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An investigation of the sublethal effects of carbon dioxide on the common sea urchin Lytechinus variegatus and the carbonate chemistry of its nearshore habitat

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AN INVESTIGATION OF THE SUBLETHAL EFFECTS OF CARBON DIOXIDE ON THE COMMON SEA URCHIN *LYTECHINUS VARIEGATUS* AND OF THE CARBONATE CHEMISTRY OF ITS NEARSHORE HABITAT

by

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ABSTRACT

Increases in CO_2 concentrations, whether from accumulation of metabolic waste under intensive culturing conditions or from increases in atmospheric levels, lead to decreases in seawater pH and carbonate saturation states. *Lytechinus variegatus* survive chronic exposure to hypercapnic conditions (pH 7.4 -7.8, partial pressure of CO_2 (pCO_2) 1738-4290 μatm) in synthetic seawater, yet aspects of growth and development are affected. Rates of embryonic and larval development were delayed and larval size and arm length reduced under high pCO_2 conditions. Fecal production rates were higher and ash absorption efficiency (%) was lower in individuals exposed to hypercapnic conditions, suggesting that the ability to process or retain dietary carbonates was affected. Sea urchins exposed to hypercapnic conditions displayed reduced total dry matter production, contributed primarily by reduced test dry matter production. Increases in neutral lipid storage in the gut were observed under hypercapnic conditions and gonads exhibited increased soluble protein storage. Conversely, organic production and energy allocation increased in the lantern in individuals exposed to hypercapnic conditions. Righting response and covering behavior were not affected by hypercapnic conditions. Carbonate system parameters (i.e., pH, pCO_2, and carbonate polymorph saturation states) of a seagrass-dominated ecosystem where individuals were collected (Eagle Harbor, Saint Joseph Bay, FL) exhibited extensive diurnal and seasonal variability. Monthly values of pH and

pCO_2 ranged from 7.36 – 8.28 and194.63 – 2536.80 μatm over one year, with mean diurnal rangess of 0.27 pH units and 517 μatm. *L. variegatus* are currently experiencing periodic hypercapnic conditions similar to experimental values employed (pH 7.8). Therefore, the experimental results in this dissertation may be more indicative of current *in situ* growth and physiology. Field conditions may facilitate acclimatization of *L. variegatus* to predicted increased hypercapnic conditions. Longer term ocean acidification studies (OA) that incorporate the natural diurnal variation in seawater chemistry into both control and experimental treatments are needed to determine the resilience of *L. variegatus* to near-future OA conditions.

Keywords: echinoid, ocean acidification, physiology, development, seagrass

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INTRODUCTION

Sources of carbon dioxide

Atmospheric carbon dioxide (CO_2) concentrations have increased rapidly due to the burning of fossil fuels and production of cement since pre-industrial times (e.g., Keeling 1960; IPCC 2007). Although estimates of palaeo-atmospheric CO_2 concentrations indicate that CO_2 levels have been much higher in the past 300 million years than current concentrations (Crowley and Berner 2001), the current rate of increase in CO_2 concentrations is much faster (approximately 100 times faster than in the past 650,000 years) (e.g., Caldeira and Wickett 2003; Siegenthaler et al. 2005). The global ocean has absorbed almost 50% of the CO_2 from these human-induced emissions (Sabine et al. 2004) and therefore, anthropogenic inputs of CO_2 to the atmosphere are a significant source of CO_2 in seawater.

The process of respiration is another source of CO_2 in seawater. In natural ecosystems such as nearshore shallow habitats with high plant biomass, macroalgae and seagrasses may be a significant source of carbon dioxide. In addition, in order for aquaculture to be economically viable, large stocking densities of marine organisms and low water exchange rates are desirable, yet intensive culturing conditions have the potential to lead to the accumulation of CO_2.

Regardless of the source of CO_2, carbonic acid (H_2CO_3) is formed when CO_2 combines with water. This acid quickly dissociates into hydrogen (H^+) and bicarbonate (HCO_3^-) ions. Under current pH conditions in the global ocean (e.g., 8.1), the H^+ ions react with carbonate (CO_3^{2-}) ions to form additional HCO_3^- and HCO_3^- is the major ionic form in seawater. There has been a 30% increase in the concentration of H^+ ions (i.e., a decrease in pH of 0.1 units) in ocean surface waters in the last 250 years (Caldeira and Wickett 2003) and it is predicted that with the continued anthropogenic input of CO_2, the pH of surface ocean waters could decrease by as much as 0.4 units by the year 2100 causing what is now known as "ocean acidification" (Brewer 1997; IPCC 2007). The historical recommendation for acceptable pH levels in aquaculture facilities has been a pH range of 7.5 – 8.4 (e.g., Spotte 1979). Yet recent ocean acidification studies on the impacts of reductions in pH suggest that the lower end of the aforementioned recommended pH range may have significant negative impacts to marine invertebrates (e.g., see Fabry et al. 2008; Ries et al. 2009; Dupont et al. 2010; Hofmann et al. 2010; Byrne 2011; and Ross et al. 2011 for review).

Impact of carbon dioxide on echinoids

The physiological impact(s) of increased concentrations of CO_2 to aquatic animals are not well understood. It has been suggested that increases in the partial pressure of CO_2 (pCO_2) in seawater affect calcification, acid-base status, and energy budgets (e.g., Melzner et al. 2012). An increase in aqueous CO_2 concentration leads not only to a reduction in pH but also to a reduction in carbonate ions (CO_3^{2-}). Because the

concentration of calcium ions (Ca^{2+}) is relatively constant in the oceans, a decrease in CO_3^{2-} directly causes a decrease in seawater carbonate saturation state (Ω), as the equation for $\Omega = CaCO_3 = ([Ca^{2+}][CO_3^{2-}])/ (K_{sp}^*)$, where $CaCO_3$ = calcium carbonate and K_{sp}^* = the stoichiometric solubility product of the form of $CaCO_3$ secreted by an organism (predominantly aragonite or calcite). On a purely thermodynamic level, when $\Omega < 1$, dissolution of $CaCO_3$ occurs. Calcification processes under hypercapnic conditions may be especially sensitive in echinoderms because the echinoderm endoskeleton is composed of a high-magnesium calcite (Weber 1969), a form of $CaCO_3$ that is more soluble in seawater than aragonite or calcite (Plummer and Mackenzie 1974; Morse et al. 2006). However, recent studies have reported than many marine organisms, including echinoids, are capable of net calcification when exposed to undersaturated conditions, primarily due to the presence of organic matrices and organic shell coverings (e.g., Ries et al. 2009; Thomsen et al. 2010; Dupont et al. 2010).

It has been hypothesized that alterations in extracellular and intracellular acid-base balances due to hypercapnic conditions elicit indirect effects due to the costs associated with restoring and maintaining appropriate pCO_2 levels in body fluids for respiratory diffusion gradients and pH levels within cells where calcification is occurring (see Melzner et al. 2009, for a discussion). As pCO_2 levels increase in the seawater, the pCO_2 of body fluids of heterotrophic metazoans become elevated to maintain concentration gradients that allow CO_2 to diffuse out into the seawater (Melzner et al. 2009). To compensate for increased pCO_2/ low pH levels, organisms actively or passively (thru shell dissolution) accumulate anionic buffers such as HCO_3^- , transport and exchange ions, metabolically consume or produce protons, and transport CO_2 via

respiratory pigments (e.g., Somero 1985; Walsh and Milligan 1989; Seibel and Walsh 2003).

