were recorded at the same time. Total dissolved inorganic carbon (DIC) concentrations were measured within a week of collection by Nutrient Analytical Services (Chesapeake Biological Laboratory, Solomons, MD, USA). DIC was determined using a Shimadzu TOC5000 gas analyzer equipped with a non-dispersive infrared sensor detector for CO_2 determination (Shimadzu Scientific Instruments, Columbia, MD, USA) calibrated with DIC standards

(Nacalai Tesque, Columbia, MD, USA) recommended by and purchased from the instrument's manufacturer. Samples were measured immediately after opening to minimize gas exchange. Three to five replicates were run for each sample, and precision of the analysis was 1% or better for the technical replicates from the same sample. Temperature, salinity and pH were measured at the time of collection and, along with the total DIC levels, were used to calculate P_{CO2}, alkalinity and the saturation state (Ω) for calcite and aragonite in seawater using co2SYS software (Lewis and Wallace, 1998). For co2SYS settings, we used the NBS scale of seawater pH constants from Millero et al. (Millero et al., 2006), the KSO_4^- constant from Dickson et al. [(Dickson et al., 1990) cited in Lewis and Wallace (Lewis and Wallace, 1998)], and concentrations of silicate and phosphate for Instant Ocean® seawater (silicate: 0.17 and 0.085 μmol kg^{-1} at salinities of 30 and 15, respectively, and phosphate: 0.04 and 0.02 μmol kg^{-1} at salinities of 30 and 15, respectively). Water chemistry data for these samples are given in Table 3.1. It is worth noting that pH and carbonate chemistry differed between salinities of 30 and 15 at the same P_{CO2} levels, reflecting changes in the DIC, buffering capacity and alkalinity associated with dilution of seawater; this situation mimics conditions naturally occurring in brackish estuarine waters where seawater and freshwater mix (Mook and Koene, 1975; Hofmann et al., 2009). In addition, total alkalinity of Instant Ocean® seawater is slightly higher (~3000μmol kg^{-1}ASW in the high salinity treatment) than values reported from the natural seawater (~2300–2500 μmol kg^{-1}seawater) (Zeebe and Wolf-Gladrow, 2001; Riebesell et al., 2010), as is typical for artificial sea salt formulations. Thus, the estimates of the effects of ocean acidification obtained in the

present study are conservative, as the CO_2-induced changes in pH and carbonate chemistry will be stronger in the natural seawater with lower alkalinity. Oxygen levels in experimental tanks were tested using Clark-type oxygen probes (YSI 5331 oxygen probe, YSI Inc.) connected to a YSI 5300A biological oxygen monitor and were >95% of air saturation throughout all exposures.

2.5. Shell and Soft tissue Mass Measurements

Following experimental exposure, approximately 50 oysters from each treatment group were stored in 70% ethanol and shipped to the University of Pittsburgh for mass measurements and mechanical testing. In addition to the four treatment groups, a set of 50 oysters that had been preserved in 70% ethanol prior to experimental exposures was also included in the shipment. These oysters are referred to as the time zero group. Only oysters with intact shells were considered in further analyses.

For mass measurements, 25 individuals were randomly selected from each treatment group, briefly rinsed in deionized water (DI), airdried for 5 days and lyophilized for approximately 16h. Lyophilized oysters were individually weighed on a microbalance (Metler-Toledo XP 26, Columbus, OH, USA) with precision of 0.01mg or better to obtain each oyster's total mass. To remove soft tissue, oysters were incubated in sodium hypochlorite (NaOCl; commercial Clorox diluted to obtain 2% v/v NaOCl and filtered through a 0.2 µm filter) on an orbital shaker at 250rpm at room temperature until all soft tissue was removed. Shells were sonicated, rinsed several times in DI, air-dried at room temperature for 3 days and finally lyophilized for approximately 16h. Lyophilized shells were weighed to determine shell mass, and soft tissue dry mass was determined for each individual by subtracting shell mass from total mass.

2.6. Micromechanical Testing of Shells

Micromechanical testing was conducted on seven shells from each treatment group. A similar distribution of shell masses was chosen for each group. Left (bottom) shell valves were used for mechanical testing, as the region of new growth during experimental exposure was most distinct in these valves. Left shell valves were mounted in epoxy resin (Epofix, ESM, Hatfield, PA, USA) and polymerized for 24h at room temperature. Embedded shells were cut longitudinally, transecting the acute apical tip (anterior) to the most distal edge (posterior), using a slow-speed water-cooled diamond saw (IsoMet, Buehler, Lake Bluff, IL, USA), as depicted in Fig.3.1A. A second cut was made parallel to the first to produce a 1- to 3- mm-thick section. Sections were ground and then polished with Metadi diamond suspensions at 6, 1 and 0.25 μm diamond particle size on a grinder-polisher (MiniMet 1000, Buehler). Grinding and polishing was conducted using a saturated $CaCO_3$ solution (pH7.8). A saturated $CaCO_3$ solution was prepared by mixing calcium and carbonate salts at very high concentrations and letting the mineral precipitate over several hours. The mixture was centrifuged and the supernatant was used to polish the samples. No etching of the shell samples was observed during grinding or polishing.

After polishing, the region of new shell growth formed during the experimental exposures was identified based on the calcein growth mark, as shown in Fig.3.1B–D. Imaging was conducted on a fluorescence microscope in the fluorescein isothiocyanate channel (Nikon TE2000, Melville, NY, USA). Although calcein staining was observed at both the anterior and posterior ends of the shell cross-sections, staining was most distinct in the anterior end, which was chosen for the microindentation testing (Fig.3.1C,D).

Dimensions of the new growth region for each shell were determined from a digital micrograph using microscopy software (NIS Elements ver. 3.20.01, Melville, NY, USA), which enabled identification of the new growth region during hardness testing.

Vickers microhardness tests were carried out using a microindentation hardness tester (IndentaMet 1104, Buehler) on polished shells at a load of 0.245N and a dwelling time of 5s. Three to six indentations per shell were made, depending on the size of the new growth region. All indents were made at least 30 μm away from the new growth region. Vickers microhardness values were averaged for each shell sample. Digital photographs were taken before and immediately after each indentation. This enabled quantification of the longest crack produced by each indent, which was measured using Adobe Photoshop (ver. 4.0, San Jose, CA, USA) as the radius of a circle radiating from the center of the indent and enclosing all visible cracks. The crack radius for a shell sample was obtained by averaging the crack radii for all indents on that sample, expressed in μm. In this study, we chose to use mean crack radius as a proxy for fracture toughness (K_c). There are a number of empirical equations used to calculate toughness from the length of cracks generated by microindentation (Anstis et al., 1981; Baldassarri et al., 2008); however, because the empirical constants used in these equations were not determined for oyster shells, we chose to use the crack length as a proxy for K_c. The term 'fracture resistance' is used in the text in place of K_c to avoid confusion. Representative indents were imaged by scanning electron microscopy (SEM) in the back-scattered electron mode. Embedded and polished shell cross-sections (Fig.3.1A, B) were carbon coated and imaged on a field emission SEM (JSM-6330F, Jeol, Peabody, MA, USA) at

10 kV with a working distance of 12.5–15.2 mm in the X500 to X3000-magnification range.

2.7. Physiological and Biochemical Traits

A separate subset of experimental animals, which had not been preserved in ethanol, was used for analyses of tissue metabolite concentrations, enzyme activities and mRNA expression. For these analyses, oyster juveniles were shock-frozen in liquid nitrogen immediately after collection and stored in liquid nitrogen to prevent metabolite, protein and mRNA degradation.

2.7.1. CA Activity

The whole soft body of juveniles was homogenized in homogenization medium (1:10 w/v) containing 250 mmol l^{-1} sucrose, 40 mmol l^{-1} TrisH_2SO_4 and 80 µgm l^{-1} phenyl methane sulfonylfluoride (PMSF), pH 7.5 using a Kontes Duall® glass-glass homogenizer (Fisher Scientific). Homogenates were centrifuged for 10min at 10,000g at 4°C. The supernatant was collected and stored at –80°C until further analysis. A pilot study showed that freezing and thawing did not affect CA activity in oyster homogenates (data not shown).

CA activity was determined as acetazolamide (AZM)-sensitive esterase activity following a standard method modified from Gambhir et al. (Gambhir et al., 2007). The assay consisted of 100µl of tissue homogenate in 1ml of assay medium containing 63mmol l^{-1} Tris-H_2SO_4, pH 7.5 and 75 µmol l^{-1} p-nitrophenyl acetate (p-NPA) as a substrate. Total esterase activity in the sample was measured as a change in absorbance at 348nm using a Cary® 50 UV-Vis spectrophotometer (Varian Inc., Cary, NC, USA). The temperature of the assay mixture was maintained at 20±0.1°C using a waterjacketed

cuvette holder (Varian Inc.). After determining the initial slope of esterase reaction, a specific CA inhibitor, AZM (7 mmol l^{-1}), was added to the assay, and CA activity was determined as the difference in the initial reaction slopes before and after AZM addition using the molar extinction coefficient for p-nitrophenol of 5l $mmol^{-1}cm^{-1}$ at 348 nm and pH 7.5. The reaction was linear for the complete duration of the assay (10–12 min). This assay allows measurement of CA activity at physiologically relevant temperatures in contrast to hydratase activity assays [such as a pH-stat Wilbur–Anderson method (Wilbur and Anderson, 1948) and its modifications)] carried out at non-physiologically low temperatures (approximately 0°C) to prevent rapid spontaneous hydration of CO_2 (Nielsen and Frieden, 1972; Smeda and Houston, 1979; Gambhir et al., 2007; Malheiro et al., 2009). CA activity determined with the AZM-sensitive esterase assay correlates with the cellular CA content (Gambhir et al., 2007). Protein concentration was measured in tissue homogenates of juvenile oysters using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. Specific CA activity was expressed as U g^{-1} protein, where 1U corresponds to the amount of enzyme catalyzing the breakdown of 1µmolp-NPA min^{-1} at 20°C and pH 7.5.

2.7.2. RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted from pooled whole-body tissues of 10–12 juveniles using Tri Reagent (Sigma-Aldrich) according to the manufacturer's protocol with a tissue to Tri reagent ratio of 1:10 (w/v) or less. Single-stranded cDNA was obtained from 5µg total RNA using 200Uμl^{-1} SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and 50µmol l^{-1} of oligo (dT) 18 primers.

Transcript expression of CA mRNA was determined using quantitative real-time PCR (qRT-PCR) using a LightCycler® 2.0 Real Time PCR System (Roche) and QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, USA) according to the manufacturers' instructions. Specific primers were designed to amplify cDNA using *C. virginica* CA, β-actin and 18S ribosomal RNA (rRNA) sequences. Gene sequences for *C. virginica* CA were obtained from the Marine Genomics database (www.marinegenomics.org, sequence accession number MGID94539); those for β-actin were obtained from GenBank (NCBI accession number X75894.1). For 18S rRNA, consensus primers were designed against highly conserved nucleotide sequences using 18S rRNA sequences from four bivalves: *C. virginica*, *Crassostrea gigas*, *Mytilus edulis* and *Mercenaria mercenaria* (NCBI accession numbers L78851.1, AB064942, L33448.1 and AF120559.1, respectively). Primer sequences were (5to 3 orientation) as follows: for CA, forward CarbAnh-F23 AGA GGA ACA CCG TAT CGG AGC CA and reverse CarbAnh-R155 ATG TCA ATG GGC GAC TGC CG; for β-actin, forward ActCv-F437 CAC AGC CGC TTC CTC ATC CTC C and reverse ActCv-R571 CCG GCG GAT TCC ATA CCA AGG; and for 18s rRNA, forward 18sRNA GGT AAC GGG GAA TCA GGG TTC GAT and reverse 18sRNA TGT TAT TTT TCG TCA CTA CCT CCC CGT.

Briefly, the qRT-PCR reaction mixture consisted of 5µl of 2x QuantiTect SYBR Green master mix, 0.3µmol l^{-1} of each forward and reverse gene-specific primers, 1µl of 10x diluted cDNA template and water to adjust to 10µl. The reaction mixture was subjected to the following cycling: 15min at 95°C to denature DNA and activate Taq polymerase and 50 cycles of 15s at 94°C, 20s at 55°C and 15s at 72°C. SYBR Green fluorescence (acquisition wavelength 530nm) was measured at the end of each cycle for

2s at the read temperature of 78°C (to melt all primer dimers but not the amplified gene product). Serial dilutions of a cDNA standard were amplified in each run to determine amplification efficiency (Pfaffl, 2001). A single cDNA sample from gills of an adult *C. virginica* was used as an internal cDNA standard and included in each run to test for run-to-run amplification variability. The CA mRNA expression was standardized relative to β-actin mRNA or 18S rRNA and against the internal standard as described elsewhere (Pfaffl, 2001; Sanni et al., 2008). The qualitative CA mRNA expression patterns were similar regardless of whether β-actin or 18S rRNA mRNA was used for normalization. However, β-actin mRNA levels were less variable between exposure conditions than 18S rRNA transcripts. Salinity had a significant effect on 18S rRNA levels (ANOVA, P= 0.009) but not on β-actin mRNA (ANOVA, P=0.649), whereas P_{CO2} of exposure did not significantly affect mRNA levels for β-actin or 18S rRNA (ANOVA, P=0.868–0.938). Therefore, we report the data on CA mRNA expression standardized to β-actin mRNA.

2.7.3. Biochemical Analyses of Juvenile Tissues

Whole-body tissues of 10–12 juveniles were pooled and immediately shock-frozen in liquid nitrogen. Frozen tissues were powdered with a mortar and pestle under liquid nitrogen and extracted using icecold 0.6moll $^{-1}$ perchloric acid (PCA) as described elsewhere (Sokolova et al., 2000). Neutralized, deproteinized PCA extracts were stored at –80°C and used for metabolic profiling using nuclear magnetic resonance (NMR) spectroscopy as well as to determine concentrations of adenylates and D-glucose using standard spectrophotometric NADH- or NADPH-linked enzymatic assays (Grieshaber et al., 1978; Bergmeyer, 1985). Briefly, the assay conditions were as follows: for ADP, 38.5 mmoll $^{-1}$ triethanolamine (TRA) buffer, pH7.6, 0.04 mmoll $^{-1}$ NADP, 7 mmoll^{-1}

$MgCl_2.6H_2O$, 50 $mmoll^{-1}$ glucose, 0.462Uml^{-1} glucose-6- phosphate dehydrogenase, 1.8Uml^{-1} hexokinase; for ADP and AMP, 58 $mmoll^{-1}$ TRA buffer, pH7.6, 3 $mmoll^{-1}$ phoshoenolpyruvate, $MgSO_4.7H_2O$ 6.2%, KCl 6.7%, 0.09 $mmoll^{-1}$ NADH, 24 Uml^{-1} lactate dehydrogenase, 18 Uml^{-1} pyruvate kinase, 16 Uml^{-1}myokinase; and for D-glucose, 38.5 $mmoll^{-1}$ TRA buffer, pH7.6, 0.04 $mmoll^{-1}$NADP, 7 $mmoll^{-1}MgCl_2.6H_2O$, 0.462 Uml^{-1} glucose-6-phosphate dehydrogenase, 1.8 Uml^{-1} hexokinase.

Glycogen concentration was measured in PCA extracts after enzymatic hydrolysis of glycogen to D-glucose by glucoamylase (Keppler and Decker, 1984) and determined by the difference in the D-glucose levels in the tissue extract before and after glucoamylase treatment. Tissue lipid content was measured using a standard method of chloroform extraction (Folch et al., 1957; Iverson et al., 2001). Whole-body tissues of 10–12 juveniles (~50mg wet mass) were homogenized in a chloroform/methanol mixture (2:1 v/v) using a tissue to chlorophorm/methanol ratio of 1:20 (w/v). Samples were sonicated for 1min (output 69W, Sonicator 3000, Misonix, Farmingdale, NY, USA), vortexed for 2min and centrifuged for 5min at 13,000g. The supernatant was transferred into a new tube and the chloroform/methanol extraction was repeated on the tissue pellet. The supernatants of two extractions were pooled, mixed with water (25% of the total volume of supernatant) and centrifuged for 5min at 13,000g. The lower phase (chloroform) was transferred to a preweighed tube and the chloroform was evaporated to determine the mass of the extracted lipids. For protein determination, whole bodies of 10–12 juveniles were homogenized in ice-cold homogenization buffer (100$mmoll^{-1}$Tris, pH7.4, 100 $mmoll^{-1}$NaCl, 1 $mmoll^{-1}$EDTA, 1 $mmoll^{-1}$EGTA, 1% Triton-X100, 10% glycerol, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 0.5µgml^{-1} leupeptin, 0.7

μgml^{-1} pepstatin, 40 μgml^{-1} PMSF and 0.5 μgml^{-1} aprotinin) using hand-held Kontes Duall tissue grinders (Fisher Scientific). Homogenates were sonicated 3X10s (output 69W, Sonicator 3000, Misonix) to ensure complete release of the proteins, with cooling on ice (1min) between sonications. Homogenates were centrifuged for 10min at 20,000g and 4°C, and supernatants were used for protein determination. Protein content was measured using the Bio-Rad Protein Assay kit according to the manufacturer's instructions (Bio-Rad Laboratories). Concentrations of glycogen, lipids and proteins were expressed in mg g^{-1}wet tissue mass, and concentrations of adenylates and D-glucose in $\mu molg^{-1}$ wet tissue mass.

2.7.4. Metabolic Profiling using ^{1}H-NMR Spectroscopy

Preparation of samples and NMR spectroscopy were performed as described by Lannig et al. (Lannig et al., 2010), with the following modifications. Freeze-dried PCA extracts were resolved in 500µl D2O containing 1% trimethylsilyl propionate (TSP) as an internal reference and concentration standard for NMR spectroscopy. Fully relaxed 1D, one pulse ^{1}H-NMR spectroscopy with F1 presaturation for water suppression was used for an analysis of metabolic profiles of the PCA extracts. All spectra were recorded with an inverse ^{1}H-broad band probe (1 H/BBI) on a 400MHz 9.4T WB NMR spectrometer with Avance electronics (Bruker Biospin GmbH, Silberstreifen, Germany). Prior to all NMR recordings, field homogeneity was optimized using TopShim (Bruker Biospin GmbH), resulting in typical line widths of 1Hz. Acquisition parameters were as follows: pulse program zgpr, TD=32k, NS=32, DS=2, SW=6k, AQ=2726s, D1=10s, RG 181, flip angle 90deg, presaturation level 60dB, resulting scan time 7.12 min.

Post-processing of spectra was performed automatically using TopSpin 2.5 (Bruker Biospin GmbH). Briefly, all data were zero filled to 64k, processed with an exponential multiplication of 0.5Hz and automated baseline and phase corrections. Quantification of signal areas was performed using a fit routine (mdcon, Bruker Biospin GmbH) and calculated relative to TSP as an internal reference standard. Specific metabolites were identified using chemical shift tables from Tikunov et al. (Tikunov et al., 2010) and as described in Lannig et al. (Lannig et al., 2010). After an operator-controlled screening of all spectra, only signals from metabolites displaying the most obvious changes were analyzed and quantified. Changes in metabolites of interest were expressed in percent change from the control group (maintained at a salinity of 30 and a P_{CO2} of ~400 µatm).

2.7.5.Calculations and Statistics

Cumulative mortality after 11weeks was compared between the different treatment groups using a chi-square test. Effects of the factors salinity, P_{CO2} and their combination on physiological parameters and shell and body mass and material properties of the shells were assessed using generalized linear model ANOVA after testing for the normality of data distribution and homogeneity of variances. Both factors were treated as fixed and had two levels each (15 and 30 for salinity, and normocapnia and hypercapnia for P_{CO2} . In the few cases where data distribution deviated from normality and/or variances were not homogenous, the data were log-transformed to ensure compliance with the ANOVA assumptions. Post hoc tests (Fisher's least square difference) were used to test the differences between the group means. Table 3.2 presents the results of ANOVA conducted on raw or log-transformed data as appropriate, but all

means and standard errors are given for the raw (non-transformed) data. Sample sizes for all experimental groups were five to nine except for lipid content (N=4) and protein content of the juveniles maintained at ~400 µatm P_{CO2} and a salinity of 15, where N=3 due to sample loss. For shell and body mass, as well as for the mechanical properties of the shells, each sample represented an individual oyster. For all other endpoints, each sample consisted of the pooled tissues of 10–12 individual juveniles. Unless otherwise indicated, data are represented as means ± s.e.m. The differences were considered significant if the probability of Type I error was less than 0.05.

Results

3.1. Mortality

At a salinity of 30, elevated P_{CO2} significantly increased mortality of juvenile oysters by almost two fold compared with normocapnia ($P<0.05$; Fig.3.2A). Low (15) salinity led to a four fold to fivefold increase in juvenile mortality compared with the controls maintained in normocapnia and high salinity ($P<0.05$; Fig.3.2A). However, no additional effect of elevated P_{CO2} on mortality of juveniles was observed at low salinity (Fig.3.2A).

3.2.Shell and Body Mass

Total body mass, shell mass and soft tissue mass did not significantly change during incubation under control conditions (ANOVA, P=0.23–0.58 for comparisons of the groups collected at time 0 and after 11 weeks of exposure at a salinity of 30 and normocapnia). This reflects relatively small shell growth increments in oysters during this period (Fig.3.1) compared with the overall variability in shell size and mass within experimental groups (data not shown). Salinity and P_{CO2} had no effect on total body mass

or shell mass of juveniles under the conditions of this experiment (Table 3.2). In contrast, soft body mass decreased significantly under elevated P_{CO2} and low salinity conditions (Table 3.2, Fig.3.2B). Overall, soft body mass was highest in juveniles maintained under control conditions of normocapnia and a salinity of 30 compared with all other groups (Fig.3.2B).

3.3. Mechanical Properties of the Shells

Vickers microhardness and fracture resistance of newly grown shells was significantly affected by interactions between salinity and P_{CO2}, indicating that the effects of elevated P_{CO2} on shell mechanical properties differ depending on exposure salinity (Table 3.2, Fig.3.2C). Elevated P_{CO2} did not affect the hardness of newly grown shells of juveniles kept at a salinity of 30, but led to a significant reduction of shell hardness at a salinity of 15 (Fig.3.2C). Similarly, an increase in P_{CO2} had no effect on the crack radius (fracture resistance) at a salinity of 30, whereas at a salinity of 15 a trend towards longer crack radius was observed in shells of juveniles grown in hypercapnia compared with their normocapnic counterparts (Fig.3.2D). Cracks resulting from indentations were considerably longer and more numerous in shells of juveniles held at low salinity and elevated P_{CO2} compared with those maintained under control conditions (Fig.3.3). Overall, shells of juveniles held at a salinity of 15 and hypercapniashowed significantly lower hardness and fracture resistance than all other experimental groups.

3.4. CA activity and mRNA Expression

Specific activity of CA in the total body extracts was lower in juvenile oysters exposed to a salinity of 15 compared with those exposed to a salinity of 30 (Table 3.2, Fig.3.4A). Elevated P_{CO2} had no significant effect on specific CA activity in whole-body

extracts of juvenile oysters (Table 3.2, Fig.3.4A). In contrast, expression of carbonic anhydrase mRNA was lower in juveniles exposed to elevated P_{CO2} and not significantly affected by salinity (Table 3.2, Fig.3.4B).

Notably, the specific activity of CA was positively correlated with CA mRNA expression in juveniles maintained under normocapnia (P_{CO2} ~400 μatm); the correlation was significant at a salinity of 15 (R=0.895, N=5–7, P=0.04) and not significant at a salinity of 30 (R=0.709, N=7, P=0.07). In juveniles maintained under elevated P_{CO2} conditions, enzyme activity of CA was not significantly correlated with CA mRNA expression (P>0.05). When all experimental groups were considered together, correlation between CA activity and mRNA expression was not significant (R=–0.07, N=22, P=0.748).

3.5.Energy-related Indices

Exposure to lower salinity and/or elevated P_{CO2} had no effect on tissue levels of ATP in juvenile oysters (Table 3.2, Fig.3.5A). In contrast, juveniles exposed to hypercapnia at a salinity of 15 had lower tissue levels of ADP and AMP compared with their counterparts maintained at a salinity of 15 and normocapnia (Fig.3.5B, C). At a salinity of 30, P_{CO2} levels had no effect on tissue concentrations of ADP and AMP (Fig.3.5B, C). Total concentrations of adenylates were not affected by salinity or CO_2 (Table 3.2), likely because the adenylate pool was dominated by ATP (with tissue ATP levels six to 10 times higher than those of ADP, and 40–186 times higher than those of AMP), and ATP levels did not change in response to exposure P_{CO2} and salinity.

At the same time, elevated P_{CO2} levels resulted in the partial depletion of tissue energy reserves (glycogen and lipids) in juveniles acclimated at a salinity of 30

(Fig.3.5D,E). A similar trend to lower glycogen concentrations at elevated P_{CO2} was seen in juveniles maintained at a salinity of 15, but it was not statistically significant (Fig.3.5D). Elevated P_{CO2} had no significant effect on the lipid content of juveniles acclimated at a salinity of 15, but tissue lipid content was reduced in juveniles acclimated at a salinity of 15 compared with their counterparts acclimated at a salinity of 30 (Fig.3.5E). Total protein content also tended to be lower in juveniles acclimated at a salinity of 15 compared with those acclimated at 30, but this trend was not statistically significant (Fig.3.5F). Concentration of free glucose in tissues of oyster juveniles did not change in response to acclimation salinity or P_{CO2} (Table 3.2) and varied between 115 and 189nmolg^{-1}wet mass in all experimental groups.

3.6. Metabolite Profile

Tissue metabolite profile of the total body homogenates determined by the ^{1}H-NMR spectra showed a significant shift in response to acclimation salinity (Table 3.2). At a salinity of 15, oyster juveniles contained significantly lower betaine, succinate and alanine levels and higher levels of lysine and acetate compared with their counterparts at a salinity of 30 (Fig.3.6). Tissue levels of metabolites were not strongly affected by exposure P_{CO2}, with the exception of acetate. At a salinity of 15, hypercapnia resulted in significantly lower acetate levels compared with those of normocapnic juveniles (Fig.3.6D), whereas at a salinity of 30 no differences were observed between hypercapnic and normocapnic animals (Fig.3.6A–E). Overall, tissue acetate levels in juveniles acclimated to normocapnia at a salinity of 15 were higher than in all other treatment groups in this study.

Discussion

Our study demonstrates that the effects of low salinity and elevated P_{CO2} , alone and in combination, have overall negative effects on juvenile eastern oysters, based on observed mortalities and tissue growth rates. Individually, low salinity and hypercapnia affect measured traits in a distinctly different manner. Under the conditions of our experiment, low salinity is a greater single stressor than high P_{CO2} , whereas the combination of these two factors produces greater changes in the physiology and shell properties of these mollusks than each of the factors alone (Table 3.3.). This result may be explained by the exacerbation of seawater acidification and other changes in seawater chemistry by low salinity, such that both stressors synergistically affect similar mechanisms. In some cases (e.g. microhardness) the effects of low salinity and hypercapnia appear to be additive, whereas their combined effect on other parameters is more complex (Table 3.3.). Overall, our data suggest that the predicted global increase in CO_2 levels would have a strongnegative effect on coastal and estuarine populations of oysters. The magnitude of this impact can be modified by changes in environmental salinity such that low salinity sensitizes oyster juveniles to the negative impacts of CO_2-induced ocean acidification.

4.1. Effects of P_{CO2} and Salinity on Juvenile Growth and Survival

Lowering seawater pH typically results in a reduction of growth in marine bivalves, with the degree of growth inhibition dependent on the magnitude of deviation in the environmental and/or body fluids pH from the organism's optimum (Ringwood and Keppler, 2002; Michaelidis et al., 2005b; Berge et al., 2006). A decrease in extracellular pH can cause metabolic depression and growth reduction; however, these effects are

typically observed only during strong acidification [see Michaelidis et al., Pörtner, and Beniash et al., and references therein (Michaelidis et al., 2005b; Pörtner, 2008; Beniash et al., 2010)]. In oysters, no reduction in the metabolic rate was observed at P_{CO2} levels as high as 3500 µatm (Beniash et al., 2010). Metabolic studies are needed to investigate whether the negative tissue growth observed in oyster juveniles in response to hypercapnia in the present study involves metabolic rate depression.

Shell deposition rates decrease with increasing P_{CO2} in mollusks, and this change has been attributed to lower $CaCO_3$ saturation levels at the calcification site, which decreases the driving force for shell deposition and increases the dissolution of existing shell (Gazeau et al., 2007; Miller et al., 2009; Ries et al., 2009; Talmage and Gobler, 2009; Beniash et al., 2010; Talmage and Gobler, 2010). Reduced salinity also lowers water $CaCO_3$ saturation levels (Cai and Wang, 1998; Miller et al., 2009) and has been shown to lead to decreased growth rates in *C. virginica* and other mollusks (Almada-Villela, 1984; Paynter and Burreson, 1991; Nagarajan et al., 2006; Heilmayer et al., 2008). In addition, negative effects of low pH and/or salinity on the organism's energy budget may also contribute to diminished shell deposition rates (Almada-Villela, 1984; Michaelidis et al., 2005b; Nagarajan et al., 2006; Heilmayer et al., 2008; Beniash et al., 2010).

In this study, the total body mass and shell mass of juvenile oysters was not affected by exposure to different salinities and/or P_{CO2} levels. This may be due to the fact that new shell growth (as indicated by calcein growth marks; Fig.3.1) was only a small fraction of the total shell volume for all exposure groups, including controls (normocapnia, salinity of 30), such that differences remain nonsignificant. Dry shell mass

of the control group did not differ from that of the time zero (no exposure) group, indicating that the mass of the new growth region was not discernable within the context of variability among individual shell masses. In a previous study, Beniash et al. (Beniash et al., 2010) exposed younger juveniles (3weeks post metamorphosis) of *C. virginica* to normocapnia (~390 µatm P_{CO2}) and hypercapnia (~3500 µatm P_{CO2}) for 20weeks and reported substantial new growth as well as differences in shell mass between juveniles kept at different P_{CO2} levels. The discrepancy in the shell growth rates between the present study and the study by Beniash et al. (Beniash et al., 2010) likely reflects differences in the age of juveniles (7 vs 3weeks post-metamorphosis, respectively), their size and the duration of experimental exposure (11 vs 20 weeks, respectively). In bivalves, the rate of shell growth decreases with increasing age and size (von Bertallanfy, 1964; Pauly, 2010), which would have been reflected in the slower shell deposition rate in the older and larger juveniles used in this study. Here, we did not individually follow shell growth of the same juveniles throughout the experimental exposures and thus our growth estimates were based on the group size means. Given the relatively slow growth rate and considerable natural variation in size within a single age cohort of oysters (Collet et al., 1999; Bayne, 2000) (I.M.S., personal observation), the small growth increment was not detectable against the background of the natural size variation within the group. This technical limitation can be overcome in future studies by individually marking oysters and following changes in individual size and mass of their shells through time.

In contrast to shell mass, soft body mass was reduced in both low salinity groups and in the hypercapnic high salinity group, indicating negative growth (i.e. partial resorption of tissues). In control juveniles, the soft body mass did not significantly

change during 11 weeks of exposure, consistent with the relatively slow growth rates discussed above. Previously, negative growth due to muscle wastage at low pH was found in a brittle star, *Amphiura filiformis*, while calcification rate was elevated to compensate for $CaCO_3$ dissolution (Wood et al., 2008). Elevated nitrogen excretion, indicative of protein breakdown expected during negative growth, was also found under low pH conditions (pH ~ 7.3) in the mussel *Mytilus edulis* (Michaelidis et al., 2005b). Notably, negative growth in juvenile oysters at low salinity and/or high P_{CO2} was associated with elevated mortality, indicating energy deficiency and supporting the notion that salinity and pH are among the key determinants of bivalve performance (including growth and survival) (Ringwood and Keppler, 2002; Heilmayer et al., 2008; Chapman et al., 2011). A caveat, applicable not only to our growth rate estimates but to all physiological and biochemical traits reported in this study, is the fact that all traits were by necessity determined in those organisms that survived experimental treatments. Therefore, a survivor effect due to the differential mortality of organisms with different physiology or growth rates cannot be ruled out. Although the potential for such selective mortality is important to consider when interpreting the mechanisms of the observed physiological effects, this effect will presumably also occur in the field. This would lead to similar shifts in physiological and biomineralization processes of the surviving population in response to elevated P_{CO2} and/or low salinity.

4.2. Effects of P_{CO2} and Salinity on the Mechanical Properties of the Shells

Combined exposure to hypercapnia and low salinity significantly affected the mechanical properties of newly deposited shell in juvenile oysters. The portions of the shells deposited during combined exposure to hypercapnia and low salinity had

significantly lower hardness and fracture resistance compared with other exposure groups. In addition, the shells of juveniles from the normocapnic low salinity treatment tended to have lower fracture resistance than the juveniles in the high salinity treatments, suggesting that salinity alone may also influence this parameter (possibly because of lower pH and/or other changes in seawater chemistry associated with low salinity seawater). Our results are consistent with mechanical testing of *C. virginica* shells by Beniash et al. (Beniash et al., 2010), which showed a significant decrease in hardness and fracture resistance of shells of juvenile oysters exposed to high P_{CO2} (~3500 µatm at a salinity of 30, $\Omega_{calcite}$=1.42). Similarly, elevated P_{CO2} resulted in the deposition of weaker, thinner and smaller shells in larvae of the California mussel, *Mytilus californianus* (Gaylord et al., 2011).

Earlier studies suggest that the differences in shell mechanical properties of oyster shell deposited under conditions of low pH and low calcite saturation are partially due to differences in shell ultrastructure (Beniash et al., 2010). The majority of *C. virginica* shell is composed of calcitic layers (laths) surrounded by an organic matrix (Carriker, 1996; Checa et al., 2007). The mechanical strength of multilayered materials such as bivalve shells is inversely related to the thickness of each layer (Anderson and Li, 1995; He et al., 1997). Thinner layers more frequently deflect cracks, hence forcing a more treacherous path and more interactions of the cracks with elastic organic material (Fratzl et al., 2007; Zhang et al., 2010). Using oyster juveniles, Beniash et al. (Beniash et al., 2010) showed that calcitic laths were significantly thicker in the shell deposited under low pH or low $\Omega_{calcite}$ conditions compared with that of oysters exposed to normocapnia. Altered ultrastructure in hypercapniaexposed mollusks has also been shown in the

developing nacre of *Pinctada fucata* (Welladsen et al., 2010) and at the growing edge of *Mercenaria mercenaria* and *Argopecten irradians* larvae (Talmage and Gobler, 2010). Further investigations, however, are needed to test this hypothesis as well as possible alternatives, such as changes in the shell organic and inorganic content and mineralogy. Irrespective of the exact mechanisms, compromised mechanical properties of the shell resulting from exposure to moderate hypercapnia and low salinity are likely to leave *C. virginica* more susceptible to predators and parasites.

4.3.Effects of Low Salinity and Hypercapnia on Activity and Expression of CA

Activity of CA, one of the key enzymes involved in carbonate chemistry regulation, acid–base homeostasis and biomineralization, was reduced during low salinity exposures in juvenile oysters, indicating a potential disturbance of biomineralization processes.CA facilitates the conversion of CO_2 into bicarbonate, supporting the maintenance of carbonate oversaturation and thus the driving force towards mineral deposition. It also supports pH regulation in both biomineralizing and non-biomineralizing tissues (Wilbur and Anderson, 1950; Wilbur and Jodrey, 1955; Nielsen and Frieden, 1972). Thus, a reduction in CA activity seen under the low salinity conditions could negatively affect shell growth and/or lead to acidosis, negatively affecting physiological processes including biomineralization, provided that activity becomes limiting for bicarbonate formation. Hypercapnia alone (~800 µatm) did not affect CA activity in juvenile oysters at high salinity.

Comparisons between the enzyme activity and mRNA expression for CA indicate that there is no consistent correlation between these two parameters across all treatment groups. This indicates that CA activity may be largely post-transcriptionally and/or post

translationally regulated. Another possible explanation for the lack of correlation between CA activity and mRNA levels could be the presence of multiple CA isoforms encoded by different genes, some of which were undetected by qRT-PCR but contributed to the total enzyme activity. Currently there is no evidence of multiple CA genes expressed in soft tissues of bivalves (Yu et al., 2006), but our knowledge about the genetic diversity of this enzyme in mollusks is very limited and requires further investigation. Overall, these data suggest that inferences about CA phenotype based on mRNA expression data should be interpreted with caution. Earlier studies in oysters and fish also showed poor correlation between enzyme activity and mRNA expression for several metabolic enzymes, including hexokinase, citrate synthase and cytochrome c oxidase (Lucassen et al., 2003; Ivanina et al., 2011). This suggests that, in aquatic ectotherms, enzyme activity may be a more reliable indicator of the metabolic phenotype than mRNA levels for several key metabolic and biomineralization enzymes.

4.4. Effects of P_{CO2} and Salinity on Energy Homeostasis of Juvenile Oysters

Exposure to moderate levels of environmental stress can lead to an increase in energy demand due to the energy costs of cellular protection systems, such as stress proteins and antioxidants, degradation and damage repair mechanisms, as well as active transport to maintain acid–base and ion homeostasis (Sokolova et al., 2011). These compensatory mechanisms allow successful acclimation to stress conditions, but can incur significant energy costs, disrupt energy homeostasis and affect cellular and whole-body energy status (Sokolova et al., 2011). Our study showed that exposure of *C. virginica* juveniles to low salinity and/or elevated P_{CO2} levels strongly affects their lipid and glycogen stores but does not affect the whole-body protein levels. This may reflect

the fact that protein reserves in bivalves are typically used up only during extreme energy deficiency such as starvation (Baghdiguian and Riva, 1985; Albentosa et al., 2007). Hypercapnic exposure at a salinity of 30 led to a partial depletion of lipid and glycogen reserves in oyster juveniles (by 56 and 31%, respectively), indicating a mismatch between energy demand and supply. Hypercapnia also led to a ~20% decrease in whole-body glycogen content of juveniles exposed to hypercapnia at a salinity of 15 compared with their normocapnic counterparts; however, this decrease was not statistically significant. Earlier studies showed high energy demand (as indicated by elevated oxygen consumption rates) in juvenile *C. virginica* exposed to high P_{CO2} levels (~3500 µatm) (Beniash et al., 2010) as well as in adult *Crassostrea gigas* exposed to P_{CO2} levels of ~1000 µatm, although in the latter case the effect of P_{CO2} on respiration was only significant at elevated temperatures (Lannig et al., 2010). More severe CO_2- induced acidification (water pH 7.3) resulted in the reduction of metabolic rates in a mussel, *Mytilus galloprovincialis* (Michaelidis et al., 2005b). Thus, the change in basal metabolic demand in response to elevated P_{CO2} may be species-specific in bivalves and depend on the magnitude of the pH/P_{CO2} change in the environment and, consecutively, in body fluids.

In juveniles maintained at low salinity (15), whole-body lipid content was reduced by ~50% regardless of P_{CO2} , compared with the normocapnic controls at a salinity of 30. These results indicate that exposure to low salinity may be associated with metabolic rearrangements that result in the preferential burning of lipids. Decrease of lipid stores in oyster juveniles kept at low salinity is consistent with earlier findings that low osmolarity changes the preferred fuel and strongly stimulates oxidation rates of acyl carnitines (C8–

C18 fatty acid derivatives) in isolated oyster mitochondria (Ballantyne and Moyes, 1987b) while inhibiting glycolytic enzymes such as hexokinase and fructose biphosphatase (Ballantyne and Berges, 1991). Lysine concentrations were elevated by 70–80% in tissues of juveniles maintained in low salinity, consistent with the proposed high input of acetyl-CoA from lipid breakdown that may reduce the need for acetyl-CoA supply from lysine degradation. High acetyl-CoA production from the lipid oxidation may also explain the elevated acetate content in tissues of juvenile oysters kept at low salinity and normocapnia. Acetate accumulation in this group is unlikely to reflect an onset of partial anaerobiosis because no accumulation of succinate or alanine was observed (which typically precedes anaerobic acetate accumulation) (Michaelidis et al., 2005a; Kurochkin et al., 2009). Notably, no acetate accumulation was observed in juveniles maintained at low salinity and hypercapnia, possibly indicating high rates of mitochondrial acetate oxidation and/or slightly lower lipid degradation rates in this group. Alternatively, reduced lipid content and accumulation of acetate in the tissues of juvenile oysters maintained at low salinity may reflect reduced rates of lipid biosynthesis. The effects of salinity on lipid biosynthesis of mollusks are not known. Studies in marine crabs, however, showed that exposure to reduced salinity either strongly enhanced (in Callinectus sapidus) or had no significant effect on lipid biosynthesis (in Libinia emarginata) (Whitney, 1974). Thus, inhibition of lipid biosynthesis appears to be a less likely explanation of the reduced lipid content in oysters maintained under low salinity conditions.

Acclimation to low salinity led to a shift in the metabolic profile in oysters, notably to a strong reduction in the levels of betaine and alanine (by approximately 70

and 40%, respectively), consistent with their role as major osmolytes in bivalves (Powell et al., 1982; Neufeld and Wright, 1996; Hosoi et al., 2003). In contrast, elevated P_{CO2} had no effect on the metabolite profile in whole bodies of oyster juveniles in this study except for the lower acetate content of tissues from hypercapnic juveniles compared with their normocapnic counterparts at a salinity of 15. This change, however, reflects elevated acetate levels in the juveniles maintained under normocapnia and low salinity conditions rather than acetate depletion in the hypercapnic group. No anaerobic end products (alanine, succinate or acetate) were accumulated under elevated P_{CO2} ,indicating that the juveniles were capable of fully maintaining their metabolic demand with aerobic pathways.

Juvenile oysters were capable of maintaining normal steady-state levels of ATP in all experimental treatments, suggesting that the metabolic adjustments to low salinity and elevated P_{CO2} are sufficient to prevent ATP depletion and severe cellular energy deficiency. This is consistent with earlier studies that have shown that intertidal mollusks including oysters effectively defend the cellular ATP pool, so that ATP depletion occurs only under conditions of severe energy limitation such as prolonged anoxia (Hochachka and Guppy, 1987; Sukhotin and Pörtner, 1999; Sokolova et al., 2000; Kurochkin et al., 2008). ADP levels were significantly elevated in juveniles from the low salinity normocapnic group, and AMP levels were reduced in juveniles kept at low salinityand hypercapnia. These changes may be indicative of higher metabolic flux and thus metabolic rate in these groups, which is typically supported by elevated ADP/ATP ratios (Pörtner et al., 1998; Hardie and Hawley, 2001; Ivanina et al., 2010); however, further investigations are required to test this hypothesis. Although cellular ATP content was not

significantly affected by salinity and CO_2 levels, a decline in the carbon energy stores went hand-in-hand with the negative growth and elevated mortality of juveniles exposed to hypercapnia and/or low salinity. This suggests that the tissue stores of fermentable substrates may be a more sensitive indicator of long-term energy deficit compared with ATP levels that are tightly regulated to ensure cellular survival (Pörtner, 1993; Pörtner et al., 1996).

Conclusions

Reduced salinity and elevated P_{CO2} levels interactively affect survival, growth, energy status and shell mechanical properties in juvenile oysters. Low salinity can strongly modify the negative effects of high P_{CO2}/low pH on the shell's material properties, weakening shells of the juveniles and making them more prone to predators, parasites and other mechanical damages. Hypercapnia and low salinity, either alone or in combination, also led to a reduction in tissue growth and survival of juveniles, possibly because of energy limitation in the stressed state, as indicated by the partial depletion of tissue energy stores. Such energy limitations can affect the organism's fitness and general stress tolerance and are likely to translate into reduced survival, growth and reproduction of oysters (Pörtner, 2008; Sokolova et al., 2011). The observed effects of hypercapnia and salinity stress on oyster physiology and the shell's material properties are especially remarkable given that oysters, like most estuarine species, can be exposed to periodical bouts of extreme P_{CO2} levels in their habitats with a reduction in seawater pH down to 6.0–7.5 (Pritchard, 1967; Burnett, 1997; Ringwood and Keppler, 2002) and thus are often considered hypercapnia tolerant. Overall, this study suggests that long-term

exposure to a modest (by estuarine standards) increase in P_{CO2} , as predicted with global climate change in the next century, will likely have negative consequences on survival and performance of oysters, especially when combined with low salinity stress in estuaries.

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Author Contributions

O.B.M measured carbonic anhydrase activitiy and participated in data analysis and interpretation, and in writing and revising the manuscript.

Figures

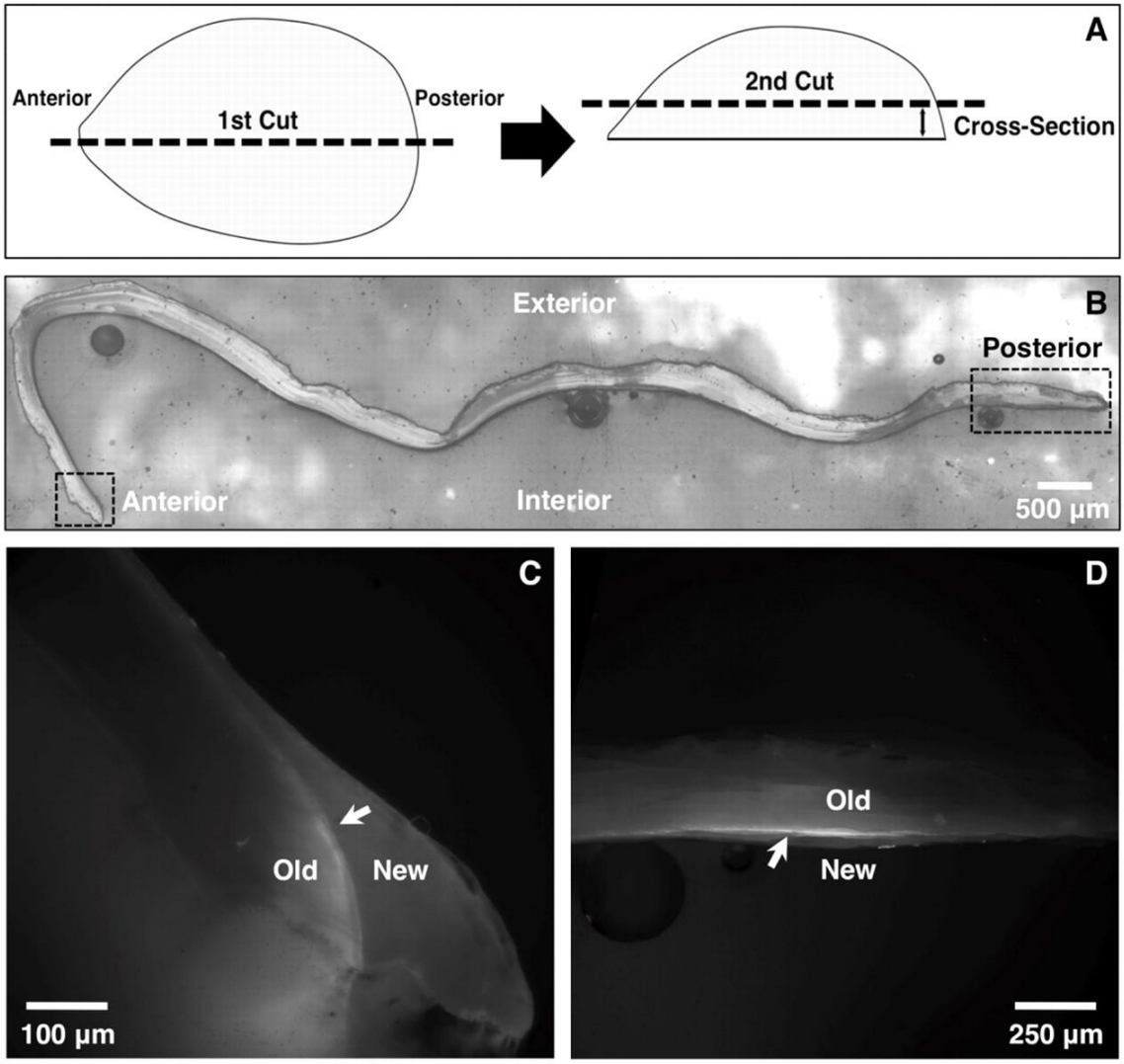

Figure.3.1: Preparation of juvenile oyster samples for mechanical testing, and identification of new shell grown during experimental exposure. (A) Embedded left shell valves were first cut longitudinally, from anterior to posterior, along their longest axis. A second cut was made parallel to the first to produce a 1–3 mm thick section. (B) Full cross-section of a juvenile shell under polarized light. New growth was observed at the far anterior and posterior ends of the shell. (C,D) Epifluorescence (FITC channel) micrographs of the anterior (C) and posterior (D) of the shell. Fluorescence micrographs correspond to regions denoted by boxes in B. Calcein staining appears as a distinct line, as indicated by arrows.

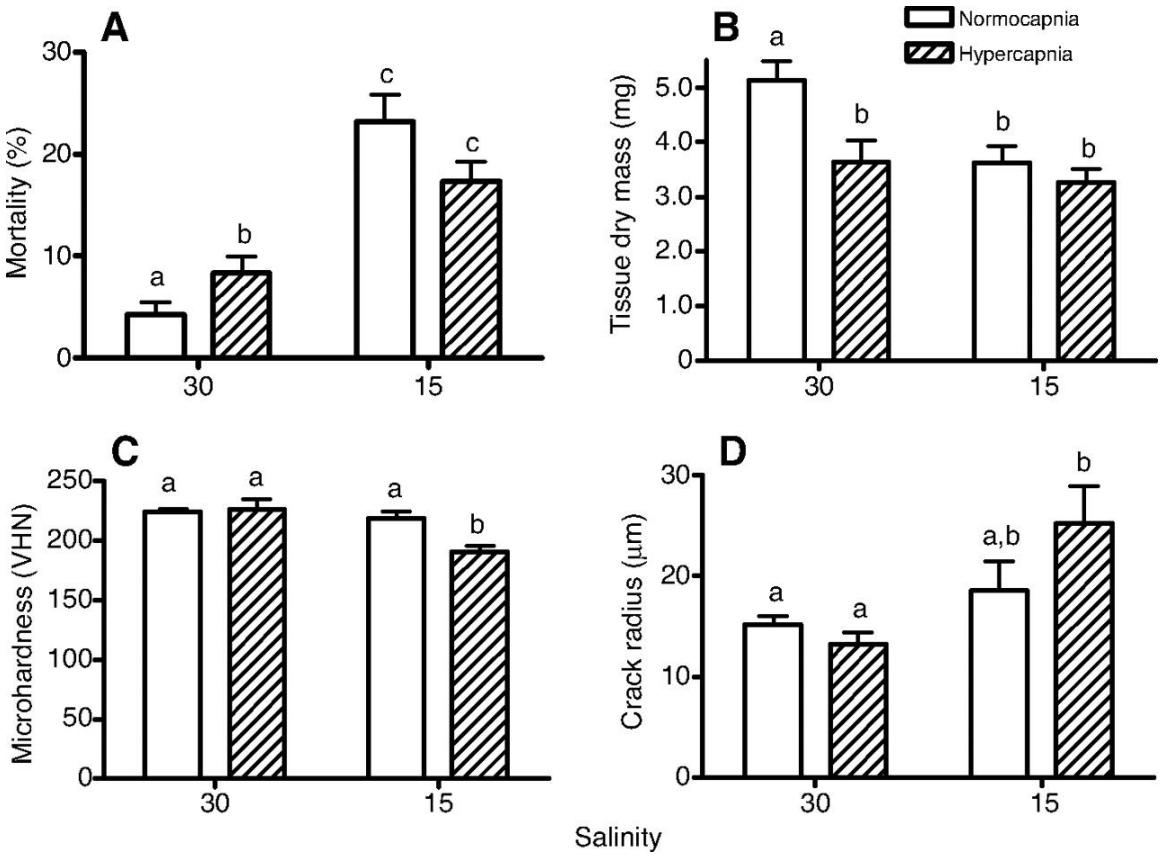

Figure.3.2: Mortality, soft body mass and mechanical characteristics of the newly grown shell in juveniles of the eastern oyster *Crassostrea virginica* maintained for 11 weeks in different salinities and P_{CO2} levels. Exposure conditions are given in Table 3.1. (A) Cumulative mortality after 10 weeks exposure; (B) soft body mass; (C) microhardness of the newly grown shell expressed as Vickers microhardness number (VHM); (D) mean crack radius in response to a mechanical stress. Within each graph, different letters indicate means that are significantly different from each other ($P<0.05$). If the columns share a letter, the respective means are not significantly different ($P>0.05$).

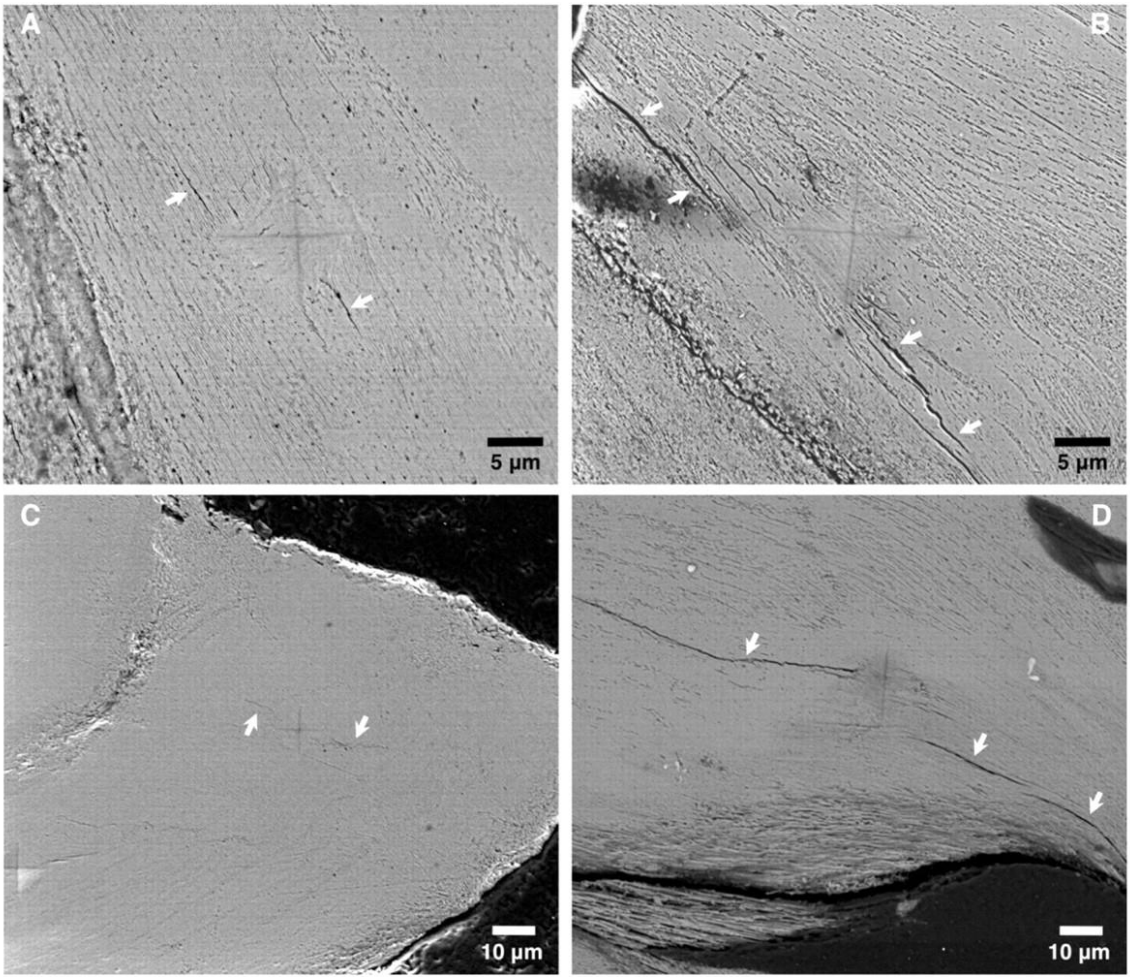

Figure.3.3:Back-scattered SEM micrographs of shells from control juveniles (maintained under normocapnic conditions at a salinity of 30) (A,C) and shells from juveniles maintained under hypercapnia at a low salinity of 15 (B,D) after indentation under a 0.245 N load. Cracks resulting from indentation are indicated by arrows. (A,B) Representative indents resulting in cracks with the length approximately equal to the mean crack diameter for the group, ×2200 magnification; (C,D) one of the longest cracks produced by indentation for each group, ×850 magnification.