As echinoderms do not have respiratory pigments and have lower capacities for acid-base regulation, they may be physiologically more vulnerable to hypercapnic conditions (Pörtner et al. 2004; Melzner et al. 2009; Hofmann and Todgham 2010). Moreover, when compensation of acid-base disturbance does take place, there may be indirect impacts from exposure to high pCO_2 conditions due to the cost of maintenance. For example, intracellular pH of primary mesenchyme cells (cells that perform calcification of spicules) of sea urchin larvae was restored within 15 minutes of exposure to low pH conditions (Stumpp et al. 2012b). This compensation was also associated with increased metabolic rate and up-regulation of genes involved with the Na^+/K^+-ATPase enzyme, indicating that exposure to hypercapnic conditions creates a higher energetic demand (Stumpp et al. 2011a,b; Stumpp et al. 2012b). Therefore, the energetic cost involved may be the source of observed changes in energy allocation (e.g., Dupont et al. 2010; Stumpp et al. 2011a). Because of these reasons, echinoderms have been identified as a group of organisms likely to be sensitive to increased pCO_2 conditions (Pörtner et al. 2004; Melzner et al. 2009; Hofmann and Todgham 2010).

Impacts of hypercapnic conditions on echinoid early development

The survival of embryos and larvae is essential to the maintenance of populations, particularly in animals with planktonic larvae (McEdward and Miner 2007). Therefore, it is important to evaluate the response of early life stages of various marine invertebrates to

hypercapnic conditions. Several studies have indicated that reductions (0.4 units) in pH due to increased pCO_2 can have significant negative effects on echinoid early development including changes in gene expression and slower larval growth rates (reviewed by Dupont et al. 2010, Byrne 2011, Ross et al., 2011; and Sewell and Hofmann 2013). How hypercapnic conditions (low pH, high pCO_2, low Ω_{Ar}) will affect the fertilization success, embryonic development, and larval development of *Lytechinus variegatus* is presently unknown.

Impacts of hypercapnic conditions on echinoid juveniles and adults

Echinoderms have discrete life stages with extensive physical and physiological differences. The echinoid juvenile stage is the period of maximal weight gain and production; therefore, the vulnerability of this stage to hypercapnic conditions may vary substantially from that of the embryonic and larval stages. The impact of increased pCO_2 exposure on juvenile and adult echinoids has not been studied extensively. However, the accumulation of CO_2 in aquaculture systems is known to affect the health of sea urchins (Grosjean et al. 1998; Timmons et al. 2001; Siikavuopio et al. 2007). Echinoid feeding may be impacted by increases in pCO_2 as the process has been shown to be affected by other abiotic factors including temperature and phosphates (Sloan and Campbell 1982; Klinger et al. 1986; Lares and McClintock 1991; Böttger et al. 2001). At extremely high pCO_2 concentrations (8000 μatm, pH 6.98), feeding rates, feed intake, and feed conversion efficiency were significantly reduced in adult *Strongylocentrotus droebachiensis* (Siikavuopio et al. 2007). Adult *S. droebachiensis* exposed to high

hypercapnic conditions (2800 – 3800 μatm, pH_{NBS} 7.25-7.19) also displayed reductions in ingestion and egestion, yet there was no difference in digestibility of organic material (absorption efficiency) (Stumpp et al. 2012a).

In addition to feeding, chronic exposure to hypercapnic conditions may affect the processing of nutrients and allocation of energy. Changes in abiotic factors including temperature and phosphate concentrations affect these processes in echinoids (Klinger et al. 1986; Lares and McClintock 1991; Böttger et al. 2001; Watts et al. 2011). In adult *S. droebachiensis* exposed to high pCO_2 conditions (2800 -3800 μatm), organic (ash-free dry mass) and inorganic (ash dry mass) matter content of body components were significantly reduced and less energy was allocated to the gonad and test tissue (Stumpp et al. 2012a). In addition to the acquisition and processing of nutrients, hypercapnic conditions affect the weight gain of echinoids. Adult *S. droebachiensis* exposed to high pCO_2 conditions have displayed reduced somatic and gonad weight gain (Siikavuopio et al. 2007; Stumpp et al. 2012a). In addition to adults, the few studies on juvenile sea urchins exposed to hypercapnic conditions have reported reductions in growth rates (Shirayama and Thornton 2005; Albright et al. 2012). Juvenile *Lytechinus variegatus* (initial weight 30.4 ± 3.5 mg) exposed to high pCO_2 conditions (800 μatm) displayed reduced growth rates and structurally degraded spines (Albright et al. 2012). Therefore, exposure to hypercapnic conditions can have significant impacts to the processing of nutrients and ultimately the growth (weight gain or production of new tissue) of sea urchins. Currently, how juvenile (pregonadal) *L. variegatus* will respond under hypercapnic conditions in terms of feeding, absorption and allocation of nutrients, and energy allocation is unknown.

Impact of hypercapnic conditions on echinoid behavior

Organismal behavior has been used as an indication of stress due to environmental changes (Eisler 1979). Righting from inversion is a well-known behavior of echinoderms (Hyman 1955; Reese 1966) and the righting response, or the time to right from inversion, has been used in relation to changes in environmental parameters to indicate the degree of physiological stress in many echinoderms (e.g., Lawrence 1973; Forcucci and Lawrence 1986; Watts and Lawrence 1986; Lawrence and Cowell 1996). In particular, righting has been used as a stress indicator in *Lytechinus variegatus* (Lawrence 1975; Böettger et al. 2001; Santos et al. 2013). Covering behavior, where regular urchins cover themselves with debris from the surrounding environment, has been well-characterized in *L. variegatus* (Millott 1955; Millott 1956; Sharp and Gray 1962; Amato et al. 2008). The function of the covering response may be to hold on to food before consumption (Péquigant 1966; Dix 1970; Douglas 1976) or for protection from sunlight/UV radiation (Lindahl and Runnström 1929; Mortensen 1943; Millott 1956; Lewis 1958; refs within Millott 1975; Adams 2001). It is possible that in addition to impacts to growth and physiology, exposure to increased pCO_2 conditions may affect organismal behavior. Yet to our knowledge, the impacts of hypercapnic conditions on echinoid behavior are unknown.

Study animal

Many echinoids have important ecological roles and/or are important to the field of aquaculture and therefore it is important to understand how they will respond to

hypercapnic conditions at various life stages. *Lytechinus variegatus* is an excellent model organism because its basic and applied biology have been studied exhaustively in the laboratory (e.g., Mazur and Miller 1971; Lawrence 1975; Klinger et al. 1986; Bishop et al. 1994; Beddingfield and McClintock 1998; Böttger and McClintock 2002; Hammer et al. 2004; Powell et al. 2004; Nelson et al. 2010; Heflin et al. 2012) and there has been a significant amount of research into its nutritional biology (e.g., Lowe and Lawrence 1976; Bishop and Watts 1992; Klinger et al. 1994; Böttger et al. 2001; Lawrence et al. 2003; Powell et al. 2004; Hammer et al. 2006a; Hammer et al. 2006b; Gibbs et al. 2009; Watts et al. 2011). Such studies often employ high urchin densities where CO_2 can become elevated and also utilize artificial sea salts. *L. variegatus* is a significant component in many coastal environments and can be found along the Western Atlantic coast from North Carolina to Brazil as well as the Gulf of Mexico and the Caribbean (see Watts et al. 2007, for a review). *L. variegatus* has a significant ecological role as it can control the amount of algal cover in nearshore environments through grazing (Valentine et al. 1997; Valentine and Heck 1999; Valentine et al. 2000; Peterson et al., 2002). In addition, the gonads of sea urchins have become a major economic resource for human consumption in the sushi industry (Keesing and Hall 1998; Andrew et al. 2002). Thus *L. variegatus* is an excellent study organism and its decline and/or loss would be detrimental both ecologically and economically.