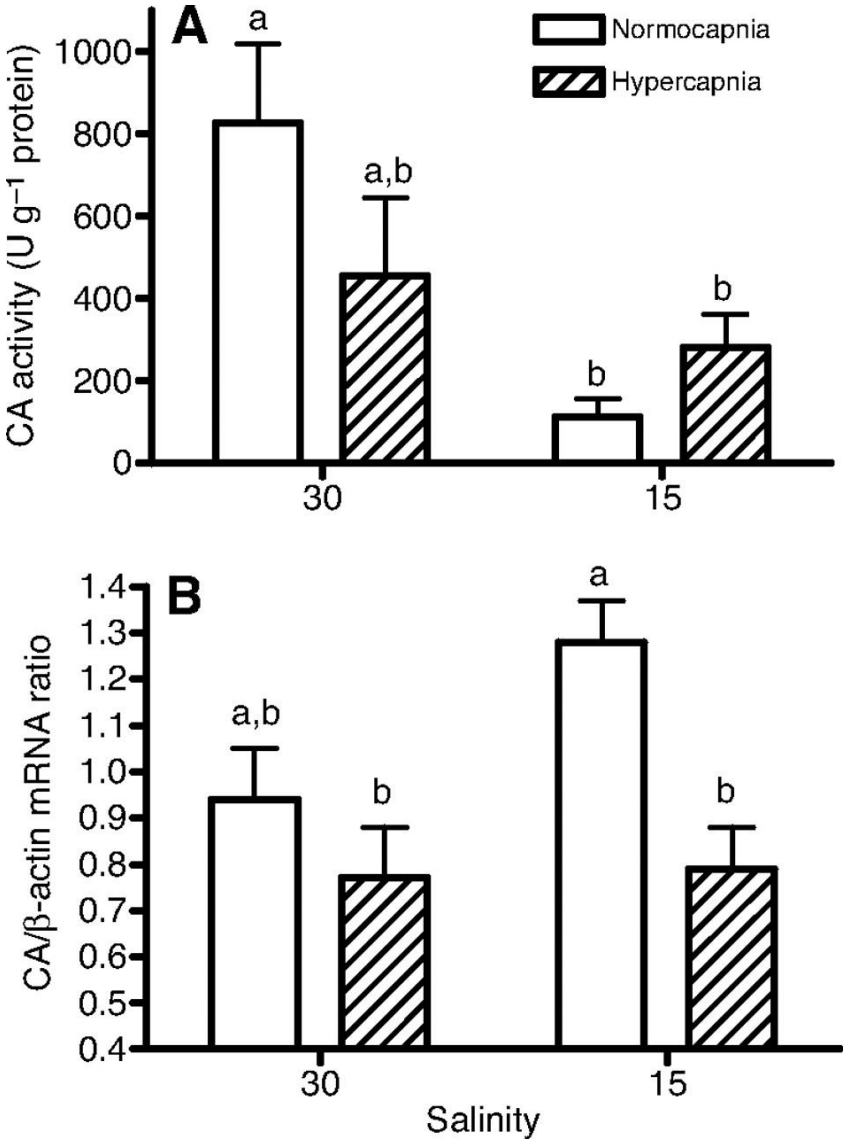

Figure.3.4: Activity and mRNA expression of carbonic anhydrase (CA) in tissues of oyster juveniles maintained for 11 weeks in different salinities and P_{CO2} levels. (A) CA activity; (B) CA mRNA expression relative to mRNA expression of β-actin. CA mRNA expression was also normalized to 18S rRNA, yielding a pattern similar to that of the β-actin-normalized expression (data not shown). Within each graph, different letters indicate means that are significantly different from each other ($P<0.05$). If the columns share a letter, the respective means are not significantly different ($P>0.05$).

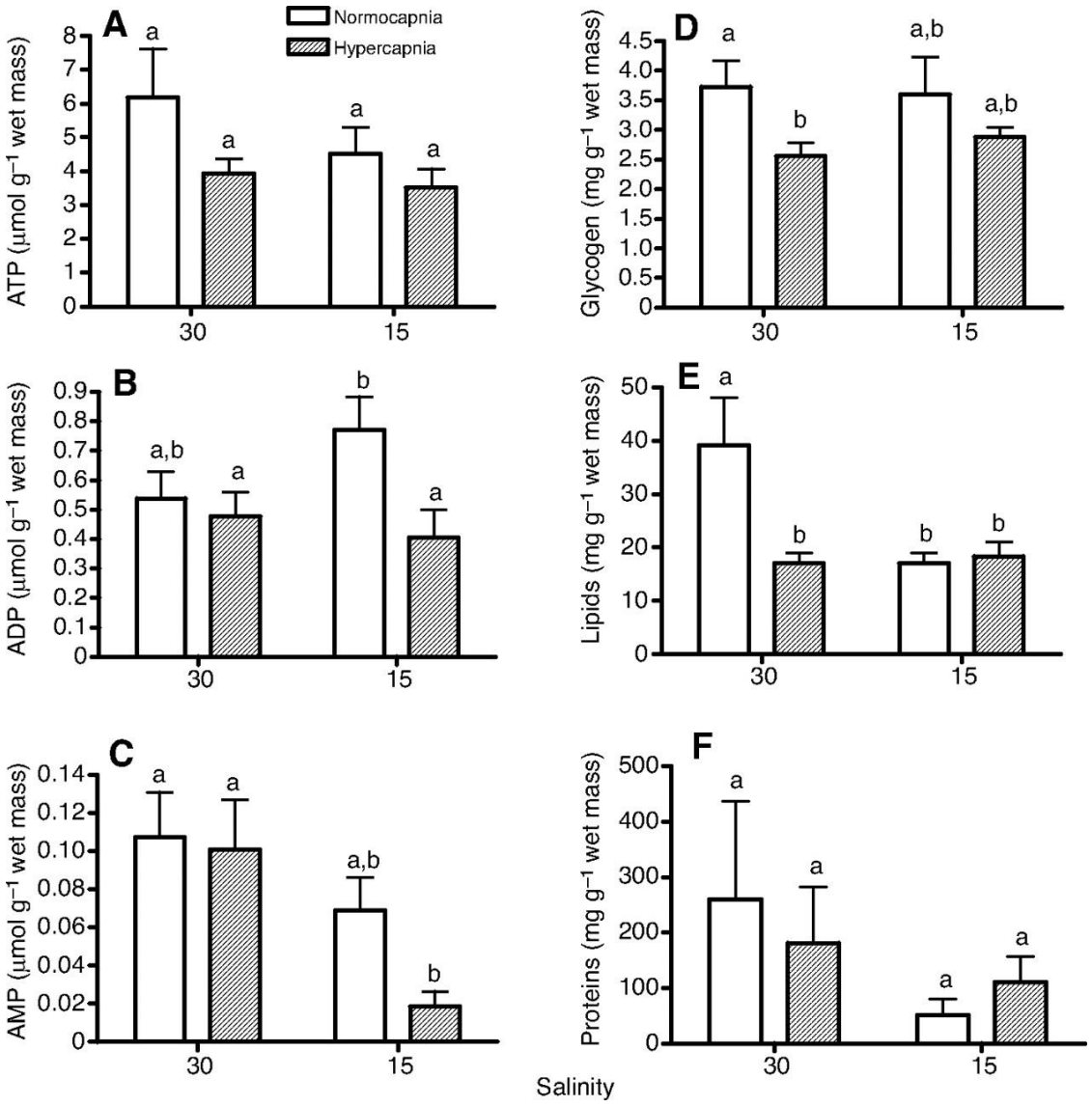

Figure.3.5:Tissue concentrations of adenylates and major energy reserves in juveniles of the eastern oyster *C. virginica* maintained for 11 weeks in different salinities and P_{CO2} levels. Exposure conditions are given in Table 3.1. (A) ATP, (B) ADP, (C) AMP, (D) glycogen, (E) lipids and (F) proteins. Within each graph, different letters indicate means that are significantly different from each other ($P<0.05$). If the columns share a letter, the respective means are not significantly different ($P>0.05$).

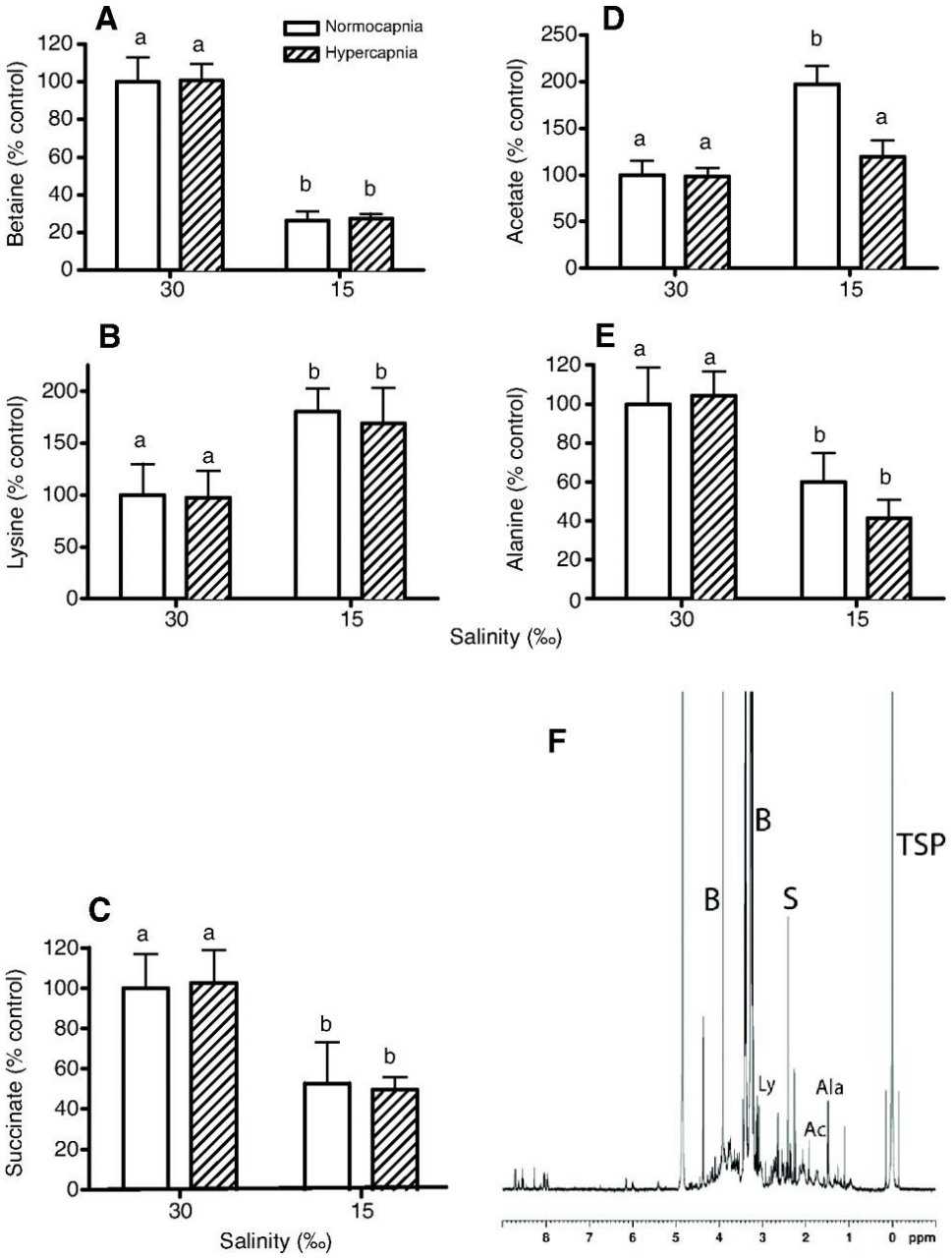

Figure.3.6: Levels of tissue metabolites in juveniles of the eastern oyster *C. virginica* maintained for 11 weeks in different salinities and P_{CO2} levels. Exposure conditions are given in Table 3.1. (A) Betaine, (B) lysine, (C) succinate, (D) acetate and (E) alanine. Data, which are given in means ± s.e.m., are presented in % relative to control conditions (normocapnia, salinity of 30). Within each graph, different letters indicate means that are significantly different from each other ($P<0.05$). (F) A typical 1H-NMR spectrum obtained from a juvenile oyster under control conditions. Signals from analyzed metabolites are indicated: Ac, acetate; Ala, alanine; B, betaine; Ly, lysine; S, succinate; TMS, standard

Table 3.1: Summary of water chemistry parameters during experimental exposures of juvenile eastern oysters, *Crassostrea virginica*. Salinity, temperature, pH and dissolved inorganic carbon (DIC) were determined in samples from experimental tanks at the beginning, middle and end of experimental exposures as described in the Materials and methods. Other parameters were calculated using co2sys software. Data are presented as means ± s.d. N=6 for the hypercapnic group at a salinity of 30 and N=11–12 for all other exposures. SW, seawater.

Exposure salinity:	15		30	
	Normocapnia	Hypercapnia	Normocapnia	Hypercapnia
pH	8.11±0.09	7.97±0.03	8.36±0.04	8.1+0.01
Temperature, °C	22.6±0.8	22.2+0.8	21.4±0.7	21.4+0.0
Salinity,	15.1±0.2	15.2±0.3	30.1±0.1	30+0.0
P_{CO2} , µatm	470.4±160.1	676.5±65.7	392.1±30.0	802.3±3.6
TA, µmol kg^{-1} SW	1628.0±163.5	1734.8±100.2	3512.3±224.2	3546.7±15. 7
DIC, µmol kg^{-1} SW	1549.9±171.1	1683.2±96.8	3058.5±171.7	3287.3±14.8
HCO_3^-, µmol kg^{-1} SW	1462.9±167.7	1602.7±91.3	2678.0±127.1	3035.2±13.6
CO_3^{2-}, µmol kg^{-1} SW	70.5±8.6	56.6±4. 8	367.6±47.7	225.4±1.0
CO_2 , µmol kg^{-1} SW	16.5±5.2	23.9±1.8	12.9±0.8	26.6±0.1
Ω Ca	1.94±0.23	1.56±0.14	9.09±1.18	5.58±0.03
Ω Arg	1.16±0.14	0.93±0.08	5.85±0.76	3.58±0.02

Table 3.2: ANOVA results of the effects of exposure salinity, P_{CO2} and their interaction on shell and soft tissue mass, mechanical shell properties, enzyme activities and energy-related indices in juvenile *C. virginica*. F-values are given with degrees of freedom for the factor and the error in subscript. Significant values (P<0.05) are highlighted in bold. AEC, adenylate energy change.

Parameters	Salinity	P_{CO2}	Salinity x P_{CO2}
Shell and body mass			
Total dry mass	$F_{1,116}$=1.29, P=0.258	$F_{1,116}$=0.94, P=0.333	$F_{1,119}$=0.42, P=0.519
Shell mass	$F_{1,116}$=1.02, P=0.313	$F_{1,116}$=0.71, P=0.400	$F_{1,116}$=0.62, P=0.431
Soft tissue dry mass	**$F_{1,116}$=6.73, P=0.011**	**$F_{1,116}$=6.33, P=0.013**	$F_{1,116}$=2.43, P=0.122
Mechanical shell properties			
Vickers Hardness	**$F_{1,31}$=17.35, P=0.002**	**$F_{1,31}$=7.43, P=0.01**	**$F_{1,31}$=9.61, P=0.004**
Crack radius	**$F_{1,31}$=12.00, P=0.002**	$F_{1,31}$=0.65, P=0.427	**$F_{1,116}$=4.43, P=0.044**
Enzyme activity and mRNA expression			
Carbonic anhydrase mRNA	$F_{1,22}$=1.62, P=0.217	**$F_{1,22}$=5.24, P=0.032**	$F_{1,22}$=1.30, P=0.267
Carbonic anhydrase activity	**$F_{1,21}$=9.27, P=0.006**	$F_{1,21}$=0.48, P=0.495	$F_{1,21}$=3.42, P=0.078
Esterase activity	**$F_{1,21}$=5.31, P=0.031**	$F_{1,21}$=0.69, P=0.414	$F_{1,21}$=3.14, P=0.091
Tissue metabolites			
ATP	$F_{1,23}$=1.03, P=0.321	$F_{1,23}$=2.62, P=0.119	$F_{1,23}$=0.37, P=0.546
ADP	$F_{1,23}$=0.73, P=0.401	**$F_{1,23}$=5.08, P=0.034**	$F_{1,23}$=2.59, P=0.121
AMP	**$F_{1,23}$=7.94, P=0.010**	$F_{1,23}$=1.76, P=0.197	$F_{1,23}$=1.06, P=0.314
Σ adenylates	$F_{1,23}$=0.93, P=0.346	$F_{1,23}$=3.24, P=0.085	$F_{1,23}$=0.18, P=0.675
ADP/ATP	$F_{1,23}$=2.82, P=0.106	$F_{1,23}$=0.49, P=0.490	$F_{1,23}$=2.23, P=0.149
AEC	$F_{1,23}$=0.73, P=0.403	$F_{1,23}$=0.05, P=0.823	**$F_{1,23}$=4.29, P=0.049**
D-glucose	$F_{1,23}$=0.01, P=0.929	$F_{1,23}$=0.34, P=0.562	$F_{1,23}$=0.38, P=0.544
Glycogen	$F_{1,23}$=0.07, P=0.791	**$F_{1,23}$=6.15, P=0.021**	$F_{1,23}$=0.34, P=0.563
Lipids	$F_{1,12}$=4.49, P=0.055	$F_{1,23}$=4.47, P=0.056	**$F_{1,23}$=5.68, P=0.034**
Proteins	$F_{1,22}$=1.48, P=0.237	$F_{1,22}$=0.01, P=0.933	$F_{1,22}$=0.37, P=0.552
Betaine	**$F_{1,23}$=53.57, P<0.0001**	$F_{1,23}$=0.01, P=0.936	$F_{1,23}$=0.00, P=0.994
Lysine	**$F_{1,18}$=6.48, P=0.022**	$F_{1,18}$=0.05, P=0.823	$F_{1,18}$=0.02, P=0.890
Succinate	**$F_{1,22}$=8.55, P=0.008**	$F_{1,22}$=0.00, P=0.979	$F_{1,22}$=0.03, P=0.872
Acetate	**$F_{1,21}$=11.38, P=0.003**	**$F_{1,21}$=5.17, P=0.035**	**$F_{1,21}$=4.78, P=0.042**
Alanine	**$F_{1,21}$=8.58, P=0.009**	$F_{1,21}$=0.17, P=0.687	$F_{1,21}$=0.44, P=0.516

Table 3.3: Summary of the effects of salinity and P_{CO2} levels on the studied physiological and biomineralization traits in *C. virginica* juveniles. Arrows represent the direction of change (F and f for an increase or decrease, respectively) for the trait values that differed significantly from the control group (maintained at a salinity of 30 and normocapnia), whereas number of signs in a cell represent the magnitude of a change., the respective differences were not statistically significant. Normocapnia and hypercapnia refer to P_{CO2} values of ~400 and ~700–800 µatm, respectively. High and low salinity refer to salinities of 30 and 15, respectively.

	High salinity/ hypercapnia	Low salinity/ normocapnia	Low salinity/ hypercapnia
Mortality	↑	↑↑↑	↑↑↑
Body Mass	↓↓	↓↓	↓↓
Microhardness	=	=	↓
Crack length	=	=	↑↑
CA activity	=	↓↓↓	↓↓
CA mRNA	=	=	=
ATP	=	=	=
ADP	=	=	=
AMP	=	=	↓↓↓
Glycogen	↓↓	=	=
Lipids	↓↓↓	↓↓↓	↓↓↓
Proteins	=	=	=
Betaine	=	↓↓↓	↓↓↓
Acetate	=	↑↑↑	=
Lysine	=	↑↑	↑↑
Alanine	=	↓↓	↓↓↓
Succinate	=	↓↓↓	↓↓↓

CHAPTER 4: INTERACTIVE EFFECTS OF ELEVATED TEMPERATURE AND CO_2 LEVELS ON METABOLISM AND OXIDATIVE STRESS IN TWO COMMON MARINE BIVALVES

The following chapter was published in the scientific journal Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology in 2013. Omera B. Matoo, Anna V. Ivanina, Claus Ullstad, Elia Beniash and Inna M. Sokolova (2013). Interactive effects of elevated temperature and CO_2 levels on the metabolism and oxidative stress in two common marine bivalves. Comparative Biochemistry and Physiology Part A. 164 (4):545-553

Abstract

Marine bivalves such as the hard shell clams *Mercenaria mercenaria* and eastern oysters *Crassostrea virginica* are affected by multiple stressors, including fluctuations in temperature and CO_2 levels in estuaries, and these stresses are expected to be exacerbated by ongoing global climate change. Hypercapnia (elevated CO_2 levels) and temperature stress can affect survival, growth and development of marine bivalves, but the cellular mechanisms of these effects are not yet fully understood. In this study, we investigated whether oxidative stress is implicated in cellular responses to elevated temperature and CO_2 levels in marine bivalves. We measured the whole-organism standard metabolic rate (SMR), total antioxidant capacity (TAOC), and levels of oxidative stress bio-markers in the muscle tissues of clams and oysters exposed to different temperatures (22 and 27°C) and CO_2 levels (the present day conditions of ~ 400 ppmv CO_2 and 800 ppmv

CO_2 predicted by a consensus business-as-usual IPCC emission scenario for the year 2100). SMR was significantly higher and the antioxidant capacity was lower in oysters than in clams. Aerobic metabolism was largely temperature-independent in these two species in the studied temperature range (22–27°C). However, the combined exposure to elevated temperature and hypercapnia led to elevated SMR in clams indicating elevated costs of basal maintenance. No persistent oxidative stress signal (measured by the levels of protein carbonyls, and protein conjugates with malondialdehyde and 4-hydroxynonenal) was observed during the long-term exposure to moderate warming (+ 5°C) and hypercap- nia (~ 800 ppmv CO_2). This indicates that long-term exposure to moderately elevated CO_2 and temperature minimally affects the cellular redox status in these bivalve species and that the earlier observed negative physiological effects of elevated CO_2 and temperature must be explained by other cellular mechanisms.

Keywords: Ocean acidification; temperature stress; mollusks; oxidative stress; total antioxidant capacity

Introduction

Estuarine and coastal zones are the most productive and biologically diverse areas of the ocean. These regions experience strong impacts of natural and anthropogenic stressors including fluctuations in temperature, salinity and dissolved levels of oxygen and carbon dioxide (CO_2). In the recent decades, global climate change and ocean acidification have been recognized as key factors affecting life in estuarine and coastal zones. Ocean acidification refers to the CO_2-driven decrease in seawater pH that is accompanied by a strong shift in the seawater chemistry including changes in dissolved inorganic carbon and calcium carbonate saturation state (Andersson et al., 2005; Doney et

al., 2009). The continued release of CO_2 into the atmosphere has caused a reduction in ocean pH value by approximately 0.1 pH units with respect to the pre-industrial levels, and a further reduction by 0.3–0.5 pH units is predicted before the end of the 21st century (Caldeira and Wickett, 2005; Raven et al., 2005; IPCC, 2007). The increasing level of carbon dioxide (CO_2) in the atmosphere also leads to other global changes, including an increase of the average air and surface ocean temperatures (IPCC, 2007). The mean global temperatures have increased by approximately 0.7°C during the last century and the recent climate models forecast a further increase of the mean global temperature by approximately 1.8 to 4°C by the year 2100 (IPCC, 2007; Mann et al., 2008).

Marine calcifiers, including bivalve mollusks, are among the most sensitive groups to ocean acidification and global climate change (review in: Pörtner, 2002, 2008b; Guinotte and Fabry, 2008; Cooley et al., 2009; Doney et al., 2009). CO_2-driven changes in the temperature and chemistry of the ocean's waters have been shown to strongly affect development, growth and metabolism of marine bivalves leading to elevated mortality and decreased growth of larvae and juveniles, impaired biomineralization, elevated costs of basal maintenance and, at extreme pH, metabolic rate depression (Michaelidis et al., 2005a; Dove and Sammut, 2007; Gazeau et al., 2007; Beesley et al., 2008; Talmage and Gobler, 2009; Beniash et al., 2010; Gazeau et al., 2010; Lannig et al., 2010; Dickinson et al., 2012). The cellular mechanisms of CO_2-induced changes in the physiology of mollusks are not yet fully understood but are likely to involve multiple pathways of metabolism, biomineralization and acid–base balance (Pörtner, 1993; Pörtner and Bock, 2000; Pörtner, 2008a). Metabolic effects of elevated P_{CO2} vary between different species (Dupont et al., 2010; Hendriks et al., 2010; Kroeker et al., 2010) and depend on the CO_2

concentration in seawater. In marine bivalves, moderate increases in P_{CO2} (<1200 μatm) have little effect on metabolic rates, while higher P_{CO2} levels have been shown to result in elevated rates of oxygen consumption (at ~2000–3500 μatm P_{CO2}) and metabolic rate depression at extremely high levels P_{CO2} levels (>5000 μatm) (Michaelidis et al., 2005b; Beniash et al., 2010; Hendriks et al., 2010; Thomsen and Melzner, 2010). Oxidative stress is a common consequence of the metabolic and acid–base disturbance in animals, and thus can be implicated in physiological responses to elevated temperature and CO_2 levels. An earlier study in the eastern oyster *Crassostrea virginica* showed that exposure to elevated CO_2 levels induces a strong oxidative stress response in gill tissues indicated by upregulation of major antioxidant proteins (Tomanek et al., 2011). Elevated temperature also often leads to oxidative stress in marine mollusks due to a mismatch between generation and detoxification of reactive oxygen species (ROS) (Abele et al., 1998, 2001, 2002; Heise et al., 2003;Pohlmann et al., 2011). This indicates that disturbances of the cellular redox balance may be a common mechanism contributing to the negative impacts of elevated temperature and CO_2 stress on marine mollusks. However, the interactive effects of elevated temperature and CO_2 levels that are relevant to the near-future global change scenarios (IPCC, 2007) are not well understood in marine mollusks and require further investigation.

The eastern oysters, *Crassostrea virginica*, and the hard shell clams, *Mercenaria mercenaria*, are common bivalve that serve as ecosystem engineers in estuaries of the southeastern U.S. (Kennedy et al., 1996; Kraeuter and Castagna, 2001). They are often exposed to variations of temperature, pH and CO_2 levels in estuarine habitats, where seawater pH can fluctuate from 8.1 to 6.9–6.5 (and P_{CO2} can change from ~400 μatm to

more than 10,000–40,000 μatm) due to biological CO_2 production and/or freshwater inflow, and temperature fluctuations can exceed 15–20°C during diurnal, tidal and seasonal cycles (Cochran and Burnett, 1996; Burnett, 1997; Ringwood and Keppler, 2002; Cherkasov et al., 2007; Chapman et al., 2011). Ocean acidification and global climate change will shift the baseline pH and temperature levels in estuarine and coastal waters and thus can exacerbate the effects of natural variations in temperature and pH in these habitats (Cooley and Doney, 2009; Doney et al., 2009; Najjar et al., 2010; Waldbusser et al., 2011). Like other marine calcifiers, clams and oysters are expected to be sensitive to ocean acidification and global climate change but physiological mechanisms of their responses to the combined effects of elevated P_{CO2} and temperature are not yet fully understood. Earlier studies have established an important role for changes in energy metabolism and cellular redox status in responses of marine bivalves to temperature stress (Viarengo et al., 1998; Pörtner, 2002; Dahlhoff, 2004; Abele et al., 2007), and recent studies indicate that metabolic and redox status changes may also be implicated in response to elevated P_{CO2} (Beniash et al., 2010; Tomanek et al., 2011). However, our knowledge of the effects of elevated CO_2 levels on oxidative status of mollusks is currently limited to extreme P_{CO2} exposures (Tomanek et al., 2011) and nothing is known about the potential involvement of oxidative stress in their responses to moderate hypercapnia such as expected in the case of ocean acidification. The aim of this study was to determine the effects of elevated temperature and CO_2 levels on oxidative stress and antioxidant defense of clams in oysters in order to assess whether shifts in the cellular redox balance are implicated in physiological response of these organisms to the combination of elevated temperature and CO_2 levels such as expected in the case of the

global climate change. We hypothesized that exposure to elevated temperature would induce oxidative stress in the bivalve tissues, and that elevated CO_2 may potentiate the pro-oxidant effects of the elevated temperature. To test this hypothesis, we have measured whole-organism oxygen consumption rates, as well as the total antioxidant capacity (TAOC), and levels of biomarkers indicative of the oxidative damage to proteins and lipids in the muscle tissues of clams and oysters exposed to different temperatures (22 and 27°C) and CO_2 levels (~400 and 800 ppmv CO_2).

Materials and Methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma-Aldrich (St Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Animal Collection and Maintenance

Eastern oysters, *C. virginica*, and hard shell clams, *M. mercenaria*, were purchased from a commercial supplier (Cuttyhunk Shellfish Farms, Cuttyhunk, MA, USA). Oysters and clams were collected subtidally to avoid the potential effects of intertidal exposure on physiological characteristics. Oysters and clams were shipped overnight to the University of North Carolina at Charlotte within 48 h of their collection and acclimated for 10 days in aerated tanks with recirculating artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 22± 1°C and 30± 1 practical salinity units (PSU) salinity. During the preliminary acclimation period, tanks were aerated with the ambient air.

After the preliminary acclimation, clams and oysters were exposed in a factorial design with two levels of PCO_2 [~400 ppmv (normocapnia) and ~800 ppmv

(hypercapnia)] and two temperature levels [22°C (control) and 27°C (elevated)], making for a total of four treatment groups. The two selected CO_2 levels were representative of the present-day conditions (~400 ppmv CO_2; normocapnia) and atmospheric CO_2 concentrations predicted by one of the moderate scenarios of the Intergovernmental Panel for Climate Change (IPCC, 2007) for the year 2100 (~800 ppmv CO_2; hypercapnia). The two selected temperatures were chosen to represent the average water temperature in clam and oyster habitats at the time of collection (22°C), and a +5°C increase predicted for the year 2100 by an IPCC scenario (27°C). It is worth noting that both experimental temperatures are within the environmentally relevant range for these bivalves that can experience temperature fluctuations of 10–20°C during summer low tides in their habitats. Two replicate tanks were set for each experimental treatment. Water in normocapnic treatments was bubbled with the ambient air whereas in hypercapnic treatments the CO_2-enriched air (certified gas mixtures containing 21% O_2, 0.08% CO_2 and balance N_2; Roberts Oxygen, Charlotte, NC, USA) was used. The flow rates were regulated to maintain the target pH of the seawater at a steady level throughout the exposures. For the bivalves exposed to elevated temperature, the water temperature in the tanks was slowly raised from 22 °C by 1°C per day until 27°C was achieved and the experimental exposures began. The interactive effects of P_{CO2} and temperature were tested after short-term (two weeks) and long-term (8–15 weeks) exposures. The two-week time point was selected because it is considered the minimum time required for full acclimation in mollusks (Prosser, 1958, 1991; Berger and Kharazova, 1997) and is a typical duration for many short-term physiological studies. Long-term exposure lasted for 15 weeks in the first experiment; however, due to the high mortality of oysters exposed to

the elevated temperature (27°C), the experiment was repeated with a new batch of clams and oysters for two and eight weeks. Clams and oysters were obtained from the same populations and matched in size to the first experimental batch. Preliminary acclimation and all exposure conditions were the same as during the first experiment. No differences in the studied traits were observed between the two batches of oysters or clams after two weeks of exposure indicating similarity in physiological parameters between the two batches. Therefore, we have combined the data from the two replicate experiments for further analyses.

Throughout the experiments, animals were fed ad libitum every other day with a commercial algal blend (5 mL per 30 L tank) containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* sp. with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Algae were added to the tanks following each water change. Water was changed every other day using ASW at the respective experimental temperature pre-equilibrated with the gas mixtures to achieve the required treatment pH. Experimental tanks were checked for mortality daily, and animals that gaped and did not respond to a mechanical stimulus were recorded as dead and immediately removed.

2.3. Seawater Chemistry

To avoid potential variations in water chemistry, ASW for all exposures was prepared using the same batch of the Instant Ocean® salt throughout the experiment. Carbonate chemistry of seawater was determined as described in an earlier study (Beniash et al., 2010). Briefly, water samples were collected periodically from the experimental tanks during the time course of the experiment in airtight containers without

headspace, preserved by mercuric chloride poisoning and stored at +4°C until further analysis. Water pH was measured at the time of collection using a pH electrode (pH meter, Pinpoint®, American Marine Inc., Ridgefield, CT, USA) calibrated with National Institute of Standards and Technology standard pH buffer solutions (NBS standards; American Marine Inc., Ridgefield, CT, USA). Water temperature and salinity were recorded at the same time. Total dissolved inorganic carbon (DIC) concentrations were measured within a week of collection by Nutrient Analytical Services (Chesapeake Biological Laboratory, Solomons, MD, USA) as described elsewhere (Beniash et al., 2010). Temperature, salinity and pH were measured at the time of collection and, along with the total DIC levels, were used to calculate P_{CO2}, alkalinity and the saturation state (Ω) for calcite and aragonite in seawater using co2sys software (Lewis and Wallace, 1998). For co2sys settings, we used the NBS scale of seawater pH, dissociation and solubility constants from Millero et al. (2006), the KSO_4^- constant from Dickson et al. (2007) (cited in Lewis and Wallace, 1998), and concentrations of silicate and phosphate for Instant Ocean® seawater (silicate: 0.17 μmol kg^{-1} and phosphate: 0.04 μmol kg^{-1} at salinity of 30 PSU). Seawater chemistry data for the experimental exposures are given in Table 4.1

2.4. Whole-organism Oxygen Consumption

Standard metabolic rate (SMR) was measured as resting oxygenconsumption ($\dot{M}O_2$) in clams or oysters at their respective acclimation temperature and CO_2 levels using microfiber optic oxygen probes (Tx-Type, PreSens GmbH, Germany, www.presens.de). Two-point calibration was performed at each temperature and CO_2 concentration. Oyster and clam shells were carefully scrubbed and cleaned of fouling

organisms. Mollusks were placed into flow-through respiration chambers and allowed to recover overnight. To avoid interference with post-prandial metabolism and feces excretion, animals were fasted for 24 h prior to the start of $\dot{M}O_2$ recordings. Water flow (20–25 mL min^{-1}) was adjusted so that animals consumed less than 25% of O_2 at all times to avoid potential inhibitory effects of low oxygen levels on respiration rate. After measurements, bivalves were dissected and wet tissue mass immediately determined. Dry tissue mass was calculated from the wet tissue mass assuming an average water content (80%) earlier measured in clam and oyster tissues (data not shown). Bivalve tissues were flash-frozen and stored in liquid nitrogen for analysis of oxidative stress markers and total tissue antioxidant capacity. SMR was calculated as follows:

$$SMR = \frac{(\Delta P_{O2} \times \beta_{O2} \times V_{fl})}{M^{0.8}}$$

where where SMR- normalized oxygen consumption (μmol O2 g^{-1}dry mass h^{-1}) normalized to 1 g dry mass, ΔP_{O2}- difference in partial pressure between in- and out-flowing water (kPa), β_{O2}—oxygen capacity of water (μmol O2 $L^{-1}kPa^{-1}$), V_{fl}— flow rate (L h^{-1}), M- bivalve dry tissue mass (g) and 0.8- allometric coefficient (Bougrieret al., 1995).

2.5. Oxidative Markers

About 200–300 mg of the adductor muscle tissue of clams or oysters from different experimental treatments was homogenized in ice cold phosphate-buffered saline (PBS) with protease inhibitors [50 μg L^{-1} aprotinin and 40 μM of phenylmethylsulfonyl fluoride (PMSF)] in tissue-to-buffer proportion of 1:5 w:v using Kontes Duall tissue grinders (Fisher Scientific, Suwanee, GA, USA). Samples were centrifuged at 15,000 g for 10 min at 4 °C. Protein concentration was measured in the supernatant using the Bio-

Rad Protein Assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Supernatants were diluted with PBS to a final concentration of 1 mg L^{-1} of protein. Protein conjugates of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) were measured as biomarkers of lipid peroxidation using enzyme immunoassays (MDA OxiSelect™ MDA adduct ELISA Kit and HNE OxiSelect™ HNE-His adduct ELISA Kit, respectively) according to the manufacturers' protocols (Cell Biolabs, Inc., CA, USA).

Protein carbonyl content was determined following the protocol of Levine et al. (2000). About 200–300 mg of adductor muscle tissue was homogenized using Kontes Duall tissue grinders (Fisher Scientific) in a homogenization buffer (50 mM HEPES, pH 7.4, 125 mM KCl, 0.5 μg mL^{-1} leupeptin, 0.7 μg mL^{-1} pepstatin A, 40 μg mL^{-1} PMSF and 0.5 μg mL^{-1} aprotinin). Ethylenediaminetetraacetic acid (1.1 mM EDTA) and $MgSO_4$ (0.6 mM) were freshly added to the buffer prior to homogenization. Homogenates were centrifuged for 10 min at 22,000 g and 4 °C. Streptomycin sulfate (1% v:v) was added to the supernatant, incubated for 15 min at room temperature and centrifuged at 6000 g for 10 min to remove possible nucleic acid contamination. Tissue extracts were stored at −80 °C.

Carbonyl content of extracted proteins was determined using the 2, 4-dinitrophenyl hydrazine (DNPH) assay as described elsewhere (Levine et al., 2000). Two technical replicates were measured for each sample, and a sample blank was prepared using 2 M HCl instead of DNPH. Proteins in the DNPH-stained samples and HCl-treated sample blanks were precipitated with trichloroacetic acid (TCA), collected by centrifugation at 22,000 g for 10 min and the pellet washed three times in ethanol:ethyl

acetate mixture (1:1 v:v). The pellets were dried, dissolved with 6 M guanidine HCl in 20 mM KH_2PO_4, pH 2.4, centrifuged at 11,000 g for 5 min, and the absorbance of the supernatants was determined at 360 nm (Cary 50 UV–Visible spectrophotometer, Cary, NC, USA). Carbonyl concentrations were calculated using an extinction coefficient of ε=22,000 $M^{-1}cm^{-1}$ for dinitrophenylhydrazone. Protein content of the samples was determined using the Bradford assay (Bio-Rad) with bovine serum albumin (BSA) standard prepared in 6 M guanidine HCl and 20 mM KH_2PO_4 (pH 2.4). Carbonyl content was normalized to the protein concentration of the tissue samples.

2.6. Total Antioxidant Capacity (TAOC)

Samples of adductor muscles were homogenized in ice cold PBS with protease inhibitors prepared as described above for the MDA and 4-HNE assays. Total antioxidant capacity was measured using colorimetric microplate assay for total antioxidant power (Oxford Biomedical Research, Oxford, MI, USA) following the manufacturer's protocol. TAOC was expressed in Cu^{2+}-reducing equivalents against a uric acid standard and normalized for the protein content of the sample.

2.7. Statistics

Effects of the factors temperature, P_{CO2}, time of exposure and their interactions were assessed for all studied physiological and biochemical traits using generalized linear model (GLM) ANOVA after testing for the normality of data distribution an homogeneity of variances. All factors were treated as fixed and had two levels for temperature and P_{CO2} (22°C and 27°C for temperature, and normocapnia and hypercapnia for P_{CO2}), and three levels for exposure time (2, 8 and 15 weeks) (Table 2.2, 2.3). Post-hoc tests (Fisher's Least Significant Difference) were used to test the

differences between the group means; only planned contrasts were used for the comparison of all studied biological traits. For unplanned comparisons of correlated variables (e.g. parameters of seawater chemistry), a sequential Bonferroni correction was used to adjust the conventional probability levels to ensure the experiment-wise error did not exceed 5%. Sample sizes were 5–11 for all measured traits and experimental groups, except for oysters after 8–15 weeks of exposure at 27°C where sample size was reduced due to mortality. Each biological replicate represents a sample from an individual clam or oyster. Unless otherwise indicated, data are represented as means± standard errors of means (SEM). The differences were considered significant if the probability of Type I error was less than 0.05.

Results

3.1. Mortality

At 22°C, clams and oysters showed low mortality rates (~ 0–5% and ~30% after 15 weeks of exposure, respectively). Elevated temperature resulted in increased mortality in both species (Fisher's exact tests, $p< 0.05$ for 22°C vs. 27°C contrasts at the respective P_{CO2}). At 27°C, mortality of oysters was significantly lower in hypercapnia than normocapnia (61% vs. 95%, $p< 0.05$), while in clams P_{CO2} did not affect mortality at 27°C (~44% in normocapnia and hypercapnia, $p>0.05$). Exposure P_{CO2} had no effect on mortality at 22°C in either species (Fisher's exact tests, $p>0.05$).

3.2. Oxidative Metabolism

Oysters had two- to three-fold higher rates of oxygen consumption ($\dot{M}O_2$) than clams regardless of the exposure conditions. In oysters, acclimation temperature and CO_2 levels had no effects on $\dot{M}O_2$ (Table 4.2; Fig.4.1A). In clams, exposure to elevated

temperature under the normocapnic conditions led to a transient increase in $\dot{M}O_2$ after 2 weeks with the subsequent return of $\dot{M}O2$ to the control levels after 8–15 weeks of exposure (Fig.4.1B). At high temperature (27°C) and hypercapnia, oxygen consumption rates of clams were elevated throughout the experimental exposure (significantly at 2 and 15 weeks) (Fig.4.1B).

3.3. Total Antioxidant Capacity (TAOC)

TAOC was higher in clam tissues compared with oysters under the control conditions (Fig.4.1.C, D). Exposure time significantly affected TAOC in both studied species, while CO_2 levels also significantly affected TAOC in clams (Table 4.2, 4.3). In oysters, there was an increase of TAOC after 8 weeks of exposure at 22°C both in normocapnia and in hypercapnia that was not observed after 15 weeks (Fig.4.1C). In clams, a similar transient increase was observed after 8 weeks of exposure at ~800 ppmv CO_2 (Fig.4.1.D). Elevated temperature had no effect on TAOC in the two studied species when maintained in normocapnia (Fig.4.1.C, D). Prolonged exposure (15 weeks) at elevated CO_2 levels led to a significant decrease of TAOC in clam tissues (Fig.4.1.D). In contrast, a 15-week exposure to elevated CO_2 resulted in elevated TAOC of oyster muscle tissues; however, this increase was only significant when hypercapnia was combined with the elevated temperature (Fig.4.1.C).

3.4. Oxidative Markers

Levels of oxidative stress biomarkers were significantly affected by interactions of the factors temperature, P_{CO2} and/or exposure time in clams and oysters thereby precluding the analysis of the effects of single factors in ANOVA (Table 4.2, 4.3). In control conditions, carbonyl levels in muscle proteins were similar in the two studied

species (Fig. 4.2.A, B). In oysters, carbonyl levels significantly but transiently increased after 2 weeks of exposure to elevated CO_2 at 22 °C, returning to the control levels after 8 and 15 weeks. In clams, carbonyl content remained at a stable low level regardless of the exposure conditions except for a transient rise at 8 weeks of exposure under the control conditions (Fig. 4.2.B).

Tissue levels of MDA–protein conjugates were considerably higher in oysters than in clams regardless of the experimental conditions (Fig. 4.2.C, D). Similar to carbonyls, tissue levels of MDA conjugates in oysters showed a significant transient increase after 2 weeks of exposure to elevated CO_2 at 22°C returning to the control levels after 8 and 15 weeks. In clams, elevated temperature (27°C) resulted in a significant increase in the levels of MDA–protein conjugates after 15 weeks of exposure at normocapnia, while hypercapnia at 27°C resulted in the suppressed levels of MDA–protein conjugates (Fig.4.2.D).

Levels of HNE–protein conjugates were similar in the muscle tissues of the two studied species under the control conditions (Fig.4.2.E, F). In oysters, exposure to elevated CO_2 led to a transient increase in the levels of HNE–protein conjugates after 2–8 weeks of exposure that returned to the control levels after 15 weeks (Fig.4.2.E). In clams, exposure to elevated temperature (27°C) led to a significant increase of HNE–protein conjugates after 15 weeks of exposure in normocapnia but not in hypercapnia. A transient increase in HNE conjugate levels was also observed after a short-term (2 weeks) exposure of clams to elevated CO_2 at 22°C that returned back to the control level after 8–15 weeks (Fig.4.2.E).

Notably, the levels of MDA and NHE conjugates in tissues of individual bivalves were significantly positively correlated (Pearson's R= 0.34, p=0.003, N= 74 and R=0.71, p< 0.0001, N= 98 for oysters and clams, respectively). In contrast, carbonyl concentration in muscle proteins did not significantly correlate with either NHE- or MDA-conjugate levels (R=−0.25–0.01, p=0.14–0.94 for oysters and R=−0.10–0.02, p=0.37–0.83 for clams).

Discussion

Clams were considerably more tolerant of chronically elevated temperatures than oyster as shown by the lower mortality at 27°C. Earlier studies showed that optimal temperature range is similar in clams and oysters (20–24 °C) but responses to chronically elevated temperatures are different (Kraeuter and Castagna, 2001; Lannig et al., 2006). Hard shell clams were capable of maintaining growth during prolonged exposures to 27–30°C despite a decrease in the feeding rates (Kraeuter and Castagna, 2001). In contrast, the growth ceased and survival dramatically declined in oysters exposed to temperatures of 28°C or above (Bushek and Allen, 1996; Surge et al., 2001; Cherkasov et al., 2006; Lannig et al., 2006; Li et al., 2007). Physiological mechanisms underlying differences in thermal tolerance of clams and oysters are presently unknown and may be related to the differences in ecology of these species. *M. mercenaria* is a typical infaunal species that lives buried below the sediment surface (Kraeuter and Castagna, 2001). In these habitats, sediment and interstitial water may buffer rapid thermal fluctuations but once the sediment warms up, evaporative cooling is limited potentially exposing clams to long-term temperature stress. In contrast, oysters are epibenthic species exposed to extreme, but typically short-term temperature fluctuations during the low tide (Kennedy et al.,

1996; Cherkasov et al., 2007). Like other epibenthic intertidal organisms, they can use evaporative cooling and metabolic rate depression to survive short-term heat exposure but may be less capable of surviving chronic temperature stress (Cleland and McMahon, 1986; Sokolova and Pörtner, 2001; Fitzhenry et al., 2004). Determination of the exact physiological mechanisms of the species-specific differences in thermal tolerance was beyond the scope of this study and requires further investigation. Regardless of the underlying mechanisms, lower tolerance of oysters to chronically elevated temperatures may have important implications for survival of their populations in the face of the global climate change.

An increase in environmental temperature typically results in elevated SMR in ectotherms reflecting rate-enhancing effects of temperature on physiological and biochemical reactions such as activities of mitochondria, metabolic enzymes, ion channels and other important oxygen- and energy-demanding processes (Hochachka, 1973, 1988; Cossins et al., 1995; Johnston, 1996). However, the absolute rates as well as temperature-dependence of metabolism are subject to acclimatory or evolutionary changes and thus can differ within and between species depending on the environment, physiological state, activity levels and history of thermal adaptation or acclimation (Somero, 1995; Johnston and Bennett, 1996; Pörtner, 2010; Seebacher et al., 2010). In the present study, a 5°C increase in temperature (from 22 to 27°C) under the current ambient CO_2 conditions had no effect on the metabolic rates of clams and oysters after prolonged (8–15 weeks) acclimation. These temperatures are well within the range of the normal temperatures experienced by clams and oysters in their intertidal and shallow water habitats (Kennedy et al., 1996; Kraeuter and Castagna, 2001; Cherkasov et al.,

2007). Notably, in clams the metabolic rate increased during the short-term (2 weeks) exposure to the elevated temperature whereas in oysters there was no change in SMR throughout the experimental exposure. A similar situation was found during the acute temperature rise in oysters where a 4 °C rise above the acclimation temperature (from 20 to 24°C) had no effect on the oxygen consumption rate (Lannig et al., 2008). Such relative temperature independence of aerobic metabolism in the environmentally relevant temperature range is commonly found in intertidal and subtidal mollusks (Branch et al., 1988; McMahon, 1992; Sokolova and Pörtner, 2003). This may reflect metabolic adaptation to fluctuating temperature regime in intertidal and shallow water habitats. Earlier studies indicate that high thermal sensitivity of aerobic metabolism in the environmentally relevant range of temperatures can be associated with long-term metabolic costs and thus may be selected against in these thermally unstable environments (Hawkins, 1995). Notably, when elevated temperature was combined with moderate hypercapnia (~800 ppmv CO_2), SMR remained elevated in clams even after the long-term acclimation indicating that a combination of these two stressors led to elevated metabolic costs in these organisms. In contrast, no significant elevation of SMR was found after prolonged exposure to hypercapnia and elevated temperatures in oysters. This may reflect a stronger metabolic regulation in oysters (a typical epibenthic species exposed to rapid and extreme thermal fluctuations) compared with hard shell clams from the more thermally buffered infaunal habitats, or may be due to the selective mortality of oysters at 27°C.

Oysters had lower total antioxidant capacity and considerably higher rates of oxygen consumption associated with higher levels of MDA–protein conjugates in

the adductor muscle tissues compared with clams. This suggests that oyster tissues may be more susceptible to lipid peroxidation than those of clams. In contrast, levels of carbonyls and HNE–protein conjugates were similar in the tissues of the two studied species. Within each species, levels of MDA- and HNE-conjugates were tightly correlated with each other but not with the carbonyl levels likely indicating different kinetics and/or susceptibilities of lipids and proteins to ROS-mediated oxidation (Avery, 2011). Lipid peroxidation biomarkers were also not linked to protein carbonyl content in tissues of aging clams and oysters (Ivanina et al., 2008). Similarly, in mussels *Mytilus gallprovincialis* an increase in carbonyl levels was induced by much lower exposures to metals (Cd and Zn) or organic pollutants (PAHs and lindane) compared with those that induced an elevation in MDA concentrations (Kaloyianni et al., 2009). In our earlier study we also found that an acute temperature rise from 20°C to 28–32°C induces a strong accumulation of protein carbonyls but not MDA in oysters (I. Sokolova, unpubl. data). This indicates that oxidative stress assessment should optimally rely on multiple biomarkers encompassing proteins, lipids and/or DNA as the oxidative damage levels can differ between different macromolecular targets (Avery, 2011). In marine bivalves, protein oxidation appears to be an earlier biomarker of oxidative stress than the lipid oxidation markers.

In this study, there was no persistent oxidative stress signal generated by the long-term exposure to moderate warming (+5°C) and hypercapnia (~800 ppmv CO_2) in the two studied bivalves. In oysters, there was a strong but transient increase in the levels of all three oxidative markers (carbonyls, MDA- and HNE-conjugates) after two weeks of exposure to hypercapnia at the normal temperature (22°C). This agrees with the

findings of an earlier study that identified an oxidative stress signature in proteome of oysters exposed for two weeks to elevated CO_2 (~3500 ppmv) (Tomanek et al., 2011). An increase in the levels of oxidative stress biomarkers after two weeks of exposure to hypercapnia (~800 ppmv CO_2) was followed by an increase in the tissue TAOC in oysters possibly reflecting a compensatory response. However, this response did not persist during the long-term (15 weeks) exposure of oysters to hypercapnia. This may indicate that ROS production and antioxidant defense reached a new steady-state after the long-term acclimation to hypercapnia in oysters. In oysters exposed to hypercapnia at the elevated temperature (27°C) the changes in oxidative stress markers and antioxidant levels were much less pronounced than at 22 °C suggesting that elevated temperature may partially counteract the effects of elevated CO_2 on the redox status of these organisms. It is worth noting that the relative stability of the levels of antioxidants and oxidative stress biomarkers during exposures of oyster to the elevated temperature may also be explained by high mortality (and thus differential selection) in this experimental group; this hypothesis could not be tested in the present experimental regimes because the oxidative stress markers and TAOC could only be measured in survivors.

In clams, exposure to moderate hypercapnia (~800 ppmv CO_2) had no effect on the oxidative status of the muscle tissue suggesting that this species is more capable of withstanding elevated CO_2 levels than oysters. Interestingly, exposure to 27°C under the normocapnic conditions led to a transient increase in metabolic rates as well as the tissue levels of MDA and HNE conjugates in clams; this increase was absent under the hypercapnic conditions indicating that elevated CO_2 may alleviate the negative effects of warming on tissue oxidative status in this species.

As a corollary, our study shows that exposure to moderately elevated temperature and CO_2 levels such as expected in the case of the global climate change does not lead to the persistent shifts in the oxidative status of tissues of clams and oysters and has a minimum impact on their energy metabolism. This may reflect adaptation of these intertidal and shallow water species to their environments that naturally experience high fluctuations in temperature and CO_2 levels. Temperature appears to have a stronger effect on metabolism and oxidative status of these species than moderate hypercapnia, especially in oysters where elevated temperature led to considerable mortality regardless of the exposure CO_2 levels. Our data indicate that cellular mechanisms underlying the negative physiological effects of elevated CO_2 and temperature in these bivalve species (including reduced growth, elevated mortality and disturbances in development and biomineralization; Michaelidis et al., 2005a; Dove and Sammut, 2007; Gazeau et al., 2007; Beesley et al., 2008; Talmage and Gobler, 2009; Beniash et al., 2010; Gazeau et al., 2010; Lannig et al., 2010; Dickinson et al., 2012) likely do not involve disturbances of the cellular redox status and must be explained by other mechanisms (such as impacts on metabolic and biomineralization enzymes, stress protection proteins and/or energy trade-offs) that are a subject of further investigations.

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Figures

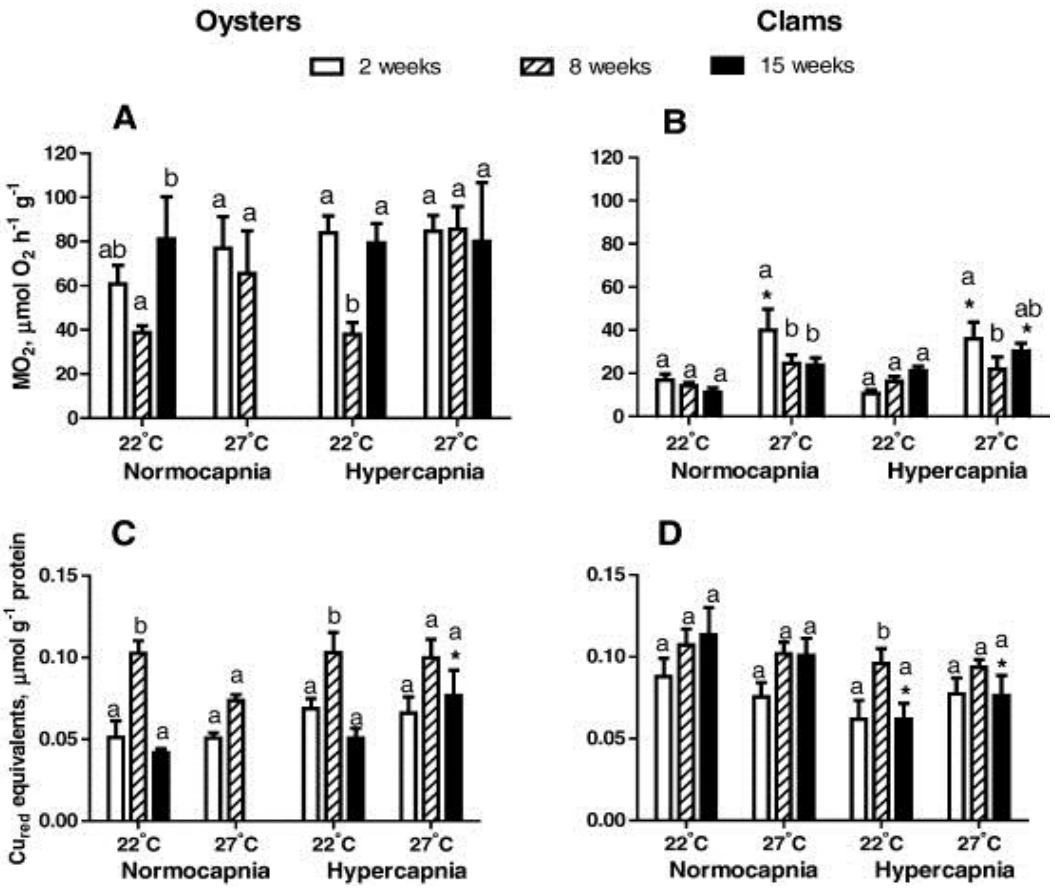

Figure.4.1: Oxygen consumption rates and total antioxidant capacity (TAOC) of oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperatures and CO_2 levels. A, B — oxygen consumption rates ($\dot{M}O_2$), and C, D — TAOC of oysters (A, C) and clams (B, D). X-axis — experimental conditions (temperature and CO levels). Different lettersindicate exposure times that are different within each experimental treatment group ($p < 0.05$). Asterisk indicates values that are significantly different from the control (normocapnia at 22°C) at the respective exposure period ($p < 0.05$). For $\dot{M}O_2$ N=8–10 except 8–15 weeks of exposure at 27°C for oysters where N=2–5. For TAOC, N=7–11except 8–15 weeks of exposure at 27°C for oysters where N=2–4.