Variation of carbon dioxide in seagrass-dominated ecosystems

Significant fluctuations in seawater carbonate chemistry have been documented for many marine ecosystems (e.g., DeGrandpre et al. 1995; Ohde et al. 1999; Hales et al. 2005; Wootton et al. 2008; Hofmann et al. 2011). Coastal data sets have primarily consisted of large-scale observations covering extensive regions of the open coastal ocean (e.g., Cai et al. 2011; Wang et al. 2013). Yet many study organisms used in OA research, such as *Lytechinus variegatus*, are collected from nearshore ecosystems. These areas are often shallow and contain dense beds of macroalgae or seagrasses and therefore have the potential to display significantly wider ranges in carbonate parameters on diurnal and seasonal timescales due to the influence of photosynthetic and respiratory processes. For example, diurnal fluctuations > 1 pH unit have been observed in seagrass meadows (Semesi et al. 2009) and increases in pH of up to 0.38 units have been associated with the presence of seagrass meadows (Unsworth et al. 2012).

Saint Joseph Bay is a shallow, subtropical lagoon located on the northwest coast of Florida (29.8°N, 85.3°W) and contains large areas of dense seagrass beds primarily comprised of *Thalassia testudium* with scattered patches of well-sorted, siliceous sand (Valentine and Heck 1993; Beddingfield and McClintock 2000). Populations of *Lytechinus variegatus* within the bay have been reported in densities as high as 35 individuals$\cdot$ m^{-2} (Beddingfield and McClintock 2000). As Saint Joseph Bay is a lagoon characterized by low-energy current regimes (Heck et al. 2000), greater variation in carbonate parameters, such as pH and pCO_2, are expected. Currently, there are no published studies on the carbonate system in Saint Joseph Bay. Characterizing the natural variation in the carbonate system of the bay will indicate *L. variegatus's* current

physiological tolerance for pH and pCO_2 variability and permit interpretations on its potential ability to acclimatize and/or adapt to changes in seawater carbonate chemistry.

Objectives

The purpose of this dissertation was to investigate the impacts of exposure to hypercapnic conditions on aspects of early and juvenile development of the common, edible sea urchin *Lytechinus variegatus*. Various metrics were used to assess fertilization success, embryonic and larval development rates as well as larval shape. Aspects of feeding, nutrient absorption efficiency, growth, and nutrient and energy allocation were examined in juveniles exposed to chronic hypercapnic conditions. To further interpret these results, the current carbonate chemistry dynamics of the nearshore habitat of *L. variegatus* were also investigated.

EFFECTS OF REDUCED CARBONATE SATURATION STATE ON EARLY DEVELOPMENT IN THE COMMON EDIBLE SEA URCHIN *LYTECHINUS VARIEGATUS*: IMPLICATIONS FOR LAND-BASED AQUACULTURE

by

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Abstract

Land-based aquaculture facilities often utilize additional bicarbonate sources such as commercial sea salts that are designed to boost alkalinity in order to buffer seawater against reductions in pH. Despite these preventative measures, many facilities are likely to face occasional reductions in pH and corresponding reductions in carbonate saturation states due to the accumulation of metabolic waste products. We investigated the impact of reduced carbonate saturation states (Ω_{Ca}, Ω_{Ar}) on embryonic developmental rates, larval developmental rates, and echinoplutei skeletal morphometrics in the common edible sea urchin *Lytechinus variegatus* under high alkalinity conditions. Commercial artificial seawater was bubbled with a mixture of air and CO_2 gas to reduce the carbonate saturation state. Rates of embryonic and larval development were significantly delayed in both the low and extreme low carbonate saturation state groups relative to the control at a given time. Although symmetry of overall skeletal body lengths was not affected, allometric relationships were significantly different between treatment groups. Larvae reared under ambient conditions had significantly greater postoral arm and overall body lengths relative to body lengths than larvae grown under extreme low carbonate saturation state conditions, indicating that extreme changes in the carbonate system affected not only developmental rates but also larval skeletal shape. Reduced rates of embryonic development and delayed and altered larval skeletal growth are likely to negatively impact larval culturing of *L. variegatus* in land-based, intensive culture situations where calcite and aragonite saturation states are lowered by the accumulation of metabolic waste products.

Introduction

There has been an increase in the demand for high-quality sea urchin roe in recent years that has led to intensive over-fishing of sea urchin populations and a resultant decline in fisheries in many countries (Keesing and Hall 1998; Andrew et al. 2002; Robinson 2003). As a result there has been rising interest in sea urchin aquaculture. In order for sea urchin aquaculture to be economically viable, large stocking densities and low water exchange rates are desirable, yet intensive culturing conditions have the potential to create several environmental conditions that can negatively impact the growth of sea urchins including the accumulation of metabolic waste products such as ammonia (Chen et al. 1989; Basuyaux and Mathieu 1999; Wickins and Lee 2002; Siikavuopio et al. 2004a), nitrite (Siikavuopio et al. 2004b), and carbon dioxide (CO_2; Timmons et al. 2001; Grosjean et al. 1998; Siikavuopio et al. 2007).

Land-based facilities that utilize static renewal systems geared towards larval culture of seed stock may also experience harmful environmental conditions as the source of the larval culture water may be from adult and juvenile culture water. An increase in aqueous CO_2 concentration leads not only to a reduction in pH but also to a reduction in the carbonate saturation state (Ω), typically described as the calcite saturation state (Ω_{Ca}) or aragonite saturation state (Ω_{Ar}), depending on whether the organism secretes a calcite or aragonite shell or endoskeleton. It is thought that many calcifying organisms that produce skeletons may be challenged under conditions of reduced carbonate saturation states, as the availability of carbonate ions (used to construct shells and endoskeletons) decreases with decreasing carbonate saturation state (Byrne 2011, refs within). Despite recent examination of reductions in the carbonate saturation state in the context of ocean

acidification (for example, see Byrne 2011, for a review), there are apparently no studies that take into account reductions in the carbonate saturation state as it relates to culturing organisms in land-based aquaculture using commercial artificial seawater recipes that are intentionally designed to increase alkalinity far beyond natural seawater levels. The historical recommendation for acceptable pH levels in facilities using additional bicarbonate sources has been a pH range of 7.5 – 8.4 (for example, see Spotte 1979). Yet recent ocean acidification studies on the impacts of reductions in pH and corresponding carbonate saturation states suggest that the lower end of the aforementioned recommended pH range may not be acceptable in terms of ideal early development and growth (Byrne 2011; Ross et al. 2011). Thus, an evaluation of the impact of reduced carbonate saturation states on organisms utilized by the aquaculture industry under chemical conditions relevant to aquaculture facilities (i.e., high alkalinity) is needed.

The common edible echinoid, *Lytechinus variegatus*, is a sea urchin that has been extensively studied as a candidate for aquaculture due to the ability to raise *L. variegatus* larvae (Mazur and Miller 1971; Watts et al. 1999; George et al. 2004; Powell et al. 2008; Gibbs et al. 2009), juveniles, and adults (Gibbs and Watts 2004; Hammer et al. 2004, 2006a, 2006b, Gibbs et al. 2007, 2009, 2011; Watts et al. 2007, 2011; Taylor et al. 2009; Nelson et al. 2010; Richardson et al. 2011; Heflin et al. 2012) in commercial artificial seawater (i.e., Instant Ocean®), the extensive knowledge of its nutritional biology based on artificial feeds (Hammer et al. 2004, 2006a, 2006b; Powell et al. 2004; Gibbs et al. 2007, 2009; Taylor et al. 2009; Watts et al. 2011), and its rapid growth (Lawrence and Bazhin 1998).

The objective of this study was to evaluate the impact of reduced carbonate saturation state under high alkalinity conditions, a common outcome of intensive land-based aquaculture using additional bicarbonate sources, on fertilization success, embryonic and larval developmental rates, and larval (echinoplutei) skeletal morphometrics of the common edible sea urchin *Lytechinus variegatus*.