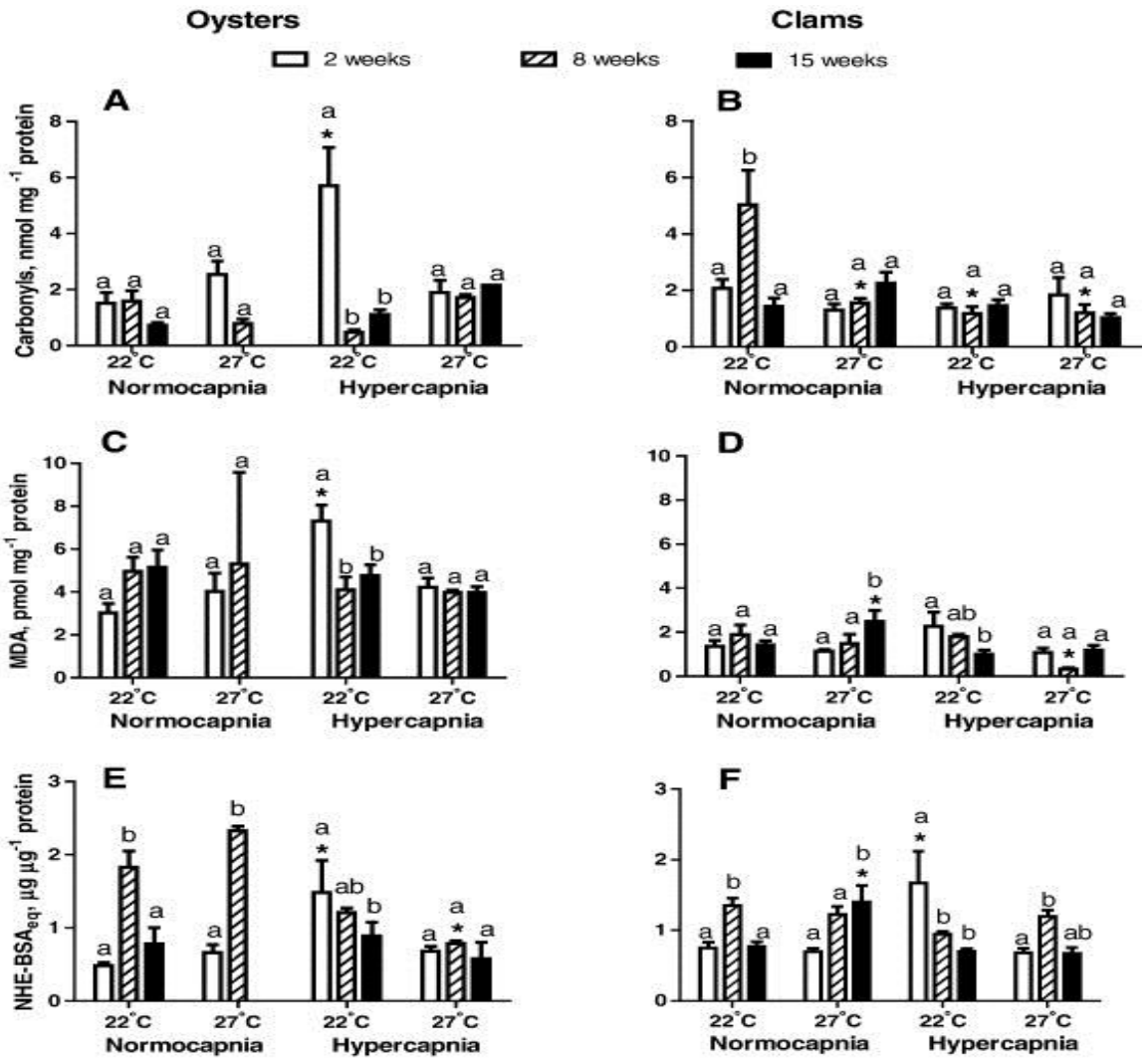

Figure. 4.2: Oxidative stress markers in gill tissues of oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperatures and CO_2 levels. A, B- protein carbonyls, C, D-MDA–protein conjugates, E, F-HNE–protein conjugates. A, C, E- C. virginica, B, D, F-M. mercenaria. Carbonyl and MDA concentrations are expressed in nmol or pmol per mg protein. HNE concentrations are expressed in relative units compared to a NHE-conjugated BSA standard that was the same for all samples. X-axis-experimental conditions (temperature and CO_2 levels). Different letters indicate exposure times that are different within each experimental treatment group ($p < 0.05$). Asterisk indicates values that are significantly different from the control (normocapnia at 22 °C) at the respective exposure period ($p < 0.05$). N=5–10 except 8–15 weeks of exposure at 27°C for oysters where N=1–4

Table 4.1: Summary of water chemistry parameters during experimental exposures. Salinity, temperature, pH, and DIC were determined in samples from experimental tanks as described in Materials and methods. Other parameters were calculated using co2sys software. P_{CO2} levels were significantly different between normocapnia and hypercapnia within each temperature treatment ($p < 0.01$ after sequential Bonferroni corrections) but not between 22°C and 27°C treatments within the respective CO_2 groups. Data are presented as means ± SEM. N = 22–27 for DIC and TA, and 95–119 for other parameters.

	Exposure temperature			
	22°C		**27°C**	
	Normocapnia	**Hypercapnia**	**Normocapnia**	**Hypercapnia**
pH	8.14 ± 0.01	7.95 ± 0.01	8.08 ± 0.01	7.94 ± 0.02
Temperature (°C)	22.4 ± 0.1	22.2 ± 0.1	26.7 ± 0.1	26.7 ± 0.1
Salinity, PSU	30.4 ± 0.1	30.3 ± 0.1	30.4 ± 0.1	30.8 ± 0.1
P_{CO2} (µatm)	429.75 ± 16.60	744.49 ± 30.63	535.52 ± 26.57	796.37 ± 47.28
DIC (µmol kg^{-1} SW)	2439.28 ± 84.29	2554.09 ± 99.41	2571.64 ± 103.26	2549.93 ± 89.09
TA (µmol kg^{-1} SW)	2683.17 ± 100.04	2735.44 ± 115.21	2824.94 ± 119.37	2725.84 ± 97.70
HCO_3^-(µmol kg^{-1} SW)	2127.67 ± 20.53	2320.04 ± 27.09	2159.68 ± 28.37	2260.54 ± 25.89
CO_3^{2-} (µmol kg^{-1} SW)	255.71 ± 6.15	182.49 ± 6.13	259.71 ± 7.03	201.16 ± 6.45
CO_2 (µmol kg^{-1} SW)	13.35 ± 0.51	23.25 ± 0.96	14.88 ± 0.74	22.03 ± 1.29
Ω_{Ca}	6.33 ± 0.15	4.52 ± 0.15	6.50 ± 0.17	5.03 ± 0.16
Ω_{Arg}	4.10 ± 0.10	2.92 ± 0.10	4.26 ± 0.11	3.29 ± 0.11

Table 4.2: ANOVA for the effects of temperature, CO_2 levels and exposure time on the studied physiological and biochemical traits of *C. virginica*. F-Values are given with degrees of freedom for the factor and the error in subscript. Significant effects are highlighted in bold.

Factors/interactions	Traits				
	Oxygen consumption rate	TAOC	Protein carbonyls	MDA conjugates	HNE conjugates
C. virginica					
P_{CO2}	$F_{1,84} = 0.89$ $p = 0.349$	$F_{1,75} = 4.32$ $p = 0.415$	$F_{1,47} = 2.70$ $p = 0.109$	$F_{1,76} = 0.04$ $p = 0.836$	$F_{1,76} = 1.96$ $p = 0.166$
Temperature (T)	$F_{1,84} = 3.39$ $p = 0.070$	$F_{1,75} = 0.02$ $p = 0.901$	$F_{1,47} = 0.01$ $p = 0.911$	$F_{1,76} = 0.35$ $p = 0.553$	$F_{1,76} = 0.28$ $p = 0.597$
Exposure time (Exp)	$F_{2,84} = 2.31$ $p = 0.107$	$\mathbf{F_{2,75} = 14.18}$ $\mathbf{p < 0.0001}$	$\mathbf{F_{2,47} = 11.64}$ $\mathbf{p = 0.0001}$	$F_{2,76} = 0.07$ $p = 0.937$	$\mathbf{F_{2,76} = 6.16}$ $\mathbf{p = 0.004}$
$P_{CO2} \times T$	$F_{1,84} = 0.02$ $p = 0.897$	$F_{1,75} = 0.62$ $p = 0.434$	$F_{1,47} = 3.27$ $p = 0.078$	$F_{1,76} = 3.00$ $p = 0.088$	$\mathbf{F_{1,76} = 5.22}$ $\mathbf{p = 0.026}$
$P_{CO2} \times Exp$	$F_{2,84} = 0.28$ $p = 0.753$	$F_{2,75} = 0.02$ $p = 0.976$	$\mathbf{F_{2,47} = 3.63}$ $\mathbf{p = 0.036}$	$\mathbf{F_{2,76} = 4.62}$ $\mathbf{p = 0.013}$	$\mathbf{F_{2,76} = 7.23}$ $\mathbf{p = 0.001}$
T × Exp	$F_{2,84} = 1.18$ $p = 0.313$	$F_{2,75} = 1.62$ $p = 0.206$	$\mathbf{F_{2,47} = 4.19}$ $\mathbf{p = 0.023}$	$F_{2,76} = 0.67$ $p = 0.513$	$F_{2,76} = 0.70$ $p = 0.500$
$P_{CO2} \times T \times Exp$	$F_{1,84} = 0.73$ $p = 0.396$	$F_{1,75} = 0.88$ $p = 0.352$	$\mathbf{F_{1,47} = 19.94}$ $\mathbf{p < 0.0001}$	$F_{1,76} = 1.88$ $p = 0.175$	$F_{1,76} = 0.00$ $p = 0.949$

Table 4.3: ANOVA for the effects of temperature, CO_2 levels and exposure time on the studied physiological and biochemical traits of *M.mercenaria*. F-Values are given with degrees of freedom for the factor and the error in subscript. Significant effects are highlighted in bold.

Factors/interactions	Traits				
	Oxygen consumption rate	TAOC	Protein carbonyls	MDA conjugates	HNE conjugates
M. mercenaria					
P_{CO2}	$F_{1,113} = 0.13$ $p = 0.716$	$\mathbf{F_{1,111} = 10.59}$ $\mathbf{p = 0.002}$	$\mathbf{F_{1,81} = 11.08}$ $\mathbf{p = 0.001}$	$F_{1,102} = 2.88$ $p = 0.093$	$F_{1,100} = 0.24$ $p = 0.628$
Temperature (T)	$F_{1,113} = 30.35$ $p < 0.001$	$F_{1,111} = 0.01$ $p = 0.937$	$\mathbf{F_{1,81} = 4.01}$ $\mathbf{p = 0.049}$	$F_{1,102} = 2.53$ $p = 0.115$	$F_{1,100} = 0.24$ $p = 0.628$
Exposure time (Exp)	$F_{2,113} = 2.16$ $p = 0.121$	$\mathbf{F_{2,111} = 5.18}$ $\mathbf{p = 0.007}$	$F_{2,81} = 2.78$ $p = 0.069$	$F_{2,102} = 0.18$ $p = 0.839$	$F_{2,100} = 2.77$ $p = 0.068$
$P_{CO2} \times T$	$F_{1,113} = 0.14$ $p = 0.714$	$F_{1,111} = 2.45$ $p = 0.121$	$\mathbf{F_{1,81} = 4.24}$ $\mathbf{p = 0.043}$	$\mathbf{F_{1,102} = 5.38}$ $\mathbf{p = 0.023}$	$F_{1,100} = 3.31$ $p = 0.072$
$P_{CO2} \times Exp$	$F_{2,113} = 2.21$ $p = 0.114$	$F_{2,111} = 2.13$ $p = 0.125$	$\mathbf{F_{2,81} = 5.04}$ $\mathbf{p = 0.009}$	$\mathbf{F_{2,102} = 3.37}$ $\mathbf{p = 0.039}$	$\mathbf{F_{2,100} = 5.11}$ $\mathbf{p = 0.008}$
T × Exp	$F_{2,113} = 3.67$ $p = 0.029$	$F_{2,111} = 0.08$ $p = 0.927$	$\mathbf{F_{2,81} = 5.03}$ $\mathbf{p = 0.009}$	$\mathbf{F_{2,102} = 5.26}$ $\mathbf{p = 0.007}$	$\mathbf{F_{2,100} = 4.56}$ $\mathbf{p = 0.013}$
$P_{CO2} \times T \times Exp$	$F_{2,113} = 0.16$ $p = 0.851$	$F_{2,111} = 0.45$ $p = 0.641$	$\mathbf{F_{2,81} = 6.84}$ $\mathbf{p = 0.002}$	$F_{2,102} = 0.02$ $p = 0.983$	$\mathbf{F_{2,100} = 3.38}$ $\mathbf{p = 0.038}$

CHAPTER 5: INTERACTIVE EFFECTS OF ELEVATED TEMPERATUE AND CO_2 ON THE ENERGY METABOLISM AND BIOMINERALIZATION OF MARINE BIVALVES *CROSSOSTREA VIRGINICA* AND *MERCENARIA MERCENARIA*

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Abstract

The continuing increase of carbon dioxide (CO_2) levels in the atmosphere leads to increases in global temperatures and partial pressure of CO_2 (P_{CO2}) in surface waters, causing ocean acidification. These changes are especially pronounced in shallow coastal and estuarine waters and are expected to significantly affect marine calcifiers including bivalves that are ecosystem engineers in estuarine and coastal communities. To elucidate potential effects of higher temperatures and P_{CO2} on physiology and biomineralization of marine bivalves, we exposed two bivalve species, the eastern oysters *Crassostrea virginica* and the hard clams *Mercenaria mercenaria* to different combinations of P_{CO2} (~400 and 800 μatm) and temperatures (22 and 27°C) for 15 weeks. Survival, bioenergetic traits (tissue levels of lipids, glycogen, glucose and high energy phosphates) and biomineralization parameters (mechanical properties of the shells

and activity of carbonic anhydrase, (CA) were determined in clams and oysters under different temperature and P_{CO2} regimes. Our analysis showed major inter-species differences in shell mechanical traits and bioenergetics parameters. Elevated temperature led to the depletion of tissue energy reserves indicating energy deficiency in both species and resulted in higher mortality in oysters. Interestingly, while elevated P_{CO2} had a small effect on the physiology and metabolism of both species, it-improved survival in oysters. At the same time, a combination of high temperature and elevated P_{CO2} lead to a significant decrease in shell hardness in both species, suggesting major changes in their biomineralization processes. Overall, these studies show that global climate change and ocean acidification might have complex interactive effects on physiology, metabolism and biomineralization in coastal and estuarine marine bivalves.

Introduction

Global change, driven by the increase in atmospheric CO_2 concentrations, has a major impact on marine ecosystems due to a concomitant increase in seawater temperatures and ocean acidification (Cicerone et al., 2004; Cao and Caldeira, 2008; Doney et al., 2009). Current models predict an average increase in global surface temperatures of 1.8–4.0°C (with some estimates reaching as high as 6.4°C) and a decline in the ocean pH by 0.3 to 0.4 units by the year 2100, depending on the CO_2 emission scenario (Intergovernmental Panel on Global Climate Change; IPCC, 2007). This change in pH is accompanied by a decrease in the carbonate concentration and saturation levels of calcium carbonate ($CaCO_3$) in seawater. Estuarine and coastal habitats, which represent the ocean's reservoirs of biological diversity and productivity, are vulnerable to ocean acidification and global climatic change. Brackish estuarine waters have lower capacity to buffer pH,

are subject to large fluctuations in pH, and carbonate chemistry due to the land run-off, upwelling of CO_2-enriched acidified waters and biological CO_2 production (Mook and Koene, 1975; Burnett, 1997; Cai and Wang, 1998; Rabalais et al., 2009). Lower capacity of thermal buffering of the shallow waters and the thermal exchanges with the land and fresh waters also predispose estuarine and coastal zones to rapid warming and temperature extremes (Helmuth et al., 2002; Gilman et al., 2006). The long-term temperature and pH recordings show that warming of the estuarine and coastal waters have outpaced those observed in the surface waters of the open ocean (Talmage and Gobler, 2011) and that rate of acidification of estuarine waters in the past 50 years closely follows the increase in atmospheric CO_2 (Cooley and Doney, 2009; Doney et al., 2009; Waldbusser et al., 2011).

Marine calcifiers such as bivalve mollusks are keystone species in estuarine and coastal ecosystems as well as an important resource for marine fisheries and aquaculture. Elevated partial pressure of CO_2 (P_{CO2}) can affect acid–base balance, physiology and biomineralization in mollusks leading to decreased rates of growth, higher mortality, as well as changes in shell structure, composition and mechanical properties (Michaelidis et al., 2005; Gazeau et al., 2007; Kurihara et al., 2007; Beesley et al., 2008; Kurihara, 2008; Ellis et al., 2009; Beniash et al., 2010; Dickinson et al., 2012). Physiological effects of elevated P_{CO2} may be modulated by the environmental temperature because of the temperature-dependent changes in solubility of gases, pH and inorganic carbon speciation, and most importantly, due to the strong effects of temperature on the rates of all physiological and biochemical reactions (Lewis and Wallace, 1998; Pörtner, 2012). Moreover, ocean acidification may reduce thermal tolerance of marine organisms by

shifting energy balance and reducing their aerobic scope (Pörtner, 2012; Sokolova et al., 2012) as was shown in crabs, bivalves and echinoid larvae (Metzger et al., 2007; O'Donnell et al., 2009; Lannig et al., 2010). However, the interactive effects of elevated P_{CO2} and temperature stress under environmentally realistic scenarios of global climate change are not well understood in marine mollusks, hampering our ability to predict the consequences of the global climate change on marine ecosystems.

The aim of the present study was to determine the interactive effects of elevated temperature and CO_2 levels on energy metabolism and biomineralization of two common ecologically and economically important bivalves, the Eastern oyster, *Crassostrea virginica* (Gmelin, 1791) and the hard clam *Mercenaria mercenaria* (Linnaeus, 1758). Clams and oysters are ecosystem engineers in the western Atlantic estuaries and common inhabitants of the intertidal and upper subtidal zones. Both species can experience large seasonal and diurnal change of temperature that can exceed 15–20°C (Helmuth et al., 2002; Cherkasov et al., 2007). Hard clams are an infaunal species periodically exposed to corrosive, acidified conditions in the sediments due to accumulation of CO_2 and other metabolic acids, while oysters, as an epifaunal species, live in the habitats less prone to extreme acidification (Kennedy et al., 1996; Kraeuter and Castagna, 2001; Green et al., 2009). Clams and oysters differ in shell composition and mineralogy; clam shells are made of a more soluble isoform of $CaCO_3$, aragonite, but their outer surface is protected by a thick proteinaceous periostracum (Kraeuter and Castagna, 2001). In contrast, shells of adult oysters consist of a much less soluble low-Mg calcite and have a poorly developed periostacum (Digby, 1968; Kennedy et al., 1996). We hypothesized that elevated P_{CO2} will lead to elevated energy costs of basal maintenance and impaired

biomineralization in clams and oysters, and that elevated temperature will exacerbate these negative effects of P_{CO2} due to the increased energy demand and reduced aerobic scope (Pörtner, 2012; Sokolova et al., 2012). We also hypothesized that the effects of elevated P_{CO2} will be stronger in oysters than in clams reflecting the adaptation of the latter species to extreme environmental acidosis common in sediments. To test these hypotheses, we determined survival, oxygen consumption rates, tissue and cellular energy status and mechanical properties of the shells in *M. mercenaria* and *C. virginica* after short-term (2 weeks) and long term (8–15 weeks) acclimation at different temperature and P_{CO2} levels corresponding to the present-day conditions and the predictions of near-future scenarios of global climate change.

Materials and Methods

2.1. Chemicals

Unless otherwise indicated, all chemicals and enzymes were purchased from Sigma Aldrich (St. Louis, MO, USA), Roche (Indianapolis, IN, USA) or Fisher Scientific (Pittsburg, PA, USA) and were of analytical grade or higher.

2.2. Animal Collection and Maintenance

The experimental animals and exposure conditions were the same as in our companion study (Matoo et al., 2013). Adult eastern oysters *C. virginica* and hard clams *M. mercenaria* were purchased from a commercial supplier (Cuttyhunk Shellfish Farms, Cuttyhunk, MA, USA), shipped on ice by an overnight delivery service to the University of North Carolina at Charlotte and acclimated for 10 days in tanks with recirculating artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, GA, USA) at 22 ± 1°C and 30 ± 1 salinity (practical salinity units), aerated with ambient air. Clams and

oysters were then randomly assigned to four treatment groups, and each group was exposed to one of the four possible combinations of two levels of CO_2 and two temperatures. The two selected CO_2 levels were representative of the present-day conditions (~400 ppm CO_2; normocapnia) and atmospheric CO_2 concentrations predicted by a moderate scenario of the Intergovernmental Panel for Climate Change (IPCC, 2007) for the year 2100 (~800 ppm CO_2; hypercapnia). The two selected temperatures were chosen to represent the average water temperature in clam and oyster habitats at the time of collection (22°C), and a +5 °C increase predicted for the year 2100 by an IPCC scenario (27°C). Both experimental temperatures are within the environmentally relevant range for these bivalves in the southeastern U.S. estuaries. Two replicate tanks were set for each experimental treatment. Water in normocapnic treatments was bubbled with the ambient air whereas for hypercapnic treatments the CO_2- enriched air (certified gas mixtures containing 21% O_2, 0.08% CO_2 and balance N_2; Roberts Oxygen, Charlotte, NC, USA) was used. The flow rates were regulated to maintain a steady pH throughout the exposures. To avoid potential variations in water chemistry, ASW for all exposures was prepared using the same batch of Instant Ocean® salt. Carbonate chemistry of seawater was determined periodically during experimental exposures as described elsewhere (Beniash et al., 2010). Seawater temperature and chemistry data are shown in Table 4.1.

For the groups exposed to elevated temperature, the water temperature in the tanks was raised by 1°C per day until 27°C was achieved. This time was considered time zero for all experimental exposures. Experimental exposures lasted for 2 and 15 weeks (short-term and long-term exposure, respectively). The two-week time point was selected

because it is considered the minimum time required for full acclimation in mollusks (Prosser, 1958, 1991; Berger and Kharazova, 1997) and is a typical duration for many short-term physiological studies. Due to the high mortality of oysters exposed to the elevated temperature (27°C) after 15 weeks of exposure, the experiment was repeated with a new batch of clams and oysters for 2 and 8 weeks (short-term and intermediate exposure, respectively). Clams and oysters were obtained from the same populations and matched in size to the first experimental batch. Preliminary acclimation and all exposure conditions were the same as during the first experiment. No differences in the studied traits were observed between the two batches of oysters or clams after two weeks of exposure indicating similarity in physiological parameters between the two batches. Therefore, the experimental data from the two exposures were pooled for analysis.

Water in experimental tanks was changed every other day using ASW pre-equilibrated to the respective temperature and pH. Throughout the experiment, mollusks were fed ad libitum every other day with a commercial algal blend containing *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* sp. with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Sycamore, IL, USA). Algae (5 mL per 30 L tank, ~15% of dry mass of animals; Espinosa and Allam, 2006) were added to the tanks following each water change. Experimental tanks were checked for mortality daily, and animals that gaped and did not respond to a mechanical stimulus were recorded as dead and immediately removed.

2.3. Micromechanical Testing of the Shells

After 15 weeks of experimental exposures, shells of experimental clams and oysters were collected and shipped to the University of Pittsburgh for micromechanical

testing. Residual tissue from the shell valves was removed by incubation in sodium hypochlorite (NaOCl; three cycles of 10 min in NaOCl followed by 5 min sonication in a saturated $CaCO_3$ solution, pH 7.8), and shell valves carefully separated. We conducted the mechanical testing along the narrow (2 mm) area of the outermost edge of the shell where the growing region is situated. However, we were not able to clearly separate shell growth that occurred during experimental exposure from what was present at the start of the experiment. Therefore, results of these analyses should be interpreted as encompassing both the newly deposited shell material and changes in the existing shell due to differences in the seawater chemistry.

Micromechanical testing was conducted on 5–8 shells of oysters and 9–11 shells of clams per treatment. Testing on shells of oysters exposed to normocapnia at 27°C could not be conducted due to high mortality in this treatment. Left valves of clam or oyster shells were manually trimmed with a bone-cutting tool. An approximately 3 x 3 cm region of the shell, which included its most distal (posterior) edge was mounted in epoxy resin (Epofix, ESM, Hatfield, PA, USA) and polymerized for 24 h at room temperature. Embedded shells were cut longitudinally; transecting the posterior edge, using a slow-speed water-cooled diamond saw (IsoMet, Buehler, Lake Bluff, IL, USA). A second cut was made parallel to the first to produce a 3-mm-thick section. Sections were ground and polished with Metadi diamond suspensions (6, 1 and 0.25 μm diamond particles in saturated $CaCO_3$ solution, pH 7.8) on a grinder-polisher (MiniMet 1000, Buehler). No etching was observed during grinding or polishing.

Vickers microhardness tests were carried out using a microindentation hardness tester (IndentaMet 1104, Buehler) on polished shells at a load of 0.245 N and a dwelling

time of 5 s. Five to ten indentations per shell were made at least 30 μm (but no more than 2 mm) away from the shell edge. Vickers microhardness values were averaged for each shell sample. Digital photographs were taken before and immediately after each indentation. This enabled quantification of the longest crack produced by each indent, which was measured using Adobe Photoshop (ver. 4.0, San Jose, CA, USA) as the radius of a circle radiating from the center of the indent and enclosing all visible cracks. The crack radius for a shell sample was obtained by averaging the crack radii for all indents on that sample. There are a number of empirical equations used to calculate fracture toughness (K_c) from the length of cracks generated by microindentation (Anstis et al., 1981; Baldassarri et al., 2008). However, because the empirical constants used in these equations were not determined for oyster shells, we chose to use the crack length as a proxy for Kc. The term 'fracture resistance' is used in place of K_c to avoid confusion.

2.4. Carbonic Anhydrase (CA) Activity

Carbonic anhydrase (carbonate hydrolyase, EC 4.2.1.1) activity was measured in the mantle (a 2–3 mm wide edge along the ventral shell margin) and gill tissues of clams and oysters exposed to different temperature and CO_2 conditions. Tissues were homogenized in Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA) in ice-cold buffer (1:10 w/v) containing 250 mM sucrose, 40 mM Tris-H2SO4 pH 7.5 and 80 μg L−1 of a protease inhibitor phenylmethylsulfonyl fluoride (PMSF) using an overhead motor (IKA Works RW 16 Basic, Fisher Scientific, Suwanee, GA, USA) at 800 rpm. The homogenate was centrifuged at 4°C and 10,000 g for 10 min to remove cell debris, and the supernatant was used to determine activity of soluble CA. Enzyme

extracts were stored at −80°C for less than two weeks before activity assays; pilot studies showed that CA activity was not affected by this storage (data not shown).

CA activity was determined at the respective acclimation temperature (22°C or 27°C) as acetazolamide (AZM)-sensitive esterase activity following a standard method (Gambhir et al., 2007). The esterase activity was monitored at 348 nm (VARIAN Cary 50 Bio UV–Vis spectrophotometer, Cary, NC, USA) in an assay medium (50 mM Tris-H_2SO_4, pH 7.5) containing 1.5 mM of p-nitrophenyl acetate as a substrate. Total activity of CA was determined as the difference in the reaction slopes in the absence and presence of a specific inhibitor of carbonic anhydrase (acetazolamide, or AZM, 20 μM) and background-corrected for spontaneous hydrolysis of p-nitrophenyl acetate. The extinction coefficient of for p-nitrophenol (5 $mmol^{-1}cm^{-1}$ at 348 nm, pH 7.5) was used in all calculations. Protein concentrations of tissue homogenates were determined using Bradford assay (Bradford, 1976), and specific activity of CA was expressed as U g_{-1} protein.

In a separate experiment, thermal sensitivity of CA activity was determined in different tissues of the two studied species. Gill, hepatopancreas, muscle and mantle tissues were collected from clams and oysters maintained under normocapnia at 20°C, and CA activity was measured in tissue homogenates at different temperatures in an environmentally relevant range (5–35°C) as described above. The apparent activation energy (E_a) was determined from an Arrhenius plot of ln (V_{max}) against 1/T (K^{-1}) and used as a measure of the temperature dependence of CA activity. Arrhenius breakpoint temperature (ABT) was determined as a point when the activation energy (i.e. the slope of the Arrhenius plot) significantly changed using an algorithm for the multi-segment

linear regression proposed by Oosterbaan (2008) (SegReg software downloaded at http://www.waterlog.info/).

2.5. Total Lipid Content

Total lipid content was determined in the mantle tissue using a chloroform extraction method (Folch et al., 1957; Iverson et al., 2001). Briefly, about 50 mg of mantle tissue was homogenized in chloroform/methanol mixture (2:1 v/v) using tissue: solvent proportion of 1:20 w/v. Samples were sonicated for 1 min (output 69 W, Sonicator 3000, Misonix, Farmingdale, NY, USA), incubated overnight at 4 °C and centrifuged for 5 min at 13,000 g. The supernatant was transferred in a new tube, mixed with ultrapure water (0.25 volumes of the supernatant), vortexed for 2 min and centrifuged for 5 min at 13,000 g. The lower phase (chloroform) was transferred into a pre-weighed microcentrifuge tube and chloroform was allowed to evaporate to determine the dry mass of extracted lipids.