Methods

Collection and experimental design

Adult *Lytechinus variegatus* (30 – 40 mm diameter) were hand-collected in August 2009 from Eagle Harbor in Saint Joseph Bay, Florida ($29^0$45'N, $85^0$24'W). This time of year has been identified as a period when *L. variegatus* are sexually mature (Watts et al. 2001). Individuals were immediately transferred to coolers supplied with air via an air pump, aquarium tubing and air stones, and transported to the University of Alabama at Birmingham in Birmingham, Alabama. Sea urchins were held for a period of two days during which daily water exchanges were made and individuals showing signs of stress (e.g., loss of spines or inability to right themselves) were removed prior to fertilization. Three males and five females were used for fertilization and gametes were obtained by injection of approximately 1 ml of 0.1 M acetylcholine into the perivisceral cavity via the peristomial membrane. Gametes were inspected for quality (sperm motility and egg maturation state) prior to collection using a light microscope. Eggs from each female were shed into each of three 100 ml glass beakers containing experimental seawater treatments (control: $\Omega_{Ca} = 7.17$, $\Omega_{Ar} = 4.68$, low carbonate saturation state: Ω_{Ca}

= 2.53, Ω_{Ar} = 1.65 and extreme low carbonate saturation state: Ω_{Ca} = 0.98, Ω_{Ar} = 0.63). Saturation states were chosen to reflect levels of pH commonly experienced in land-based culture situations that utilize additional bicarbonate sources (i.e., control pH = 8.2, low group pH = 7.8, extreme low group pH = 7.4; Spotte 1979). Eggs from all five females were thus pooled in each beaker and then rinsed three times in seawater from their respective experimental treatment over a period of 30 minutes. Sperm were collected dry, pooled, and diluted just prior to fertilization with acidified artificial seawater (1 drop sperm per 100 ml) for each of the three treatments. Once fertilization had taken place (detected by taking samples from each beaker and verifying the presence of fertilization membranes under a light microscope), eggs were placed in 15 x 1 L glass beakers containing acidified artificial seawater (five replicates per treatment) such that no more than a monolayer of cells covered the bottom of the beaker (approximately 200 eggs ml^{-1}). This density was not maintained; once embryos hatched (within 24 h) densities were reduced to approximately 2 per ml to simulate extremely high densities. We did not provide food to the developing larvae as such an addition would have confounded the carbonate chemistry within the control group by potentially adding more CO_2 and/or metabolic waste from microbial activity or respiration of algae.

Seawater chemistry

Prior to fertilization, commercial artificial sea salts were purchased (Instant Ocean® Spectrum Brands) and combined with purified water (tap water filtered through a Four-Stage Barracuda RO/DI three part filter system, AQUAfx). We chose to use artificial seawater as it would be a likely choice for land-based, intensive culture situations where the high buffering capacity (high alkalinity) of the seawater would

buffer against reductions in pH and corresponding carbonate saturation states due to the build-up of metabolic wastes. CO_2 was used to alter the carbonate saturation state by continuously bubbling seawater with a combination of pure gaseous (United States Pharmacopeia grade) CO_2 from a canister (Airgas) and air (from a Whisper 300 aquarium air pump using room air) via a gas proportioning rotameter (Omega engineering). We used ambient (room) CO_2 as our control (Ω_{Ca} = 7.17, Ω_{Ar} = 4.68), an experimental 'low' Ω (Ω_{Ca} = 2.53, Ω_{Ar} = 1.65) and 'extreme low' Ω (Ω_{Ca} = 0.98, Ω_{Ar} = 0.63). Daily water exchanges (< 15 % volume) were conducted and pH and temperature monitored continuously during water exchange. Control beakers were continuously aerated with a Whisper 300 aquarium air pump (room air). Air and the mix of CO_2 and air were delivered via standard aquarium tubing capped with a glass pipette tip on the end to control bubble rate and beakers were covered with Saran® wrap to help maintain salinity and establish a headspace of approximately 2 to 5 cm. Temperature, salinity and pH_{NBS} of each container were measured twice daily using a Fischer Scientific Accumet © basic model AB15 pH meter and ACCUTU pH electrode (Cat. # 13-620-183) and a handheld refractometer for salinity measurements. Room air temperature was controlled to maintain larval culture temperatures. The pH electrode was calibrated twice daily with Fisher brand pH 4.0, 7.0, and 10.0 buffers (NBS standards). Total alkalinity was measured by potentiometric titration using the method of the American Public Health Association (APHA) (APHA, 1992) modified for use with a Hach digital titrator at the beginning of the experiment (Table 1). This method was tested for accuracy against certified reference materials provided by the laboratory of Andrew G. Dickson (University of California, San Diego, Scripps Institute of Oceanography) and was found

to have an error of ± 66 μmol kg^{-1} (standard error of the mean; n = 6). Dissolved oxygen of each container was measured using a YSI 85 oxygen meter just prior to the start of the experiment and every three days thereafter.

Calcite and aragonite saturation states and pCO_2, were determined from TA, pH_{NBS}, temperature and salinity using the Microsoft Excel spreadsheet 'co2sys.xls' based on work by Lewis and Wallace (1998) and provided by Pelletier et al. (2007) with the CO_2 constants of Millero et al. (2006) and the $KHSO_4$ constant of Dickson (1990) (Table 1). In order to ensure that our single-point measure of carbonate saturation state for each treatment was representative of seawater over the four-day experiment, we conducted a separate analysis. Under identical experimental conditions we measured total alkalinity in three replicate samples per treatment per day and obtained carbonate saturation state values for the four-day time course. As samples were taken, water was replaced with previously acidified water. Using a one way analysis of variance (ANOVA) it was determined that the carbonate saturation states in each group did not vary significantly within treatment groups over the course of four days (all $p > 0.05$, df = 2, 11, Ω_{Ca} control = 5.96 ± 0.48, Ω_{Ar} control = 3.93 ± 0.32, Ω_{Ca} low = 2.43 ± 0.21, Ω_{Ar} low = 1.60 ± 0.14, Ω_{Ca} extreme low = 1.01 ± 0.17, Ω_{Ar} extreme low = 0.67 ± 0.11; values given are means ± standard deviations).

Embryonic development

To assess fertilization success, 30 ml subsamples were taken from each replicate 30 min after the time of fertilization and immediately fixed in 10% buffered formalin. Fifty (50) eggs from each replicate were evaluated under a light microscope for the

presence of a fertilization envelope. Subsamples were also taken from each replicate at 75, 107, 146, and 1553 min and fixed in 10 % buffered formalin to assess embryonic developmental rates at various stages of development. The development stage of 50 randomly selected embryos from each subsample was evaluated at each time sampling using a light microscope. We used a multinomial Cochran-Mantel-Haenszel (CMH) test to determine whether changes to the carbonate saturation state affected development while controlling for time and replicate. If significant, post-hoc CMH tests were performed for each time while controlling for replicate. Analyses were performed using SAS 9.2 software.

Echinoplutei development and morphometrics

Within 24 h larval densities were reduced to approximately 2 larvae ml^{-1}. Excess larvae were removed by gently pouring off a portion of culture water and any particulate matter that had collected on the bottom of the beakers (e.g., dead cells) was removed by pipette to maintain water quality. Any water removed was replaced with previously acidified water. Fifty randomly selected larvae from each replicate from each day were evaluated for developmental stage (e.g., no arms = prism/very early pluteus, 2 arms = 2 postoral arms, or 4 arms = 2 postoral arms and 2 anterolateral arms) and for the presence of abnormalities. We used a multinomial Cochran-Mantel-Haenszel (CMH) test to determine whether carbonate saturation state affected development while controlling for day and replicate. If significant, post-hoc CMH tests were performed for each day while controlling for replicate.

To evaluate echinoplutei skeletal morphometrics, 50 ml subsamples from each replicate were taken on each day (day one = 24 h post-fertilization) and fixed in 10 %

buffered formalin. Digital photographs of 20 randomly selected larvae from each replicate per day were taken using a digital camera mounted on a dissecting microscope and were analyzed using an image analysis program (Image Tool) to determine overall skeletal body length (both left and right side), post-oral skeletal arm length of the right side, and body length (Figure 1). Larvae were manipulated such that the post-oral arms were dorsal to the rest of the body and lay flat for consistent orientation. Extremely abnormal larvae (e.g., without arms or severely deformed skeletal arms; Figure 2) were not measured.

Symmetry of overall skeletal body lengths for all replicates on each day in each treatment group was evaluated by subtracting the overall body length of the left side (OLL) minus the overall body length of the right side (OLR). A Levene's test for equal variances was then used to determine if carbonate saturation state affected symmetry of overall body length for each day. Replicates were first tested for significant differences (none was found, all $p > 0.12$) and then pooled within each treatment per day using R 2.13.1.

To determine whether carbonate saturation state had an effect on the growth of various skeletal morphometric parameters, two linear mixed effect models were created in R 2.13.1 using the lme4 package. The dependent variables were morphological measurements including body length (BL), postoral arm length (PL) and overall body length left (OLL), whereas carbonate saturation state and time post fertilization (TPF) were independent variables. In the first model, carbonate saturation state and TPF were defined as categorical fixed effects and replicate as a random effect (since fertilized eggs were placed at random into replicate beakers). In the second nested model, carbonate

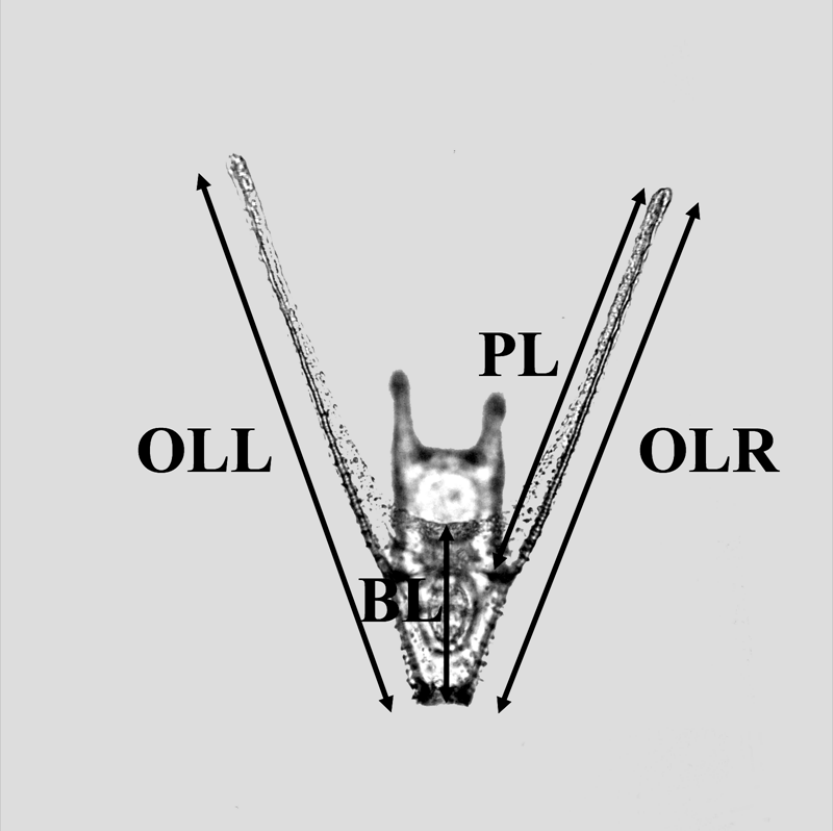

Figure 1. *Lytechinus variegatus*. Echinoplutei measurements. (OLL) overall arm length left side; (OLR) overall arm length right side; (BL) body length; (PL) postoral arm length. Individuals were manipulated such that the postoral arms were always dorsal to the rest of the body to ensure the larva would lay flat and were in the same orientation. No abnormal larvae, such as those seen in Figure 2, were used in the morphometric analysis.

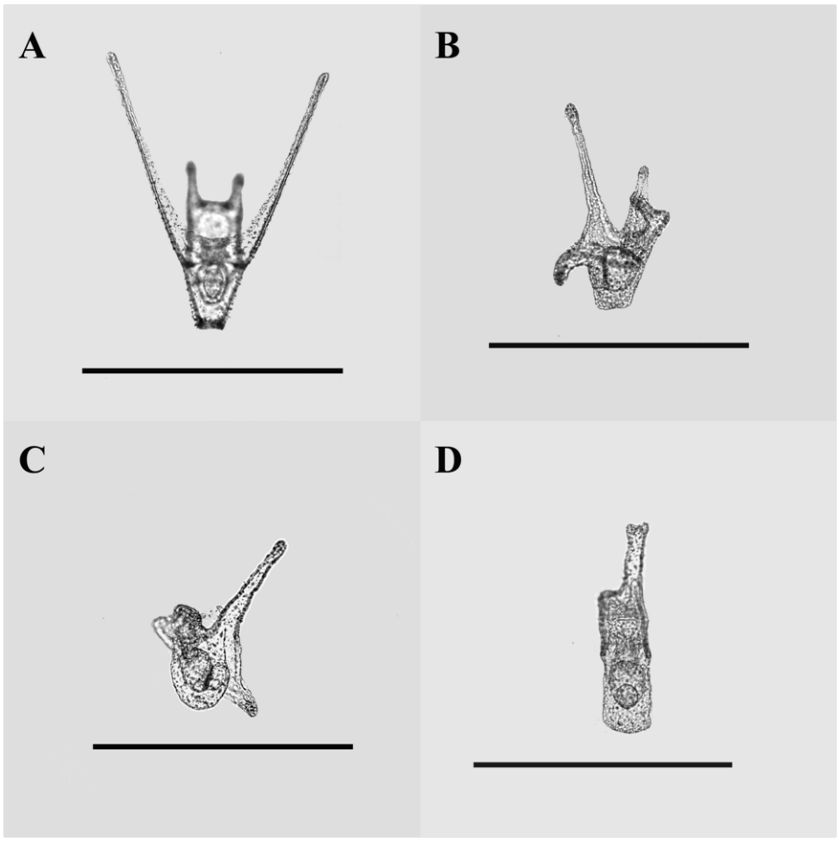

Figure 2. *Lytechinus variegatus*. Photomicrographs of two-day-old normal (a) and abnormal (b, c, d) echinoplutei larvae from the control (Ω_{Ca} = 7.17, Ω_{Ar} = 4.68) and extreme low (Ω_{Ca} = 0.98, Ω_{Ar} = 0.63) treatments, respectively. Bar = 0.5 mm.

saturation state was removed and an analysis of variance (ANOVA) was conducted to compare the two models to determine whether carbonate saturation state was significantly associated with the dependent variable.

The two linear mixed effect models described above were then used to determine whether carbonate saturation state had an effect on the allometric relationships of BL x PL and BL x OLL, where body length (BL) was defined as an independent variable as body length has been shown to be a reliable indicator of larval size in early development (McEdward and Herrera 1999). Due to a complex relationship between PL and BL, the natural spline of BL with 4 degrees of freedom was substituted in both models. An analysis of variance (ANOVA) was done to compare the nested models (with and without carbonate saturation state) to determine whether carbonate saturation state was significantly associated with either independent variables (i.e., BL or TPF). All model assumptions were assessed by examining residual histograms and residual vs. fitted plots.

Results

Seawater carbonate chemistry

Means and standard deviations for each parameter measured are presented in Table 1.

Table 1. Measured and calculated parameters of seawater carbonate chemistry. Values are means; values in parentheses are standard deviations. The total number of observations per carbonate saturation state group are as follows: pH_{NBS}, n = 60; pCO_2, n = 1; dissolved inorganic carbon, n = 1; salinity, n = 60; dissolved oxygen, n = 15; temperature, n = 60; total alkalinity, n = 1; Ω_{Ca} = saturation state of calcite, n = 1; Ω_{Ar} = saturation state of aragonite, n = 1.