2.6. Total Protein Content

Mantle tissue (50) mg was homogenized in ice-cold homogenization buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 10% glycerol, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 0.5 µg mL^{-1} leupeptin, 0.7 µg mL^{-1} pepstatin, 40 µg mL^{-1} PMSF and 0.5 µg mL^{-1} aprotinin) using Kontes® Duall® tissue grinders (Fisher Scientific, Suwanee, GA, USA). Homogenates were sonicated three times for 10 s each (output 69 W, Sonicator 3000, Misonix), with cooling on ice between sonications, centrifuged for 10 min at 20,000 g and 4°C, and supernatants were used for protein determination. Protein content was measured using the Bio-Rad Protein

Assay kit according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Tissue Levels of Metabolites

Mantle tissue was powdered with mortar and pestle under liquid nitrogen and homogenized with five volumes of ice-cold 0.6 M perchloric acid (PCA) with 150 mM EDTA to maximize ATP recovery (Sokolova et al., 2000). An aliquot of the homogenate was reserved for glycogen determination, and the remaining homogenate was centrifuged to remove precipitated protein and neutralized with 5 M potassium hydroxide to pH 7.2–7.5. Precipitated potassium perchloride was removed by a second centrifugation and extracts were stored at−80°C. Concentrations of metabolites were measured in neutralized PCA extracts using standard NADH- or NADPH-linked spectrophotometric tests (Bergmeyer, 1985). Briefly, assay conditions were as follows:

ATP: 38.5 mM triethanolamine (TRA) buffer, pH 7.6, 0.04 mM $NADP^+$, 7 mM $MgCl_2$.6H2O, 50 mM glucose, 0.462 U mL^{-1} glucose-6-phosphate dehydrogenase, 1.8 U mL^{-1} hexokinase.

ADP and AMP: 58 mM triethanolamine buffer, pH 7.6, 3 mM phoshoenolpyruvate (PEP), 0.09 mM NADH, 24 U mL^{-1} lactate dehydrogenase (LDH), 18 U mL^{-1} pyruvate kinase (PK), 16 U mL^{-1} myokinase (MK).

D-glucose: 38.5 mM TRA buffer, pH 7.6, 0.04 mM $NADP^+$, 7 mM $MgCl_2.6H_2O$, 0.462 U mL^{-1} glucose-6-phosphate dehydrogenase, 1.8 U mL^{-1}hexokinase.

Glycogen concentration was measured in PCA extracts after enzymatic hydrolysis of glycogen to D-glucose by glucoamylase (Keppler and Decker, 1984) and determined by the difference in the D-glucose levels in the tissue extract before and after

glucoamylase treatment. Concentrations of glycogen, lipids and proteins were expressed in mg g^{-1} wet tissue mass, and concentrations of adenylates and D-glucose in μmol g^{-1} wet tissue mass.

Adenylate energy charge (AEC) was calculated using the formula

$$AEC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]}$$

where [AMP], [ADP] and [ATP] are concentrations (μmol g^{-1} wet weight) of the corresponding compounds.

2.8. Statistics

Experimental data sets were tested for the presence of potential outliers using Grubbs' test (extreme studentized deviate, or ESD, method), as implemented in GraphPad Prism ver. 5.03 (GraphPad Software, Inc., La Jolla, CA, USA). A small number of statistically significant outliers was detected and removed from the analysis; the outlier occurrence was random among the treatment groups.

Effects of the factors “Temperature”, “P_{CO2}”, “Time of exposure” and their interactions were assessed for all studied physiological and biochemical traits using a generalized linear model (GLM) ANOVA after testing for the normality of data distribution and homogeneity of variances. All factors were treated as fixed and had two levels for temperature and P_{CO2} (22°C and 27°C for temperature, and normocapnia and hypercapnia for P_{CO2}), and three levels for exposure time (2, 8 and 15 weeks). Post-hoc tests (Fisher's Least Significant Difference) were used to compare the group means; only planned contrasts were used for the post-hoc comparisons. For micromechanical tests, only factors “Temperature” and “P_{CO2}” were assessed since all tests were performed with

animals exposed for 15 weeks. Cumulative mortalities were compared among treatments using Fisher's exact test. The best fit and significance of linear regressions for the Arrhenius plots of the temperature-dependent CA activity were determined using GraphPad Prism ver. 5.03. Each of CA was compared between different tissues and species by comparing the slopes of the respective Arrhenius plots using an algorithm implemented in GraphPad Prism 5.03. Sample sizes for all experimental groups were 5–11 except for oysters after 8–15 weeks of exposure at 27°C where sample size was reduced due to mortality. Each biological replicate represented a sample from an individual bivalve. Unless otherwise indicated, data are represented as means ± standard errors of means.

Pearson correlation and principal component (PC) analyses were conducted using Origin 8.6 software package (OriginLab, Northhampton, MA, USA) on values of all biochemical traits including concentrations of adenylates (ATP, ADP, AMP, total adenylate content) parameters of energy status (ADP/ADP ratio, AEC), tissue levels of glucose, glycogen, lipids and proteins, carbonic anhydrase activities in mantle and gill tissues and mortality. We also included standard metabolic rates ($\dot{M}O_2$) and parameters of tissue oxidative status (total antioxidant capacity, TAOC; and levels of oxidative markers–protein carbonyls, malondialdehyde (MDA)–protein adducts, and 4-hydroxynonenal (HNE)-protein adducts) that were determined in the same experimental animals in our companion study (Matoo et al., 2013). Since all studied parameters cannot be measured in the same individual, we used mean parameter values for the respective treatments and exposure time points. Seawater parameters (pH, P_{CO2}, calcite (Ω_{Cal}) and

aragonite saturation (Ω_{Arg}) and temperature), exposure duration and species were included into the correlation and PCA analyses as potential explanatory variables.

Results

3.1. Survival

Temperature had a significant effect on survival of clams and oysters (Fig. 5.1A, B). In normocapnia, mortality was higher at 27°C compared to 22°C in both studied species ($p < 0.05$ after 5, 8 and 15 weeks of exposure). Survival was more strongly compromised by elevated temperatures in oysters compared to clams (Fig. 5.1A, B). Hypercapnia did not affect survival of clams or oysters at 22°C ($P > 0.05$), while at 27°C, hypercapnic exposure improved survival in oysters ($p = 0.02$) but not in clams ($p > 0.05$) (Fig. 5.1A, B).

3.2. Shell Mechanical Properties

Mechanical properties of the shells differed substantially between the two studied species. Clam shells (aragonite) had considerably higher hardness and fracture resistance than oyster shells (predominately calcite). On average, shell microhardness (measured as Vicker's hardness number) was 219.8 ± 2.2 in oysters and 331.2 ± 1.8 in clams (Fig. 5.1C, D). Crack radius formed at 0.245 N load and 5 s dwelling time was >50% larger in oysters compared to clams (15.1 ± 0.63 μm vs. 9.27 ±0.30 μm, respectively). After 15 weeks exposure, fracture resistance (estimated by the crack radius) was not significantly affected by exposure to different CO_2 and temperature levels in clams or oysters (Supplement Table 1). In contrast, there was a significant decrease in the shell microhardness in clams and oysters exposed to hypercapnia at 27°C (Fig. 5.1C and D). Microhardness of clams exposed to normocapnia at 22°C was 334.2 ± 3.6 versus 323.2 ±

2.9 in animals exposed to hypercapnia at 27°C ($p = 0.025$). For oysters, microhardness of animals exposed to normocapnia at 22°C was 225.4 ± 2.9 versus 212.1 ± 2.1 in those exposed to hypercapnia at 27 °C ($p = 0.018$).

3.3. Carbonic Anhydrase (CA) Activity

In clams, the apparent Ea of CA from all studied tissues was 40– 43 kJ $mol^{-1}K^{-1}$ indicating the temperature dependence typical for metabolic enzymes of ectotherms, and no Arrhenius breakpoint temperature, indicative of a significant change in E_a, was detected in the range of 5–35°C (Table 5.2). In oysters, the apparent E_a of CA was significantly lower than in clams in all studied tissues except hepatopancreas, and CA activity from the oyster gills and adductor muscles was essentially temperature-independent (Table 5.2).

Long-term acclimation to different temperatures exerted a strong effect on CA activity in mantle and gill tissues of the studied bivalves leading to a notable increase in the specific CA activity at 27°C (Fig. 5.2). This effect was observed in gills and mantle tissues of both species but was more pronounced in clams than in oysters (Fig. 5.2). Notably, in oysters there was a slight decrease in CA activity after 8 and 15 weeks of exposure under the control conditions (22°C, normocapnia) but in clams CA activity was stable throughout to the control exposures (Fig. 5.2). Elevated CO_2 levels alone had little or no effect on the specific CA activity in gills and mantle of the studied bivalves (Fig. 5.2; Supplemental Table 1).

3.4. Tissue Composition and Energy Reserves

In oysters, glycogen levels in the mantle tissues were maintained during acclimation under the control conditions (Fig. 5.3A). Hypercapnia at 22°C resulted in a significant

increase in glycogen levels after 2 weeks of exposure followed by a gradual decline after 8 and 15 weeks (Fig. 5.3A). Acclimation at 27°C led to a significant reduction of the tissue glycogen content of oysters, and this reduction was especially pronounced when elevated temperature was combined with hypercapnia (Fig. 5.3A). In clams, glycogen content was maintained at the normal levels regardless of the exposure temperature and P_{CO2}, except for a slight but significant transient increase after 8 weeks of exposure at 22°C in both normo- and hypercapnia (Fig. 5.3B).

Tissue glucose levels varied over time and in response to elevated temperature in the two studied species (Fig. 5.3C, D). In clams, significantly elevated tissue glucose levels were found after 8 and 15 weeks of exposure to elevated temperature (27°C) at both normo- and hypercapnia indicating enhanced glycolysis (Fig. 5.3D). A similar but less pronounced increase was found after 8 weeks of exposure to 22°C at normo- and hypercapnia in clams and oysters but it disappeared after 15 weeks (Fig. 5.3C, D).

The total lipid content of mantle tissue was not affected by acclimation temperature or P_{CO2} in oysters (Fig. 5.3E). The lipid content in oyster mantle was reduced after 15 weeks of exposure in all treatments (no data available for normocapnia at 27°C) (Fig. 5.3E). In clams, tissue lipid levels were elevated after 15 weeks at 27°C and hypercapnia (Fig. 5.3F). At other exposure times, no significant effects of temperature or P_{CO2} on the lipid content of clam mantle was observed (Fig. 5.3F).

The protein content of mantle tissues was not significantly affected by acclimation time, temperature or P_{CO2} in oysters except for a significant increase after 15 weeks at 22°C and hypercapnia (Fig. 5.3G). No significant effects of temperature or P_{CO2} on the protein content were found in clam tissues (Fig. 5.3H) but protein content of mantle

tissues slightly decreased over time in all experimental treatments except hypercapnia at 27°C (Fig. 5.3H).

3.5. Cellular Energy Status

In oyster tissues, ATP content was stable over time and among different treatments, except for a significant decline after 8 and 15 weeks of exposure to hypercapnia at 22°C (Fig. 5.4A). A transient decrease in ADP and increase in AMP levels were observed in oyster tissues after 8 weeks of exposure in all treatments except elevated temperature and P_{CO2} (Fig. 5.4C & E). In the latter treatment, ADP and AMP concentrations declined over time during the 15 weeks exposure period (Fig. 5.4C). A similar decline in AMP levels over time was also observed in oysters exposed to elevated P_{CO2} at 22°C (Fig. 5.4E).

In clams, tissue ATP levels declined over time in the control exposure but increased in both high temperature treatments; however, the variations in ATP levels among treatments and experimental exposure times were modest (Fig. 5.4B). In contrast, tissue concentrations of ADP and AMP remained stable over time and among the experimental treatments except a moderate increase of ADP levels after 15 weeks of exposure at 27°C and normocapnia and a transient increase in AMP concentrations after 8 weeks at 22°C and elevated P_{CO2} (Fig. 5.4D and F).

Adenylate energy charge (AEC) decreased after 2 weeks of exposure to elevated temperature and P_{CO2} (both in clams and oysters) and after 2 weeks of exposure to normocapnia at 27°C (in clams only) (Fig. 5.4G,H). At later time points AEC returned to the control levels in both species and all experimental treatments (Fig. 5.4G, H).

3.6. Correlation and principal component (PCA) analyses

As expected, different parameters of seawater chemistry (pH,P_{CO2}, Ω_{Cal} and Ω_{Arg}) were significantly and strongly correlated with each other (Pearson correlation coefficient >0.9; Supplementary Table 2). Therefore, we used P_{CO2} as a potential explanatory variable and a proxy for seawater chemistry in all further analyses. Based on the correlation analysis, species-specific differences were largely responsible for variations in mortality, tissue levels of proteins, adenylates (including ATP, ADP, AMP and total adenylate content) as well as the standard metabolic rate and tissue levels of MDA-protein conjugates (Supplementary Table 2). Notably, tissue levels of ATP were also strongly and positively associated with ADP and AMP content as well as the standard metabolic rate and MDA levels. Exposure time did not significantly correlate with any of the studied biological traits, while exposure temperature correlated with mortality, CA activity in gill and mantle tissues and tissue glycogen content (Supplementary Table 2). Exposure P_{CO2} significantly and negatively correlated with AEC.

PCA including all studied physiological and biochemical traits as well as P_{CO2}, temperature, exposure duration and species as potential explanatory variables, identified five principal components explaining 76% of the total variation of the studied data set (Supplementary Table 3). Tissue levels of adenylates (except AMP), protein content, overall metabolic rate ($\dot{M}O_2$) and MDA levels were associated with the 1st principal component (24% of variance) which reflects species-specific differences between clams and oysters (Supplementary Table 3). The 2nd and 3rd principal components (19 and 14% of variance, respectively) were associated with exposure temperature and had high loadings of the majority of the studied biological traits including CA activity in gills and

mantle tissues, tissue levels of glucose, glycogen, ATP, AMP, total adenylates, AEC and ADP/ATP ratio, lipid content, as well as mortality, TAOC and tissue levels of HNE-protein conjugates. The 4th principal component (10% of variance) was correlated with P_{CO2} and exposure time and linked to the parameters of cellular energy status (levels of AMP, ADP/ATP ratio and AEC). Lastly, the 5th principal component (9% of variation) was linked to the exposure duration and correlated with mortality, tissue content of lipids and protein carbonyls, and the parameters of cellular energy status (levels of AMP, ADP, as well as ADP/ATP ratio and AEC) (Supplementary Table 3).

Pearson correlation analysis conducted separately for each species revealed tight correlations between tissue levels of adenylates and energy-related indices (ADP/ATP ratio and AEC) in both studied species (Supplementary Table 4). Acclimation temperature significantly and positively correlated with CA activity in mantle and gill tissues of both clams and oysters, as well as with SMR of clams and mortality of oysters. In both species, oxidative stress biomarkers (tissue levels of carbonyls, MDA- and/or HNE-protein conjugates) were positively associated with the parameters of carbohydrate metabolism (glucose and glycogen content). Exposure P_{CO2} was not strongly associated with any of the studied parameters except TAOC levels in clams where a significant positive correlation with P_{CO2} levels was observed (Supplementary Table 4).

Discussion

4.1. Bioenergetic Responses to Temperature and P_{CO2}

The effects of elevated temperature and P_{CO2} on survival and energy metabolism of the studied bivalves were species-specific indicating different sensitivity of clams and oysters to these factors. Oysters were especially susceptible to elevated temperatures showing

high mortality after prolonged exposure at 27°C. This is consistent with earlier findings that oysters have no "scope for growth" and do not deposit shell material at and above 28°C, and cannot withstand exposures to sublethal levels of pollutants such as cadmium at this temperature (Surge et al., 2001; Lannig et al., 2006). These data suggest that chronic warming such as expected in the case of global climate change may be deleterious for oyster populations, especially in the low-latitude parts of their distribution range. Of the two studied factors, temperature exerted much stronger effects on metabolism and survival of clams and oysters than P_{CO2}. This is consistent with the results of earlier studies that showed the predominant effects of temperature on metabolic physiology and survival of marine invertebrates in different temperature/P_{CO2} combinations within the environmentally relevant range (Lannig et al., 2010; Chapman et al., 2011; McElroy et al., 2012). While the effects of moderate hypercapnia on energy metabolism are usually less pronounced, P_{CO2} can modulate metabolic responses to elevated temperature in bivalves and other aquatic ectotherms (Lannig et al., 2010; Wood et al., 2011; Catarino et al., 2012; McElroy et al., 2012; present study).

Earlier studies showed that oysters (*C. virginica*) and clams (*M. mercenaria*) strongly differ in the rates as well as the temperature sensitivity of basal metabolism (Shumway and Koehn, 1982; Kraeuter and Castagna, 2001; Matoo et al., 2013 and references therein). Standard metabolic rates (SMR) of adult oysters are, on average, 2–4 times higher than in adult clams and do not change with acclimation to elevated temperature (27°C) while in clams a significant temperature induced increase in SMR is observed during long-term acclimation to 27°C (Matoo et al., 2013). The higher SMR of oysters (as compared to clams) was associated with elevated steady-state levels of ATP

and ADP (but not AMP) and higher total adenylate content in the former species. This indicates differences in the equilibrium constants of arginine kinase and/or adenylate kinase between these two species (Hardie and Hawley, 2001; Igamberdiev and Kleczkowski, 2006) and suggests that the overall metabolic activity may play a role in determining the set-points for cellular adenylate homeostasis of bivalves. As expected, tissue levels of different adenylates are tightly linked within each of the two studied species (Supplementary Fig. 1) reflecting physiological and biochemical coordination of adenylate metabolism. Despite the higher ATP and ADP levels as well as total adenylate content in oysters, the adenylate energy charge (AEC) was similar in the two studied bivalve species (~0.6) and within the range of the values reported for bivalves (Wijsman, 1976; Rainer et al., 1979; Barthel, 1984; Isani et al., 1997; Ivanina et al., 2011) . AEC was not significantly affected by elevated temperature and P_{CO2} in clams or in oysters (except for a modest and transient decrease after two weeks of exposure), suggesting that it may not be a sensitive index to detect bioeneregetic shifts during chronic stress exposures in these species.

Temperature- and P_{CO2}-induced changes in bioenergetics were considerably more pronounced in oysters than in clams. In oysters, long-term exposure to elevated temperature (27°C) resulted in the depletion of glycogen reserves indicating energy deficiency that goes hand-in-hand with elevated mortality. Hypercapnia partially alleviated the negative effects of elevated temperature on mortality but not on the glycogen depletion in oysters. Notably, exposure of oysters to hypercapnia at 22°C resulted in an initial increase of the tissue glycogen content after two weeks of exposure followed by a gradual decline, so that after 15 weeks the tissue glycogen content in this

group reached the control levels. This early hypercapnia-induced rise in glycogen content could have gone undetected at elevated temperatures due to the opposing effects of high temperature and P_{CO2} on glycogen stores but may have contributed to the better survival of hypercapnia-exposed oysters at 27°C. In fish (the rainbow trout *Salmo gairdneri*) and mammals (the brown rat Rattus norvegicus), external hypercapnia induced a rapid depletion of glycogen in the liver and the cerebral cortex, respectively; however, P_{CO2} levels used in these studies were selected to mimic the respiratory rather than environmental hypercapnia and were much higher than in the present study (~10,000 and 100,000 μatm P_{CO2} for fish and rats, respectively) (Folbergrová et al., 1975; Mommsen et al., 1988; Perry et al., 1988). The effects of environmental hypercapnia on glycogen turnover in invertebrates have not been studied, and so the mechanisms of the hypercapnia-induced accumulation of glycogen in oysters remain presently unknown. Rapid changes in the glycogen content over time and in response to elevated temperature and P_{CO2} in oysters (as opposed to much more stable levels of lipid and protein content) are in agreement with the role of glycogen as a rapidly mobilizable fuel that can be used to buffer excessive energy demand and is quickly replenished when energy assimilation exceeds consumption (Kooijman, 2010; Sokolova et al., 2011).

In the less temperature-sensitive of the two studied species, *M. mercenaria*, the levels of tissue energy reserves (including glycogen, lipids and proteins) were not affected by temperature and P_{CO2} exposure, and a transient decline in ATP level after 2 weeks of exposure to elevated temperature and/or hypercapnia was restored or overcompensated after 8–15 weeks. In contrast to the present study that used adult clams and oysters, an earlier study in juvenile clams showed that moderate hypercapnia (~800

μatm) results in elevated mortality and a significant depletion of glycogen and lipid reserves after 11 weeks of exposure at the control temperature (22 °C) (Dickinson et al., 2012). Similarly, juvenile oysters were more sensitive to extreme hypercapnia (~3500μatm) than adults as shown by a strong increase in standard metabolic rates and elevated mortality in hypercapnia-exposed juveniles (Beniash et al., 2010). This is in agreement with the generally higher sensitivity of early life stages of marine invertebrates to acidification and temperature stress (Kurihara, 2008; Dupont and Thorndyke, 2009; Ellis et al., 2009; Gazeau et al., 2010; Suwa et al., 2010; Zippay and Hofmann, 2010) which may reflect a priority of energy demands for growth and development over the basal maintenance during the early life stages (Sokolova et al., 2012).

4.2. Temperature Modulates the Effects of P_{CO2} on Biomineralization

Mechanical testing of the shells showed that aragonitic shells of *M. mercenaria* are considerably stronger (in terms of both hardness and fracture resistance) than calcitic oyster shells. Such mechanical differences in bivalves that produce different polymorphs of $CaCO_3$ have been well established (Currey and Taylor, 1974; Currey, 1976) and reflect differences in the mechanical properties of calcite and aragonite and the nano- and microstructural organization of the shells. Moderate hypercapnia alone (~ 800 μatm) did not significantly affect shell mechanical properties (microhardness and fracture toughness) or carbonic anhydrase (CA) activity in the mantle tissues of the two studied bivalve species acclimated under the control temperature conditions (22°C). In contrast, hypercapnia combined with the elevated temperature (27°C) led to an increase in CA activity in the mantle tissues of clams and oysters. While in clams this increase may reflect the direct rate-enhancing (Q10) effects of the temperature, this is unlikely to be the

case in oysters because of the low thermal sensitivity of CA enzyme from the mantle tissue (indicated by low Ea, Table 5.2) and the absence of a comparable increase in CA activity in the mantle tissues of oysters acclimated to 27°C in normocapnia. The increase in CA activity in the mantle may indicate a compensatory response to increase the driving force towards calcium carbonate deposition and/or facilitate the gas exchange and CO_2 release at the mantle surface in the bivalves exposed to the combination of elevated temperature and P_{CO2}.

The temperature-augmented increase in CA activity, however, appears insufficient to fully prevent disturbance of biomineralization processes in the studied bivalves as indicated by the reduced hardness of the shells of clams and oysters exposed to hypercapnia at the elevated temperature. It is worth noting that the impact of elevated temperature and P_{CO2} on the mechanical properties of the bivalve shells is not likely due to simple chemically induced shell dissolution because Ω_{Arg} and Ω_{Cal} remained well above saturation in all experimental treatments and were in fact higher in the hypercapnic treatments at 27°C than at 22°C (Table 5.1). It is therefore likely that hypercapnia, under our experimental conditions, influences the biological processes underlying shell deposition rather than physical chemistry of mineral precipitation, although at this point the exact mechanism remains elusive. Notably, synergistic negative effects of elevated temperature and hypercapnia on biomineralization were also described in a brittlestar *Ophiopecten sericeum*, where combined exposure to hypercapnia and elevated CO_2 levels reduced the rates of arm regeneration (Wood et al., 2011).

In summary, elevated temperature (such as can be expected in the case of the global climate change) negatively affects bioenergetics of intertidal bivalves such as

clams and oysters leading to energy deficiency that can have an impact on their survival, growth, reproduction, immunity and other fitness-related functions. Compared to the dominant effects of temperature, metabolic consequences of moderately elevated P_{CO2} appear mild and in some cases can slightly improve survival of temperature-stressed animals as was found in oysters exposed to elevated temperature and hypercapnia. On the other hand, the combination of elevated temperature and P_{CO2} had an overall negative effect on biomineralization leading to a decrease in shell mechanical properties, suggesting a synergistic relationship between these environmental factors. These changes may have important implications for survival and performance of the populations of clams and oysters in the face of the global climate change especially under the conditions when the ambient temperatures are at the species-specific thermal tolerance limits (e.g. during summer heat waves and/or in the southernmost populations of the studied bivalves).

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Author Contributions

O.B.M. conducted the experimental exposures, protein and lipid analyses, measurement of carbonic anhydrase activities and participated in data analysis and interpretation, and in writing and revising the manuscript.

Figures

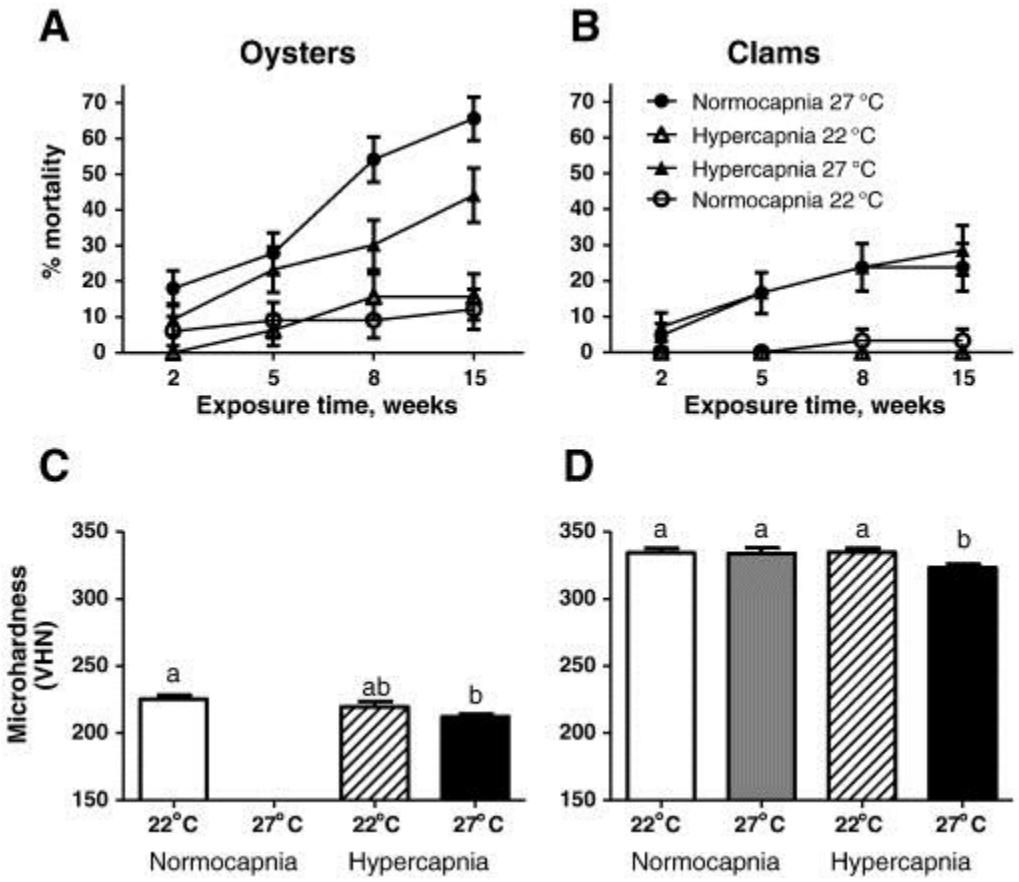


Figure.5.1: Mortality and mechanical characteristics of the growing edge of oyster (*C. virginica*) and clam (*M. mercenaria*) shells after 15 weeks of exposure to different temperature and CO_2 levels.A, B –Mortality of oysters and clams, respectively. C, D – microhardness of the growing edge of shell expressed as Vickers microhardness number (VHM) in oysters and clams, respectively.Different letters in C and D denote significantly different values (p b 0.05) while the columns that share the same letter are not significantly different ($p > 0.05$). N = 10 – 51 for mortality and 5–11 for microhardness. Testing of microhardness on shells of oysters exposed to normocapnia at 27°C could not be conducted due to high mortality in this treatment.