	Control	Low Ω	Extreme Low Ω
pH (NBS)	**8.22 (0.037)**	**7.81 (0.044)**	**7.41 (0.039)**
***p* CO2 (µatm)**	**413.6**	**1795.11**	**4289.88**
CO_2 (mg/L)	**0.8**	**3.48**	**8.31**
Dissolved inorganic carbon (µmol/kg)	**2647.38**	**2954.88**	**2879.62**
Temp (Celsius)	**22.0 (0.25)**	**22.12 (0.21)**	**22.08 (0.18)**
Dissolved oxygen (%)	**89.52 (0.37)**	**89.59 (0.48)**	**89.39 (0.52)**
Salinity (‰)	**34 (1)**	**34 (1)**	**34 (1)**
Total alkalinity (µmol/kg)	**2997.302**	**3077.23**	**2837**
Total alkalinity (mg/L $CaCO_3$)	**149.8**	**153.8**	**141.8**
Ω Ca	**7.17**	**2.53**	**0.98**
Ω Ar	**4.68**	**1.65**	**0.63**

Embryonic development

Percent fertilization was high across all groups (≥ 96%) and the presence of abnormalities in recently fertilized eggs were not significantly different between treatment groups (Fisher's exact test, p = 0.3094). However, development of embryos reared at the experimental carbonate saturation states were both significantly different from one another and from the control group at all time samplings (with the exception of the low carbonate saturation state group sampled at 146 min, Figure 3, Table 2). The number of fertilized eggs that had not reached the first cleavage (one-cell) increased with decreasing carbonate saturation state, whereas the number of embryos at the two-cell stage increased with increasing carbonate saturation state at the first two time samplings

(Figure 3). At 107 minutes post-fertilization, the low carbonate saturation state group had less fertilized eggs at the four-cell stage and more fertilized eggs at the two-cell stage than the control group. Several fertilized eggs had yet to go through the first cleavage and more abnormalities were observed in the extreme low carbonate saturation state group than either the low carbonate saturation state group or the control. At 146 min post-fertilization, the state of the fertilized eggs reared at the low carbonate saturation state did not differ significantly from the control, whereas there were more abnormalities and a large amount of fertilized eggs found at the two-cell stage in the embryos exposed to an extreme low carbonate saturation state. The number of later-stage embryos (e.g., gastrula versus prism versus early pluteus) also differed significantly between groups with the number of early plutei increasing with increasing carbonate saturation state and the number of gastrula increasing with decreasing carbonate saturation state. At almost all time samplings significantly slower developmental rates were associated with exposure to reduced carbonate saturation state.

Larval development

Development rates of larvae reared at lower carbonate saturation states were significantly longer than those reared at a control carbonate saturation state at all time samplings (Figure 4, $p < 0.001$ for all comparisons by CMH association tests). On the first day (24 h post-fertilization), more larvae were without arms (somewhere between

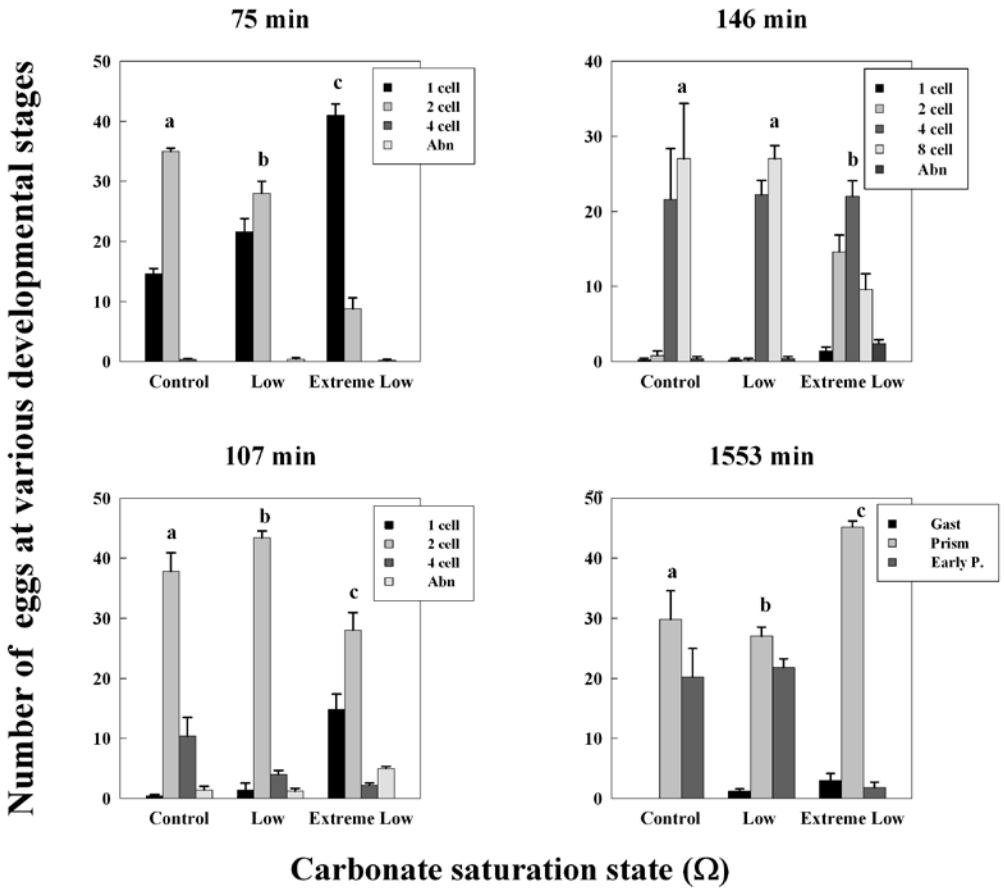

Figure 3. *Lytechinus variegatus.* Number of fertilized eggs out of 50 at the one-cell, two-cell, four-cell, eight-cell, gastrula, prism or early pluteus stage in each treatment at various sampling times (mean ± standard error of the mean, n = 5 replicates per treatment, 50 fertilized eggs were sampled from each replicate). Carbonate saturation state group differences (as indicated by different lower case letters) were tested using Cochran-Mantel-Haenszel (CMH) general association tests and p-values are given in Table 2. Minutes given represent minutes post-fertilization.

Table 2. *Lytechinus variegatus.* Summary of statistical analysis on embryonic development**.** Comparisons and associated p-values generated by Cochran-Mantel-Haenszel (CMH) association tests.

Time (min)	Control vs Low Ω p-value	Control vs Extreme Low Ω p-value	Low Ω vs Extreme Low Ω p-value
75	**0.0017**	**<0.0001**	**<0.0001**
107	**<0.0001**	**<0.0001**	**<0.0001**
146	**0.7653**	**<0.0001**	**<0.0001**
1553	**0.0297**	**<0.0001**	**<0.0001**

the prism and very early pluteus stages) in the lower carbonate saturation state groups than the control and there were higher numbers of larvae without arms than those with two arms in the extreme low carbonate saturation state group (Figure 4). The trend of slower development continued throughout the rest of the experiment with greater numbers of abnormalities present in reduced carbonate saturation state groups (Figure 4). Day 5 results are not shown as by that time only two replicates from the extreme low carbonate saturation state group contained larvae in the water column.

Echinoplutei morphometrics

Symmetry (determined as the difference between overall body lengths measured on each side of the larvae, Figure 1) between treatments was not significantly different at any time post-fertilization when the nominal alpha of 0.05 was adjusted using a Bonferroni correction alpha of 0.0125 (Day 1, $F_{2,97} = 0.462$, $p = 0.631$; Day 2, $F_{2,97} = 0.462$, $p = 0.682$; Day 3, $F_{2,97} = 0.462$, $p = 0.035$; Day 4, $F_{2,97} = 0.462$, $p = 0.024$). However, the Bonferroni method may be overly conservative and / or underpowered, thus future experiments that incorporate more replicates or longer experimental times may detect differences in symmetry at later time points (i.e., day 5 or later).

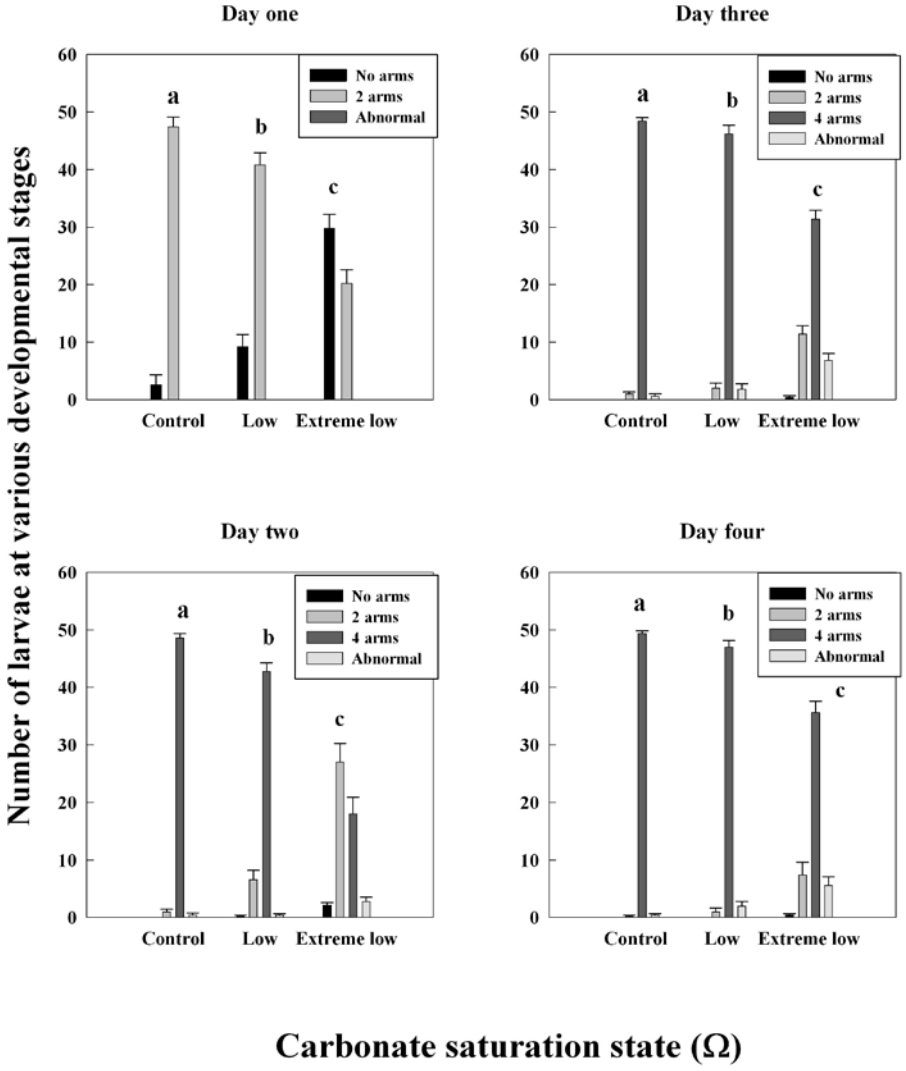

Figure 4. *Lytechinus variegatus*. Number of larvae (out of 50) without arms (prism or very early pluteus stage), or possessing two arms (postoral arms), four arms (postoral arms and anterolateral arms), or significant abnormal morphologies in each treatment (control, low Ω, or extreme low Ω) on each of the four days sampled (mean ± standard error of the mean, n = 5 replicates, 50 larvae were sampled from each replicate). Treatment group differences (as indicated by different lower case letters) were tested using CMH general association tests; all comparisons were significantly different ($p < 0.001$).

The measurements of the right side of the larvae (overall body length right, or OLR) were not included in the morphometric analysis, as the symmetry analysis indicated no significant differences in overall body lengths. Larval morphometrics measured included overall body length left (OLL), body length (BL), and postoral arm length (PL); carbonate saturation state significantly affected each skeletal morphometric (Table 3). For all skeletal morphometrics, decreasing carbonate saturation states yielded smaller measurements (Figure 5). Not all of these reductions were statistically significant (i.e., BL and PL of larvae reared at a low carbonate saturation state were not statistically different from those of larvae reared at an ambient carbonate saturation state), although all morphometrics were statistically smaller in the extreme low carbonate saturation state compared to the control (Table 3). Thus, reduced carbonate saturation state had a significantly negative effect on larval morphometric size between days 1 and 4 post-fertilization. In addition to being significantly smaller in length, many more larvae in the extreme low carbonate saturation state group were at the two-armed stage than in the control group (Figure 6).

Allometric relationships between skeletal body length (BL) and skeletal postoral arm length (PL) and BL and overall skeletal body length (OLL) were significantly different between those reared in the control versus extreme low carbonate saturation state (Table 3 and Figure 7). BL x PL was not significantly different in those larvae reared under a low carbonate saturation state (versus the control), yet the relationship between BL and OLL was. Thus, the more severe the reduction in carbonate saturation state, the greater the effect not only on larval size but larval skeletal shape as well, with larval skeletal postoral arm lengths and overall skeletal body lengths relative to skeletal

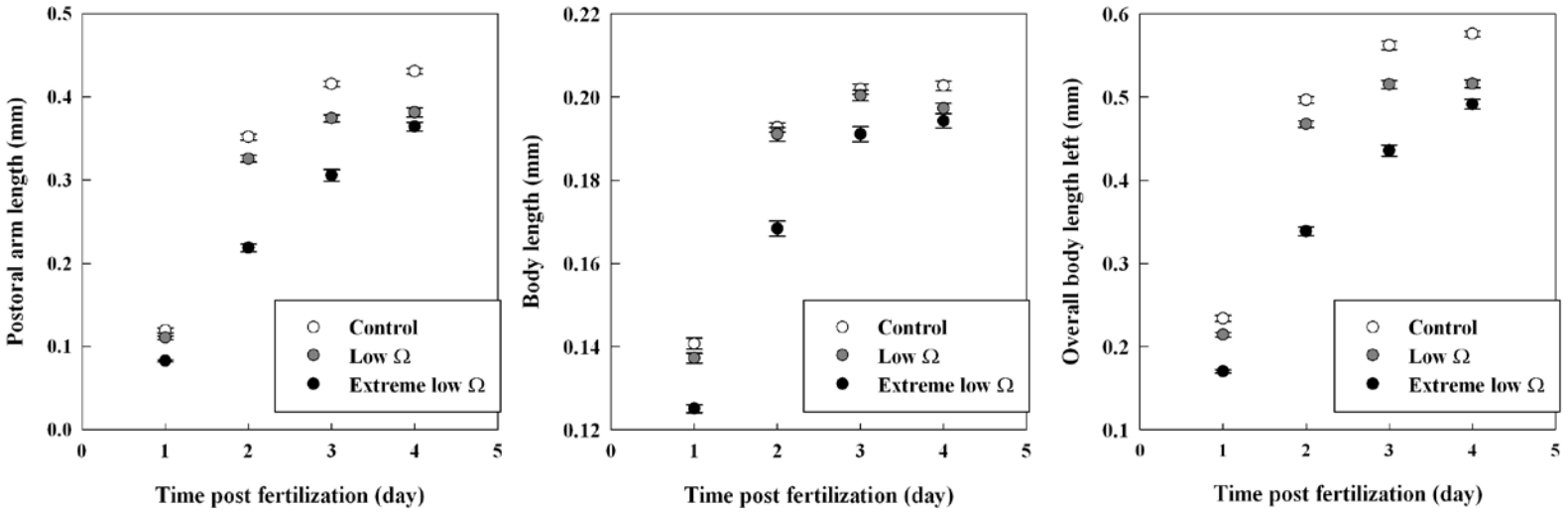

Figure 5. *Lytechinus variegatus*. Larval morphometric growth over time showing measurements for both two-armed and four-armed echinoplutei sampled each day and exposed to different carbonate saturation states. Values are mean ± standard error of the mean, n = 5 replicates per treatment group with 20 larvae sampled from each replicate.