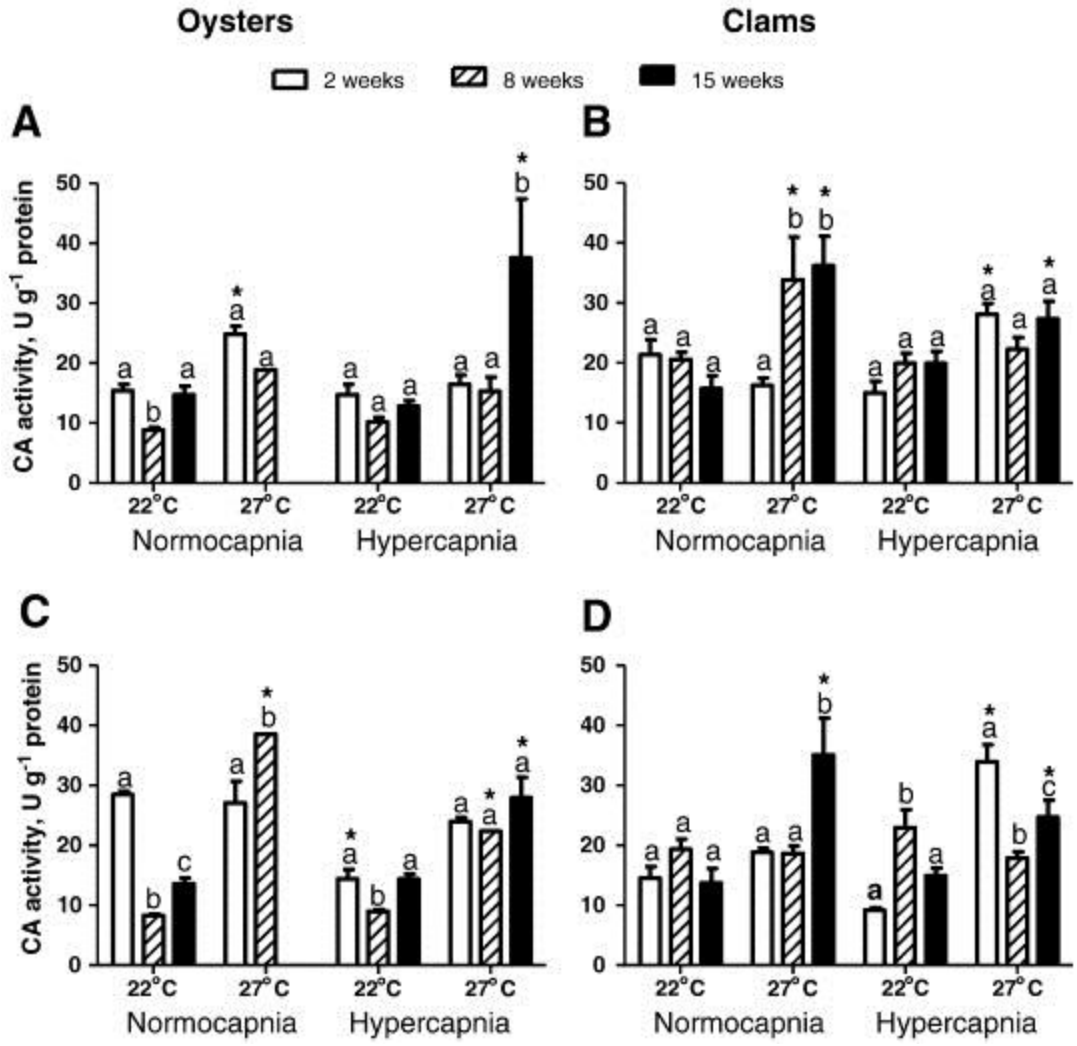


Figure.5.2: Carbonic anhydrase (CA) activity in mantle edge and gill tissues of oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperature and CO_2 levels. A, B – CA inmantle edge, C, D – CA in gills, A, C – *C. virginica*, B, D – *M. mercenaria*. X-axis – experimental conditions (temperature and CO_2 levels). CA activities were measured at the respective acclimation temperatures. Different letters indicate exposure times that are different within each experimental treatment group (p b 0.05). Asterisk indicate values that are significantly different from the control (normocapnia at 22°C) at the respective exposure period (p b 0.05).N = 5–10 except 8–15 weeks of exposure at 27°C for oysters whereN = 1–4.

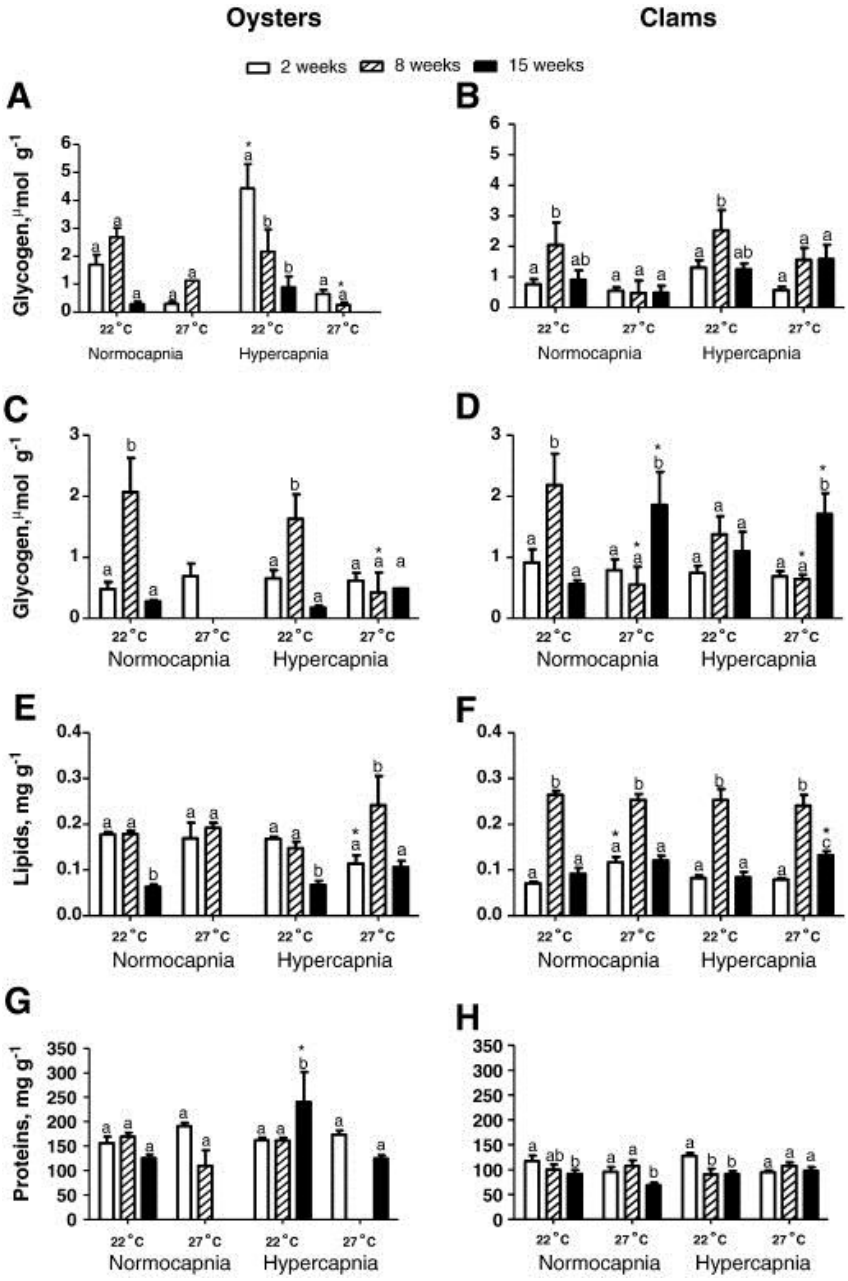


Figure.5.3: Tissues concentration of major energy reserves in oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperature and CO_2 levels. A,B – glycogen , C,D –glucose, E,F – lipids, G,H – proteins, A,C,E,G– *C. virginica*, B,D,F,H – *M. mercenaria*. X-axis – experimental conditions (temperature and CO_2 levels). Different letters indicate exposure times that are different within each experimental treatment group (p b 0.05). Asterisks indicate values that are significantly different from the control (normocapnia at 22°C) at the respective exposure period (p b 0.05). N = 5–10 except 8–15 weeks of exposure at 27°C for oysters where N = 1–4.

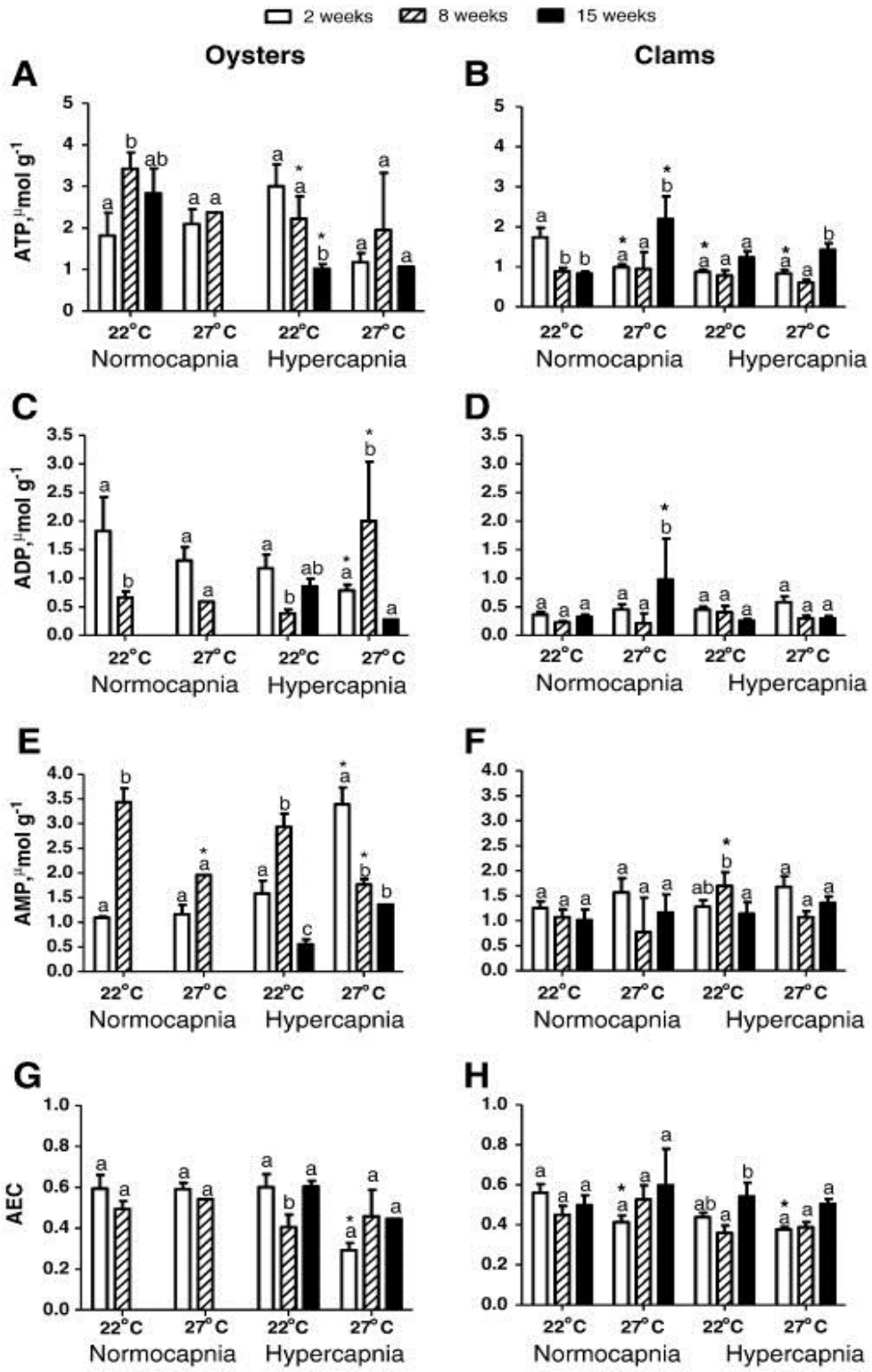


Figure.5.4: Tissues concentration of adenylates in oysters (*C. virginica*) and clams (*M. mercenaria*) exposed to different temperature and CO_2 levels. A, B –ATP, C, D – ADP, E, F –AMP, G, H –adenylate energy charge (AEC). A, C, E, G– *C. virginica*, B, D, F, H– *M. mercenaria*. X-axis–experimental conditions (temperature and CO_2 levels). Different

letters indicate exposure times that are different within each experimental treatment group (p b 0.05). Asterisk indicate values that are significantly different from the control (normocapnia at 22°C) at the respective exposure period (p b 0.05).N = 5–10 except 8–15 weeks of exposure at 27°C for oysters where N = 1–4.

Table 5.1: Summary of water chemistry parameters during experimental exposures. The exposure conditions are the same as in our companion study (Matoo et al., 2013). Salinity, temperature, pH, and dissolved inorganic carbon (DIC) were determined in samples from experimental tanks as described in Materials and Methods. Other parameters were calculated using co2sys software. Data are presented as means ± S.E.M. N = 22–27 for DIC and total alkalinity (TA), and 95–119 for other parameters.

	Exposure temperature			
	22 °C		**27 °C**	
	Normocapnia	**Hypercapnia**	**Normocapnia**	**Hypercapnia**
pH	8.14 ± 0.01	7.95 ± 0.01	8.08 ± 0.01	7.94 ± 0.02
Temperature (°C)	22.4 ± 0.1	22.2 ± 0.1	26.7 ± 0.1	26.7 ± 0.1
Salinity	30.4 ± 0.1	30.3 ± 0.1	30.4 ± 0.1	30.8 ± 0.1
P_{CO2} (µatm)	429.75 ± 16.60	744.49 ± 30.63	535.52 ± 26.57	796.37 ± 47.28
DIC (µmol kg^{-1} SW)	2439.28 ± 84.29	2554.09 ± 99.41	2571.64 ± 103.26	2549.93 ± 89.09
TA (µmol kg^{-1} SW)	2683.17 ± 100.04	2735.44 ± 115.21	2824.94 ± 119.37	2725.84 ± 97.70
Ω_{Cal}	6.33 ± 0.15	4.52 ± 0.15	6.50 ± 0.17	5.03 ± 0.16
Ω_{Arg}	4.10 ± 0.10	2.92 ± 0.10	4.26 ± 0.11	3.29 ± 0.11

Table 5.2: Activity, activation energy and Arrhenius breakpoint temperature (ABT) for carbonic anhydrase (CA) in different tissues of clams and oysters. Ea values highlighted in bold and marked with an asterisk are significant after the sequential Bonferroni corrections ($p < 0.05$). Q10 temperature coefficients were calculated for the complete range of the studied temperatures (5–35°C). Specific enzyme activities measured at 22 and 27°C are shown for the enzymes isolated from clams and oysters acclimated at 20°C and subjected to temperature rise in vitro; this was done to enable comparison of the acute response of CA to the temperature rise with the effects of the long-term acclimation to the respective temperatures (shown in Fig. 4.2). Different letters denote the specific activities and/or apparent Ea values that are significantly different between different tissues of the same species ($p < 0.05$). Comparisons of E_a between species for the respective tissues are given in the lower portion of the table; "=" sign indicates that the respective E_a values are not significantly different ($p > 0.05$), ">" or "<" signs indicate significant differences ($p < 0.05$). Cv – *C. virginica*, Mm – *M. mercenaria*

Tissue	**Carbonic anhydrase**					
		E_a, kJ mol^{-1} K^{-1}		Q_{10}	Specific activity, U g^{-1} protein	
	ABT, °C	Below ABT	Above ABT		22 °C	27 °C
C. virginica (eastern oysters)						
Mantle	N/a	**18.46*[a]**	–	1.32	10.05 ± 2.30[a]	11.64 ± 0.85 [a]
Gills	17.0	25.77[b]	− 8.73	1.01	15.15 ± 3.37[a]	12.67 ± 1.80 [a]
Hepatopancreas	N/a	**30.84*[b]**	–	1.62	49.53 ± 6.45[b]	54.67 ± 10.28 [b]
Muscle	23.5	− 13.96[a]	27.60	1.00	4.58 ± 1.34[a]	4.90 ± 0.93 [a]
M. mercenaria (hard shell clam)						
Mantle	N/a	**42.31*[a]**	–	1.78	18.92 ± 1.54 [a]	24.43 ± 2.22 [a]
Gills	N/a	**43.06*[a]**	–	1.69	15.93 ± 0.65 [a]	19.13 ± 1.09 [a]
Hepatopancreas	N/a	**39.65*[a]**	–	1.69	42.54 ± 3.99 [b]	49.80 ± 5.54 [b]
Muscle	N/a	**43.48*[a]**	–	1.78	14.37 ± 1.06 [a]	17.92 ± 1.27 [a]
Between-species comparisons of E_a					***Between-species comparisons of CA activity***	
Mantle	Cv < Mm ($p < 0.05$)				$p = 0.088$	$\mathbf{p = 0.015}$
Gills	Cv = Mm				$p = 0.880$	$p = 0.213$
Hepatopancreas	Cv = Mm				$p = 0.179$	$p = 0.348$
Muscle	Cv < Mm ($p < 0.05$)				$p = 0.060$	$\mathbf{p = 0.013}$

Supplementary Materials

Supplementary Table 1: ANOVA results testing for the effects of temperature, CO_2 levels and exposure time on the studied traits in *C.virginica* and *M.mercenaria*. *F*-values are given with degrees of freedom for the factor and the error in subscript. Significant values (p<0.05) are highlighted in bold. CA – carbonic anhydrase, AEC - adenylate energy change.

	Factors/interactions						
	P_{CO2}	Temperature (T)	Exposure time (Exp)	P_{CO2} x T	P_{CO2} x Exp	T x Exp	P_{CO2} x T x Exp
C.virginica							
Vickers microhardness	$F_{1,19}$=1.65 p=0.216	$F_{1,19}$=2.32 p=0.146	N/a	N/a	N/a	N/a	N/a
Crack radius	$F_{1,19}$=0.04 p=0.838	$F_{1,19}$=0.12 p=0.737	N/a	N/a	N/a	N/a	N/a
CA activity in mantle edge	$F_{1,77}$=3.74 p=0.057	**$F_{1,77}$=52.85 p<0.0001**	**$F_{2,77}$=19.73 p< 0.0001**	$F_{1,77}$=3.22 p=0.08	$F_{2,77}$=0.76 p=0.473	**$F_{2,77}$=16.75 p<0.0001**	$F_{1,77}$=0.17 p=0.684
CA activity in gills	**$F_{1,54}$=19.18 p< 0.0001**	**$F_{1,54}$=102.53 p<0.0001**	**$F_{2,54}$=3.41 p=0.042**	$F_{1,54}$=1.00 p=0.323	**$F_{2,54}$=4.28 p=0.020**	**$F_{2,54}$=20.20 p<0.0001**	**$F_{1,54}$=21.18 p<0.0001**
Glycogen	$F_{1,60}$=0.45 p=0.507	$F_{1,60}$=15.43 p=0.0003	**$F_{2,60}$=7.73 p=0.0012**	$F_{1,60}$=1.54 p=0.221	$F_{2,60}$=2.19 p=0.1228	$F_{1,60}$=0.61 p=0.439	$F_{1,60}$=0.83 p=0.357
D-glucose	$F_{1,59}$=0.38 p=0.543	$F_{1,59}$=0.27 p=0.607	**$F_{2,59}$=3.60 p=0.035**	$F_{1,59}$=0.18 p=0.669	$F_{2,59}$=0.57 p=0.568	$F_{2,59}$=1.55 p=0.222	N/a
Lipids	$F_{1,67}$=0.19 p=0.666	$F_{1,67}$=1.31 p=0.258	**$F_{2,67}$=15.78 p< 0.0001**	$F_{1,67}$=0.33 p=0.568	$F_{2,67}$=1.24 p=0.297	**$F_{2,67}$=4.48 p=0.016**	$F_{1,67}$=4.32 p=0.042
Proteins	$F_{1,60}$=0.92 p=0.343	$F_{1,60}$=3.70 p=0.060	$F_{2,60}$=0.93 p=0.4001	$F_{1,60}$=0.11 p=0.737	**$F_{2,60}$=3.38 p=0.046**	**$F_{2,60}$=3.60 p=0.034**	N/a
ATP	$F_{1,55}$=3.31 p=0.075	$F_{1,55}$=0.93 p=0.339	$F_{2,55}$=0.77 p=0.468	$F_{1,55}$=0.56 p=0.459	$F_{2,55}$=2.43 p=0.099	$F_{2,55}$=0.36 p=0.703	$F_{1,55}$=2.57 p=0.116
ADP	$F_{1,53}$=0.00 p=0.957	$F_{1,53}$=0.20 p=0.653	$F_{2,53}$=2.78 p=0.073	$F_{1,53}$=4.25 p=0.0504	**$F_{1,53}$=6.51 p=0.014**	**$F_{2,53}$=4.79 p=0.013**	$F_{1,53}$=3.00 p=0.0902
AMP	$F_{1,49}$=3.81 p=0.058	$F_{1,49}$=0.01 p=0.931	**$F_{2,49}$=10.34 p=0.0002**	$F_{1,49}$=3.94 p=0.054	**$F_{1,49}$=10.94 p=0.002**	**$F_{2,49}$=9.62 p=0.0004**	$F_{1,49}$=1.92 p=0.174
AEC	**$F_{1,49}$=5.42 p=0.025**	$F_{1,49}$=1.61 p=0.212	$F_{2,49}$=1.00 p=0.377	$F_{1,49}$=2.31 p=0.137	$F_{1,49}$=0.36 p=0.551	$F_{2,49}$=2.15 p=0.129	$F_{1,49}$=2.40 p=0.129
ADP/ATP	$F_{1,52}$=0.11 p=0.745	$F_{1,52}$=0.17 p=0.685	$F_{2,52}$=2.38 p=0.104	**$F_{1,52}$=5.58 p=0.023**	**$F_{1,52}$=4.73 p=0.035**	**$F_{2,52}$=3.33 p=0.045**	$F_{1,52}$=0.05 p=0.816

Supplementary Table 1 continued							
∑ adenylates	$F_{1,49}$=0.00 p=0.988	$F_{1,49}$=0.34 p=0.564	**$F_{2,49}$=6.23 p=0.004**	$F_{1,49}$=1.90 p=0.176	$F_{1,49}$=2.60 p=0.115	$F_{2,49}$=0.32 p=0.729	$F_{1,49}$=2.58 p=0.116
M. mercenaria							
	P_{CO2}	Temperature (T)	Exposure time (Exp)	P_{CO2} x T	P_{CO2} x Exp	T x Exp	P_{CO2} x T x Exp
Vickers microhardness	$F_{1,39}$=2.25 p=0.142	$F_{1,39}$=2.97 p=0.094	N/a	$F_{1,39}$=2.71 p=0.108	N/a	N/a	N/a
Crack radius	$F_{1,39}$=0.79 p=0.379	$F_{1,39}$=1.19 p=0.283	N/a	$F_{1,39}$=0.07 p=0.799	N/a	N/a	N/a
CA activity in mantle edge	$F_{1,98}$=1.46 p=0.229	**$F_{1,98}$=30.32 p< 0.0001**	**$F_{2,98}$=3.64 p=0.0304**	$F_{1,98}$=0.38 p=0.538	$F_{2,98}$=2.65 p=0.076	**$F_{2,98}$=3.79 p=0.026**	$F_{2,98}$=10.91 **p<0.0001**
CA activity in gills	$F_{1,94}$=0.14 p=0.706	**$F_{1,94}$=34.53 p< 0.0001**	$F_{2,94}$=1.47 p=0.236	$F_{1,54}$=0.24 p=0.626	**$F_{2,94}$=3.32 p=0.041**	**$F_{2,94}$=14.72 p<0.0001**	**$F_{2,94}$=9.67 p=0.0002**
Glycogen	**$F_{1,81}$=4.43 p=0.039**	**$F_{1,81}$=4.41 p=0.039**	$F_{2,81}$=3.06 p=0.0534	$F_{1,81}$=0.24 p=0.629	$F_{2,81}$=0.35 p=0.709	$F_{2,81}$=1.41 p=0.252	$F_{2,81}$=0.61 p=0.548
D-glucose	$F_{1,85}$=0.26 p=0.613	$F_{1,85}$=0.29 p=0.592	$F_{2,85}$=2.93 p=0.059	$F_{1,85}$=0.06 p=0.812	$F_{2,85}$=0.64 p=0.531	**$F_{2,85}$=9.14 p=0.0003**	$F_{2,85}$=1.27 p=0.288
Lipids	$F_{1,104}$=1.15 p=0.287	**$F_{1,104}$=5.35 p=0.023**	**$F_{2,104}$=209.41 p< 0.0001**	$F_{1,104}$=0.59 p=0.444	$F_{2,104}$=0.47 p=0.624	**$F_{2,104}$=4.35 p=0.016**	$F_{2,104}$=2.18 p=0.119
Proteins	$F_{1,100}$=0.91 p=0.344	$F_{1,100}$=2.13 p=0.148	**$F_{2,100}$=5.95 p=0.004**	$F_{1,100}$=0.84 p=0.363	$F_{2,100}$=1.11 p=0.335	**$F_{2,100}$=4.90 p=0.009**	$F_{2,100}$=1.34 p=0.268
ATP	**$F_{1,95}$=5.77 p=0.019**	$F_{1,95}$=0.72 p=0.398	**$F_{2,95}$=7.39 p=0.001**	$F_{1,95}$=0.85 p=0.359	$F_{2,95}$=0.73 p=0.487	**$F_{2,95}$=8.65 p=0.0004**	**$F_{2,95}$=5.37 p=0.006**
ADP	$F_{1,84}$=0.50 p=0.483	**$F_{1,84}$=4.06 p=0.048**	$F_{2,84}$=2.83 p=0.066	$F_{1,84}$=2.97 p=0.089	**$F_{2,84}$=6.66 p=0.002**	$F_{2,84}$=3.05 p=0.053	$F_{2,84}$=2.53 p=0.086
AMP	$F_{1,85}$=2.76 p=0.101	$F_{1,85}$=0.03 p=0.858	$F_{2,85}$=2.09 p=0.131	$F_{1,85}$=0.04 p=0.849	$F_{2,85}$=0.65 p=0.525	$F_{2,85}$=2.91 p=0.061	$F_{2,85}$=0.20 p=0.821
AEC	**$F_{1,78}$=4.57 p=0.036**	$F_{1,78}$=0.05 p=0.832	**$F_{2,78}$=3.86 p=0.026**	$F_{1,78}$=0.22 p=0.641	$F_{2,78}$=0.56 p=0.571	$F_{2,78}$=2.12 p=0.128	$F_{2,78}$=1.00 p=0.375
ADP/ATP	**$F_{1,80}$=6.73 p=0.012**	$F_{1,80}$=0.78 p=0.3801	**$F_{2,80}$=4.32 p=0.017**	$F_{1,80}$=0.00 p=0.959	**$F_{2,80}$=4.05 p=0.022**	$F_{2,80}$=2.56 p=0.085	$F_{2,80}$=0.72 p=0.489
∑ adenylates	$F_{1,78}$=0.69 p=0.410	$F_{1,78}$=2.25 p=0.138	**$F_{2,78}$=7.33 p=0.001**	$F_{1,78}$=3.02 p=0.087	$F_{2,78}$=2.37 p=0.101	**$F_{2,78}$=10.37 p=0.0001**	**$F_{2,78}$=4.37 p=0.016**

Supplementary Table 2: Pearson correlation coefficients between the experimental conditions and the studied physiological and biochemical parameters of oysters and clams**.**

Species were designated as 1 (*C. virginica*) or 2 (*M.mercenaria*). Time – exposure time, Temp – temperature, Mort – mortality, CAmant and CAgill – CA activity in the mantle and gill, respectively, Σ aden – total adenylate concentrations in the gill tissues, MDA – concentrations of malondialdehyde-protein conjugates in the muscle tissues, HNE - concentrations of 4-hydroxynonenal-protein conjugates in the muscle tissues, carbonyls – concentrations of protein carbonyls in the muscle tissues, MO2 – standard metabolic rates (measured as the whole-organism oxygen consumption). The data were combined from the present study and our companion paper (Matoo *et al.*, 2013). Significant correlations ($p<0.05$) are highlighted in bold and marked with asterisks.

	Species	Time	Temp	PCO2	pH	Ω_{Cal}	Ω_{arg}	Mort	CAmant	CAgill	Glycogen	Glucose	Lipids	Proteins
Time	0.000													
Temp	0.000	0.000												
PCO2	0.000	0.000	-0.023											
pH	0.000	0.000	-0.180	**-0.968***										
Ω_{Cal}	0.000	0.000	0.226	**-0.974***	**0.916***									
Ω_{arg}	0.000	0.000	0.262	**-0.967***	**0.900***	**0.999***								
Mort	**-0.482***	0.377	**0.531***	-0.015	-0.095	0.122	0.141							
CAmant	0.377	0.235	**0.614***	-0.040	-0.085	0.164	0.186	0.205						
CAgill	-0.024	-0.033	**0.668***	-0.109	-0.027	0.244	0.268	**0.460***	**0.597***					
Glycogen	-0.140	-0.144	**-0.507***	0.272	-0.175	-0.373	-0.389	-0.181	-0.368	-0.319				
Glucose	0.294	0.128	-0.141	-0.157	0.171	0.121	0.116	-0.175	0.040	-0.032	0.378			
Lipids	0.010	-0.172	0.178	-0.086	0.042	0.117	0.123	0.149	0.057	0.144	0.309	0.286		
Proteins	**-0.763***	-0.204	-0.238	0.156	-0.116	-0.206	-0.213	0.107	**-0.528***	-0.288	0.145	-0.312	-0.133	
ATP	**-0.620***	-0.026	-0.184	-0.306	0.325	0.257	0.248	0.128	-0.324	-0.088	0.304	0.207	-0.017	0.305
ADP	**-0.587***	-0.300	0.080	-0.049	0.039	0.069	0.071	0.082	-0.234	0.261	-0.056	-0.318	0.156	0.408
AMP	**-0.443***	-0.267	0.012	0.134	-0.108	-0.111	-0.111	0.098	**-0.425***	-0.126	0.246	0.337	0.032	0.221
AEC	-0.180	0.208	-0.211	**-0.427***	0.416	0.342	0.333	-0.005	0.082	0.059	0.050	-0.062	-0.134	0.220
ADP/ATP	-0.321	-0.413	0.086	0.153	-0.139	-0.113	-0.110	-0.066	-0.190	0.245	-0.165	**-0.567***	0.180	0.303

Σ aden	**-0.654***	-0.245	0.027	-0.108	0.105	0.113	0.113	0.149	-0.357	0.076	0.253	0.267	0.086	0.342
	Species	Time	Temp	PCO2	pH	Ω_{Cal}	Ω_{arg}	Mort	CAmant	CAgill	Glycogen	Glucose	Lipids	Proteins
MO2	**-0.874***	-0.065	0.224	0.141	-0.172	-0.086	-0.077	**0.463***	-0.152	0.218	-0.062	**-0.522***	-0.088	**0.651***
TAOC	0.398	0.135	0.060	-0.116	0.121	0.139	0.140	-0.071	0.138	-0.099	0.154	**0.529***	**0.478***	**-0.464***
Carbonyls	-0.008	-0.276	-0.127	-0.056	0.083	0.031	0.025	-0.273	0.110	0.008	**0.486***	0.185	0.246	0.043
MDA	**-0.884***	-0.044	-0.170	0.018	-0.005	-0.066	-0.072	0.299	-0.373	-0.048	0.341	-0.171	-0.031	**0.647***
HNE	-0.066	-0.058	-0.093	-0.173	0.132	0.115	0.113	0.178	-0.179	-0.047	0.384	**0.489***	0.316	-0.039

Supplementary Table 2 (continued)

	ATP	ADP	AMP	AEC	ADP/ATP	Σ aden	MO2	TAOC	Carbonyls	MDA
ADP	**0.452***									
AMP	**0.479***	0.042								
AEC	**0.464***	0.345	**-0.496***							
ADP/ATP	-0.074	**0.802***	-0.095	-0.013						
Σ aden	**0.883***	**0.578***	**0.719***	0.123	0.149					
MO2	**0.429***	**0.637***	0.230	0.147	**0.467***	**0.481***				
TAOC	-0.128	-0.323	0.170	-0.280	-0.365	-0.008	**-0.551***			
Carbonyls	0.136	0.156	-0.138	0.228	0.007	0.096	0.102	0.097		
MDA	**0.735***	**0.489***	0.405	0.295	0.132	**0.684***	**0.784***	-0.335	0.249	
HNE	0.378	-0.150	0.247	0.122	-0.411	0.313	-0.160	0.273	0.122	0.332

Supplementary Table 3: Principal component analysis of the studied physiological and biochemical parameters of oysters and clams and the respective experimental conditions.

Eigenvalues and % of variance are given for the first 14 principal components that explain 100% of the variance in the data set, as well as loadings of the studied parameters on the first 8 principal components. Loadings exceeding 0.20 (both positive and negative) are highlighted in bold. Time – exposure time, Temp – temperature, Mort – mortality, CAmant and CAgill – CA activity in the mantle and gill, respectively, Σ aden – total adenylate concentrations in the gill tissues, MDA – concentrations of malondialdehyde-protein conjugates in the muscle tissues, HNE - concentrations of 4-hydroxynonenal-protein conjugates in the muscle tissues, carbonyls – concentrations of protein carbonyls in the muscle tissues, MO2 – standard metabolic rates (measured as the whole-organism oxygen consumption).

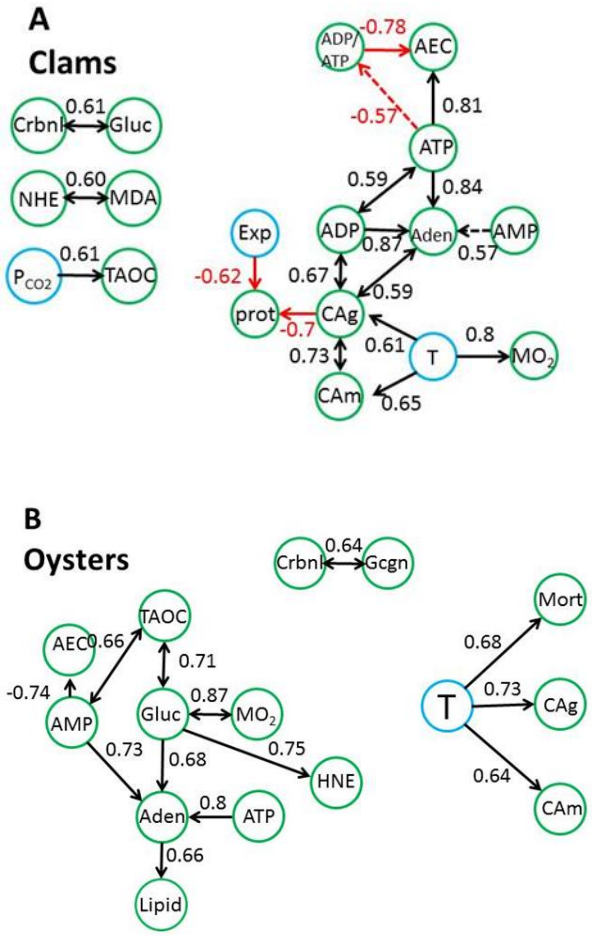
Principal component (PC)	Eigenvalue	Percentage of Variance	Cumulative
1	4.89	24%	24%
2	3.74	19%	43%
3	2.74	14%	57%
4	2.05	10%	67%
5	1.71	9%	76%
6	1.29	6%	82%
7	0.98	5%	87%
8	0.64	3%	90%
9	0.47	2%	93%
10	0.40	2%	95%
11	0.37	2%	96%
12	0.26	1%	98%
13	0.19	1%	99%
14	0.16	1%	100%

Supplementary Table 3 continued

	Loadings							
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Species	**-0.39**	0.05	-0.07	0.02	0.15	-0.10	0.10	-0.02
Time	-0.09	-0.03	0.13	**-0.38**	**-0.33**	**0.22**	**-0.22**	**0.53**
Temp	-0.05	**-0.28**	**0.41**	0.18	-0.03	-0.01	0.12	-0.13
pCO2	0.01	-0.05	-0.18	**0.32**	-0.19	**0.48**	**0.22**	**0.28**
Mort	0.11	-0.17	**0.39**	-0.05	**-0.29**	**0.25**	**-0.25**	-0.13
CAmant	**-0.21**	**-0.21**	**0.31**	-0.10	0.12	0.16	**0.32**	0.09
CAgill	-0.01	**-0.26**	**0.40**	0.06	0.17	0.00	**0.20**	-0.02
Glycogen	0.09	**0.36**	-0.09	0.06	0.14	**0.42**	0.06	0.08
Glucose	-0.11	**0.39**	**0.22**	0.00	0.03	-0.08	0.13	**0.20**
Lipids	-0.01	0.12	**0.24**	0.27	**0.32**	**0.22**	-0.52	-0.01
Proteins	**0.31**	-0.03	**-0.21**	-0.02	-0.10	0.13	-0.10	**-0.20**
ATP	**0.30**	**0.21**	0.16	-0.20	0.01	**-0.21**	0.12	0.15
ADP	**0.30**	-0.16	0.02	0.07	**0.32**	-0.16	-0.13	**0.29**
AMP	0.18	**0.24**	0.12	**0.36**	**-0.29**	-0.21	0.20	0.04
AEC	0.11	-0.02	0.01	**-0.57**	**0.27**	-0.02	-0.05	0.06
ADP/ATP	0.17	**-0.28**	-0.14	**0.27**	**0.30**	-0.09	**-0.22**	0.17
Σ aden	**0.32**	0.18	**0.21**	0.10	0.01	**-0.26**	0.16	**0.21**
MO2	**0.35**	**-0.21**	0.06	0.02	-0.06	0.17	0.08	0.01
TAOC	-0.19	**0.26**	**0.21**	0.17	0.05	-0.01	**-0.27**	**0.26**
Carbonyls	0.05	0.14	0.00	-0.04	**0.47**	**0.35**	**0.34**	-0.04
MDA	**0.38**	0.08	0.06	-0.11	-0.06	0.18	0.07	-0.08
HNE	0.05	**0.33**	**0.24**	-0.08	0.00	0.09	-0.14	**-0.49**

Supplementary Figure 1: A matrix showing significant Pearson correlation coefficients between different studied parameters in clams (A) and oysters (B).

Black and red connector lines represent relationships with positive and negative correlations, respectively. Solid lines represent significant correlations (based on Pearson correlation analysis, $p<0.05$). Dashed lines represent marginally significant relationships ($p<0.10$). Arrows identify putative causality and numbers by the connector lines are Pearson correlation coefficients (R). Abbreviations: Crbnl – carbonyls, Gluc – glucose, Aden – total adenylate content, Exp – exposure duration, prot – protein content, Cag and Cam – CA activitiy in gills and mantle, respectively, T – temperature, Gcgn – glycogen, Mort – mortality.



CHAPTER 6: SUMMARY

The results of my dissertation research significantly contribute to our understanding on the interactive effects of ocean acidification with multiple stressors on the physiology of marine mollusks. Our studies demonstrated that elevated P_{CO2} either alone or in combination with other stressors such as elevated temperature and reduced salinity have negative effects on the survival, growth bioenergetics and calcification of juvenile and adult life stages of the eastern oyster, *Crassostrea virginica* and the hard shell clam, *Mercenaria mercenaria*. We also found that within each species, earlier life stages (juveniles) were more sensitive to the impacts of ocean acidification, and that the two studied species of bivalves considerably differed in their responses to ocean acidification, salinity and temperature stress indicating that they will differ in their susceptibility to the global change in marine environments.

5.1. Differential Sensitivity of Selected Bivalves to Elevated CO_2 across Life Stages

The sensitivity in oysters and hard clams to elevated P_{CO2} (hypercapnia) were dependent on the life cycle stage. Hypercapnia reduced the survival of juvenile clams and oysters whereas the survival of adults was largely unaffected. In juvenile oysters, reduction in tissue growth and a partial depletion of energy reserves was observed under the hypercapnic conditions, while in the adult bivalves hypercapnia had only mild effects on growth and metabolism. These findings are consistent with the results of earlier studies showing that early life stages of marine mollusks are more sensitive to elevated

P_{CO2} than adults (Kurihara 2008; Kroeker et al 2010, 2013;Beniash et al 2010; Green et al 2009; Gazeau et al 2007; Shirayama et al., 2005). The higher sensitivity of early life stages to elevated P_{CO2} may reflect the underdeveloped mechanisms of ion and acid-base regulation (Melzner et al., 2009), higher metabolic rates and thus higher energy costs of basal maintenance (Sokolova et al., 2011; Sokolova et al., 2012). In the case of the larvae, higher susceptibility to the ocean acidification may be also due to the presence of a more soluble polymorph of $CaCO_3$ (amorphous calcium carbonate) in larval shells (Weiss et al., 2002; Ross et al., 2011). In juveniles, elevated sensitivity to hypercapnic stress may also reflect limited energy reserves and competing demands of development versus basal maintenance, in agreement with the energy-limited concept of tolerance to environmental stress (Sokolova et al., 2012).

In clams, the mechanical properties of shells were also differently affected by hypercapnia in juveniles and adults. In juvenile clams, moderate hypercapnia stimulated the shell growth but reduced the mechanical properties (microhardness) of the shells. Similarly, reduced hardness of the shells has been reported earlier in juvenile oysters exposed to extreme hypercapnia (~ 3500µatm) (Beniash et al., 2010). In contrast, hypercapnia did not affect the shell mechanical properties of adult clams. It is possible that juvenile clams have poorly developed protective periostracum compared to the adults that makes juvenile shells prone to corrosion under hypercapnia. Another explanation for the higher susceptibility of juvenile shells to elevated P_{CO2} may be the ongoing changes in the structural organization of organic and mineral components and their relative proportions in the rapidly glowing shells of juveniles in contrast to the shells of the adults that are fully formed and grow more slowly. Elevated P_{CO2} may therefore change

ultrastructure of the rapidly growing juvenile shells (Welladsen et al 2010; Talmage and Gobler, 2010), which in turn can affect their mechanical properties. Juveniles with weakened shells in the environment are more susceptible to predators and mechanical damage that may result in increased mortality. Higher susceptibility of early life stages like larvae and juveniles to elevated P_{CO2} may result in changes in the abundance and distribution of the population.

5.2. Temperature Effects and Interaction with Elevated P_{CO2}

Elevated temperature exerted a much greater effect on the bioenergetics, oxidative stress and survival in the two studied bivalve species compared to moderate hypercapnia. In adult clams and oysters, elevated temperature induced an increase in the basal metabolism. Temperature had an especially strong impact on oysters where prolonged exposure to elevated temperature led to significant mortalities and a depletion of energy reserves. This is consistent with the results of an earlier study showing that oysters have increased basal metabolism, lose the aerobic scope for growth and cannot deposit shells at and above 28°C (Surge et al, 2001; Lannig et al., 2006). Other studies have also shown predominant effects of temperature on metabolic physiology and survival in different marine invertebrates under different P_{CO2}/temperature combinations (Lannig et al., 2010; Chapman et al., 2011; McElroy et al., 2012).

Temperature is an important abiotic factor affecting physiology of ectotherms including bivalves. It affects rates of all biochemical and physiological processes and stability of biomolecules (Hochacka and Somero, 2002). Deviation of temperature from the population's evolutionary optimum results in disturbance of energy homeostasis and oxidative stress (Abele et al., 2001; Pörtner and Lannig, 2009; Wittmann et al., 2008). A

fundamental physiological concept explaining the thermal stress response in aquatic organisms is the concept of oxygen- and capacity-limited thermal tolerance (OCLTT) (Pörtner 2001, 2002). According to this model, a positive aerobic scope (the fraction of energy available for fitness-related functions after the basal maintenance costs are met) is critical in the long-term survival of populations. In ectotherms, deviations from the optimum temperature results in diminishing aerobic scope due to the imbalance between the ventalitory/circulatory capacity, ATP supply and basal maintenance costs (Pörtner 2001, 2002). Zero and/or negative aerobic scope characterized by the partial transition into anaerobiosis leaves the organism with no energy supply for processes such as growth and reproduction. This is bioenergetically unsustainable situation that eventually leads to mortality and affect persistence of the population. Decreased survival, performance and reproduction in population can also lead to changes in trophic interactions and geographical distribution during the climate change (Childress and Seibel, 1998; Pörtner et al., 2001, 2008; Edwards and Richardson, 2004; Perry et al., 2005; Pörtner and Farrell, 2008).

Temperature and elevated P_{CO2} also interact in a complex way and affect different physiological processes antagonistically or synergistically in the studied bivalves. In adult oysters, the negative effects of elevated temperature on glycogen reserves and survival were partially offset by hypercapnia. Hypercapnia induced an increase in tissue glycogen content, an effect opposite to that of the elevated temperature alone. This may have contributed to better survival of hypercapnic oysters at elevated temperature compared to their normocapnic counterparts exposed to the elevated temperature. Modulation of metabolic responses to elevated temperature by P_{CO2} has also been

reported in other ectotherms including other bivalve species and echinoderms where elevated P_{CO2} diminished the effects of temperature of oxygen consumption rate and arm regeneration,(Lannig et al., 2010; Wood et al., 2011; Catarino et al., 2012; McElroy et al., 2012) . On the other hand, elevated temperature enhanced the negative effects of elevated CO_2 on biomineralization in adult clams and oysters leading to decrease in shell mechanical properties and suggesting a synergistic relationship between the two factors. Most of the studies show a synergistic effect of elevated temperature and P_{CO2} on a number of physiological process including fertilization, embryonic development, survival and calcification in mollusks (Rosa et al., 2008; Lischka et al., 2010; Parker et al., 2012) as well as in invertebrates including corals and echinoderms (Anthony et al., 2008; Gooding et al., 2009; Byrne et al., 2009). It has been proposed that the thermal window of the organism determines the interactive effects between elevated temperature and P_{CO2} (Pörtner and Farrell, 2008). Elevated temperature may exacerbate the negative effects of elevated P_{CO2} if the thermal window is exceeded. Conversely, if the thermal window is not exceeded elevated temperature can mitigate or suppress the negative effects of elevated P_{CO2} . Hard shell clams have a broader performance window than the eastern oysters. Activity of the adult clams have a greater thermal optimum of 21 to 31°C whereas for the adult oysters the optimum lies between 24 to 26 °C (Loosanoff, 1958; Tenore et al., 1973). The negative impact of elevated CO_2 may be small in the thermal optimum range but the effects become stronger as thermal optimum limits are reached. In the case of our selected species, the thermal optimum limit is lower for oysters than clams and may partially explain the greater sensitivity of oysters to temperature and hypercapnia. In addition, the temperature optima of different physiological processes also

vary due differential Q_{10} effect of temperature on reaction rates and enzyme kinetics. Shell growth optimum for oysters and clams are around 20-25°C (Kraeuter and Castagna, 2001; Shumway, 1996 as cited in Kennedy, 1996). In our study, elevated temperature (27°C) was outside the calcification optima for both the species. The presence of elevated CO_2 as an additional stressor acts synergistically and further reduces this physiological window resulting in reduced mechanical shell properties in bivalves exposed together to hypercapnia and elevated temperature.

5.3. Salinity Effects and Interactions with Elevated P_{CO2}

Reduced salinity had a strong effect on the physiology of juvenile clams and oysters leading to elevated basal maintenance costs, reduced soft tissue growth, lower levels of energy reserves, decreased activity of a key biomineralization enzyme, carbonic anhydrase, reduced growth and increased mortality . For estuarine organisms including clams and oysters, salinity is an important factor that can profoundly affect physiology and limit population distribution (Gunter 1961). When salinity deviates from the optimum in dynamic estuarine environments, metabolic adjustments play a key role in the survival of organisms (Sokolova et al., 2000). In osmoconformers like estuarine bivalves, hypoosmotic stress can incur shifts in energy allocation and elevated costs of water and ion homeostasis, cell volume regulation and/or damage and repair (Hilbish, 1982; Nelson et al., 1996; Hochachka and Somero, 2002; Sokolova et al., 2012). Elevated basal maintenance costs results in reduced aerobic scope (Pörtner 2001, 2002). According to the energy-limited concept of stress tolerance, reduced aerobic scope with depletion of energy reserves may be a sign of transition into bioenergitically unsustainable conditions

and has important fitness-related implications including reduced growth and survival (Sokolova, 2012).

Salinity also strongly modulates the effects of hypercapnia on the bioenergetics and calcification in juvenile clams and oysters. Combined exposure to low salinity and hypercapnia led to further reduction of energy reserves and altered the mechanical properties of the newly deposited shells in oysters. Moderate hypercapnia (~800 µatm) stimulated shell and tissue growth and reduced mortality in juvenile clams; however, this effect was abolished during exposure to low salinity. Combined exposure to reduced salinity and hypercapnia negatively affected the shell mechanical properties (microhardness and fracture toughness) and altered the shell ultrastructure in juvenile clams. Elevated P_{CO2} lowers the saturation state (Ω) of carbonate minerals used in shell formation and this decrease is further compounded by reduced salinity. This may explain our finding that etching and pitting of the shell surface and corrosion of the hinge regions was most severe and extensive in clams simultaneously exposed to hypercapnia and reduced salinity. Previous studies have also shown altered calcification rates in many marine species including corals, coccolithophores, foraminifera and pteropods under undersaturated carbonate concentration ($\Omega < 1$) (Fabry et al., 2008). Weakened shells of juveniles make these bivalves more prone to parasites, predation and mechanical damage in their natural estuarine habitats where both chronically reduced salinities and large salinity fluctuations are seen. This in turn can have consequences for the survival of the population.

5.4. Species-specific Responses to Temperature and CO_2 levels

The effects of elevated temperature and P_{CO2} on the survival, energy metabolism and biomineralization differed between clams and oysters indicating differential sensitivities of these species to ocean acidification and global change in the estuaries. Oysters showed more pronounced changes in survival and bioenergetics in response to elevated temperature and P_{CO2} . Prolonged exposure to elevated temperature resulted in decreased survival and reduction in tissue energy reserves of oysters. In contrast, clams were less sensitive and showed no changes in tissue and cellular energy reserves under the conditions of elevated temperature and P_{CO2}. Standard metabolic rate (SMR) of adult clams was 2-4 times lower than in oysters and increased in response to prolonged acclimation to higher temperature, while in oysters SMR did not change during the acclimation to elevated temperature (27°C). Earlier studies have also shown that oysters and clams strongly differ in the rates as well as temperature sensitivity of basal metabolism (Shumway and Koehn, 1982 as cited in Kennedy 1996; Kraeuter and Castagna, 2001). Mechanical testing (microhardness and fracture resistance) also showed that aragonitic shells of adult clams are stronger compared to the calcitic shells of adult oysters and less prone to the negative effects of hypercapnia and elevated temperature.

Species- specific differences in responses to ocean acidification found in our study and in many other species (Fabry et al., 2008; Melzner et al., 2009; Reis et al., 2009) can be explained in terms of the local adaptations to their habitat, inherent physiological and phenotypic plasticity of the species (Melzner et al., 2009; Hoffmann et al., 2010). Many species occupy environmental niches that are naturally hypercapnic at least during a part of the life cycle. This may result in adaptation to elevated P_{CO2} and

make these species more resilient to future ocean acidification. In this context, it is important to note that oysters are epifaunal intertidal species and typically do not experience extreme environmental hypercapnia during and after settlement. Settlement occurs on previous oyster beds and other suitable substrates with biofilms (Kennedy, 1996) and can be reversed in the absence of the favorable physical conditions (salinity, temperature, oxygen) or appropriate chemical cues. Once settled, the spat firmly attaches to the substrate and cannot move for the rest of the life. In contrast, clams are infaunal species buried deep in sandy and muddy sediments, with living specimens taken from depths as great as 12 m (Kraeuter and Castagna, 2001). Bacterial respiration and organic matter degradation in upper sediment layers produces significant amounts of CO_2 and other metabolic acids. These conditions lower the pH , make the sediments corrosive and undersaturated with respect to aragonite and calcite (Aller et al., 1982; Green and Aller, 1998, 2001, Green et al., 2009; Waldbusser et al., 2011) compared to the overlaying water. Post-larval clams actively borrow the sediment and choose sites based on the properties of the sediment-water interface and the particle size and chemical composition of the sediment (Kraeuter and Castagna, 2001). Initial burrowing is reversible and if the conditions turn unfavorable secondary relocation is possible for a short window of time (Kraeuter and Castagna, 2001; Green et al., 2013). However, after this the settled clam juveniles are confined in the sediment layers where, unlike oysters, they are subjected to extreme environmental hypercapnia. Significant size-dependent mortalities in newly settled cohorts of clams due to dissolution in the corrosive sediments have been reported (Green et al., 2009).

One of the evolutionary adaptations of clams to the natural hypercapnia of their habitats is the development of a thicker proteinous covering (periostracum) covering their shell surface compared to oysters. This may partially explain the resilience of clam biomineralization to elevated P_{CO2} found in our study that can make them more tolerant to future acidification conditions than oysters. Earlier studies of different mussel species from volcanic vents and fjords have also shown successful survival and high tolerance of elevated P_{CO2} in the species from the naturally acidified environments, which was associated with the presence of a thick periostracum and physiological adaptations to handle high H^+ load (Tunnicliffe et al 2009; Thomsen et al 2010). Our study suggests that some estuarine species (especially sediment dwelling) may also be pre-adapted to better tolerate the future ocean acidification conditions; however, the tolerance may be limited by additional stressors especially low salinity.

5.5 Conclusions and Perspectives

Ocean acidification is an urgent problem that strongly affects marine ecosystems. The present dissertation demonstrates that two ecologically and economically important marine bivalve species will experience a reduction in fitness during the juvenile and adult life cycle-stages as shown by the reduced survival, growth, impaired metabolism and calcification in response to ocean acidification, either alone or in combination with temperature and salinity stress. Several meta-analyses of literature on ocean acidification also came to similar conclusions concerning a range of marine calcifying taxa including mollusks (Dupont et al., 2010b; Kroeker et al., 2010; Chan and Connolly, 2013; Harvey et al., 2013; Kroeker et al., 2013).

The results of the present study show the importance of considering the complex, non-linear interactions between the multiple drivers of global change on the physiology of marine organisms. Understanding the dynamics and outcome of the effects of multiple stressors in the environmentally realistic settings is complicated due to the potential non-linear and context-specific interactions among stressors, variable frequency and intensity of the stressors as well as their pattern of temporal occurrence (simultaneous vs. consecutive) as well as the effects of the species' evolutionary or ecologically derived tolerance and interactions between species in an ecosystem (Crain et al., 2008). Taking into account the factorial combination and direction of each individual stressor, the outcome of interactions between the stressors vary but conceptually falls into one of the three following categories:

1. Additive, where the magnitude of cumulative effect of the two or more stressors acting together is equal to the sum of the individual stressors,
2. Antagonistic, where the magnitude of cumulative effect of the two or more stressors acting together is less than the sum of the individual stressors, or
3. Synergistic, where the magnitude of cumulative effect of the two or more stressors acting together is greater than the sum of the individual stressors.

The present study also showed the patterns of interaction between elevated P_{CO2}, temperature and reduced salinity on the survival, metabolism and biomineralization in the two bivalve species. In adult oysters, higher mortality at 27°C was partially alleviated by hypercapnia suggesting an antagonistic relationship. In both oysters and clams, elevated temperature and hypercapnia act antagonistically and lower oxidative damage after prolonged exposure of 15 weeks. For some other physiological traits measured though,

the relationship between P_{CO2} and temperature/ salinity follows synergism. In adult clams, elevated temperature and hypercapnia increases the standard metabolic rate compared to either elevated temperature or hypercapnia alone. Negative synergistic effects of are P_{CO2} with salinity/temperature are especially pronounced for biomineralization in both juvenile and adult clams and oysters. Extensive itching and pitting and reduced mechanical properties of shells (microhardness and fracture resistance) is seen in juvenile and adult bivalves. This provides important physiological and ecological context to the present study. Most of the research on ocean acidification till date, however, are focused on single factor, single species studies with a relatively short time (hours to days) of exposure. Only small number of articles has focused on more than one life-cycle stage, including carry-over effects from one life-cycle stage to the next (Dupont et al., 2012; Troedsson et al., 2012; Parker et al., 2012) . In nature, ocean acidification co-occurs with other natural and anthropogenic stressors like warming, eutrophication, hypoxia and pollution. The interactive effects of multiple stressors may significantly differ from the effects of a single stressor which limits our current ability to make reliable predictions of the consequences of ocean acidification in most marine environments (where temperature and seawater pH will change simultaneously) and more specifically, in the coastal and estuarine habitats that experience large natural variations in many key physico-chemical factors including temperature, salinity, pH, and oxygen concentration. To fully understand the consequences and close critical knowledge gaps, research in ocean acidification needs to move beyond mere cataloging and simplistic experimental designs. Multi-stressor, multi-factorial complex experimental designs on longer time scales taking multi-generational

approach across taxa and species from geographically distinct locations are needed. This will help gain mechanistic insights into physiological responses, identify susceptible species, access evolutionary potential to cope and unravel ecological interactions.

Ocean acidification studies are still in infancy but the threat is real and has potential for significant ecological and economic impacts. Marine ecosystem services are estimated to be US$21 trillion per annum, a staggering 63% of the total value of earth's ecosystem services (US$33 trillion) (Costanza et al., 1997). Researchers need to streamline and collaborate to communicate the concerns to media, general public and stakeholders at the national, international and intergovernmental levels to ensure effective change. In the 15th Conference of Parties at Copenhagen (COP15) to UN Framework Convention on Climate Change (UNFCC) in December 2009, no consensus was reached to reduce CO_2 emissions. Ocean acidification and climate change and has the same cause-increasing anthropogenic CO_2 emissions. Both of these global issues have solutions that are possible only through active action on the part of individuals as members of a responsible society, political will and global actions.

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