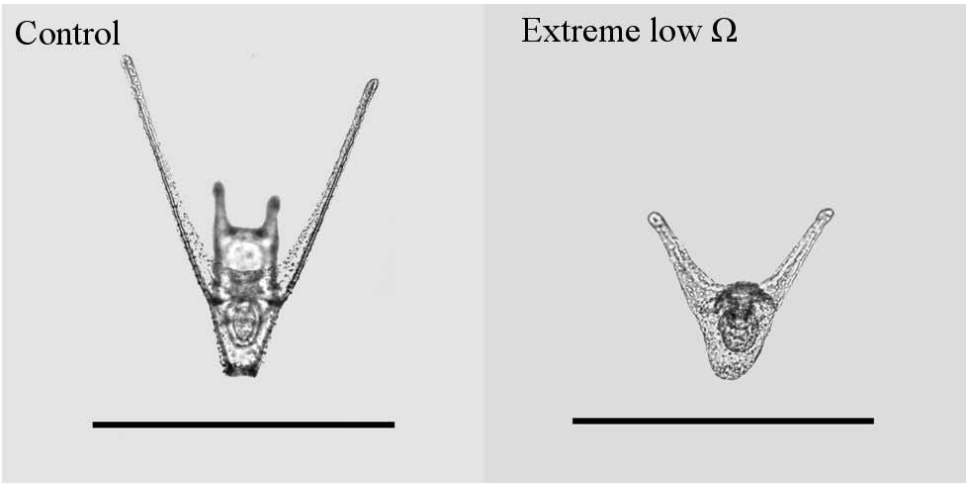

Figure 6. *Lytechinus variegatus.* Photomicrographs showing developmentally normal echinoplutei larvae on day two from two of the three treatments. Each larva is representative of the developmental and size differences found between larvae held in a given treatment. Bar is 0.5 mm.

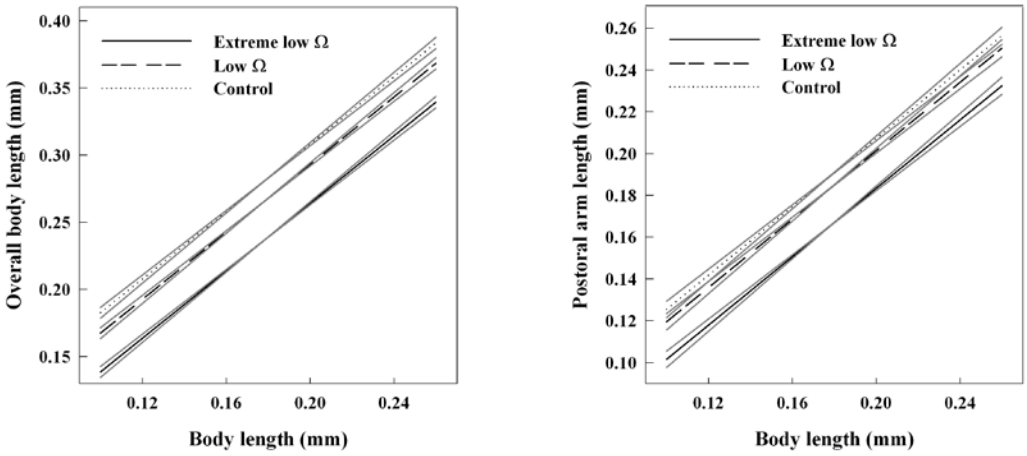

Figure 7. *Lytechinus variegatus.* Allometric relationships between body length (BL) and postoral arm length (PL) and overall body length left (OLL) as estimated from the linear mixed effect model that incorporated carbonate saturation state. This model explained 90% (left) and 92% (right) of the variance observed in the data, with carbonate saturation state accounting for 8% (left) and 9.7% (right). Statistical results on comparisons between each reduced carbonate saturation state group and the control can be found in Table 3.

Table 3. *Lytechinus variegatus.* Significance tests for carbonate saturation state as a categorical predictor across various measurements. (OLL) overall body length left; (PL) postoral arm length (BL) body length.

				Morphometric analyses					
	Overall Ω test			**Low Ω vs Control**			**Extreme low Ω vs Control**		
	X^2	**DF**	**p**	**t**	**DF**	**p**	**t**	**DF**	**p**
OLL	933.9	8	$<2.2e^{-16}$	3.07	1184	0.0022	10	1185	$<2.2e^{-16}$
PL	794.5	8	$<2.2e^{-16}$	1.5	1184	0.13	6.2	1185	7.80E-10
BL	279.5	8	$<2.2e^{-16}$	1.76	1184	0.078	7.9	1185	6.30E-15
				Allometric analyses					
	X^2	**DF**	**p**	**t**	**DF**	**p**	**t**	**DF**	**p**
BL vs PL	530.4	8	$<2.2e^{-16}$	0.93	1180	0.35	3.49	1181	5.00E-05
BL vs OLL	641.2	8	$<2.2e^{-16}$	2.33	1180	0.02	5.66	1181	1.90E-08

body lengths being significantly smaller in larvae raised at an extreme low carbonate saturation state.

Discussion

The use of artificial seawater

In this study, commercial artificial sea salts with high alkalinity were used to assess the impacts of reduced carbonate saturation state on early development of the sea urchin *Lytechinus variegatus*. Land-based, intensive aquaculture facilities utilize artificial seawater or additional sources of bicarbonate (i.e., Loyless and Malone 1997) in order to create seawater with a greater total alkalinity than what is found in nature. Studies have shown that the use of synthetic sea salts, in general, does not impact early development in echinoids (Hovanec et al. 2005) and the use of commercial artificial

seawater has many benefits such as cost and logistics, particularly for those facilities not located near the coast. The average total alkalinity of seawater is approximately 2300 μmol kg^{-1} (116 mg L^{-1} $CaCO_3$, Boyd 2000). In this study, the total alkalinity was 2970 ± 70 μmol kg^{-1} (148 ± 3 mg $^{-1}$ $CaCO_3$). To our knowledge, there are no studies that have compared the impact of reduced carbonate saturation states in natural versus artificial seawater, or a combination of the two, on echinoid development. Previous studies comparing various types of commercial sea salts have found that the pH buffering system and therefore the carbonate saturation state can vary significantly with sea salt brand (Atkinson and Bingman 2005). Total alkalinity did vary between our experimental groups but not significantly when water was repeatedly tested after the experiment. Because we observed some negative impacts to early echinoid development, more studies are needed to further determine whether the impacts of reduced pH and carbonate saturation states under artificial seawater conditions will have significant impacts to aquaculture production. Culture facilities should consider performing pilot studies on their specific sea salts of choice to assess the potential for drops in pH and carbonate saturation states. Water quality (nitrate, nitrite, and ammonia) was not assessed in this study as the density of embryos and larvae was low and the water used was freshly prepared. The global impact of nutrients and organic matter on total alkalinity has been assessed (Wolf-Gladrow et al. 2007), yet fluctuations between containers and facilities is likely to occur under various conditions; future studies should investigate whether the impact these factors have on the carbonate chemistry is of significance. Although not assessed in this study, an additional aspect to consider when using additional carbonate sources is the potential for alterations to the calcium or magnesium concentrations in the

water. Commercial sea salts have also been found to vary in the concentration of some major ions (Atkinson and Bingman 2005). Calcium and magnesium concentrations are needed to calculate the carbonate saturation state and therefore should be quantified in future studies in order to accurately determine the carbonate saturation state.

Impacts to development

Fertilization success was not significantly affected in either experimental treatment yet embryonic and larval development rates were negatively impacted and various morphometric measurements of plutei at a given time were significantly smaller under chronic exposure to reduced carbonate saturation states. Embryonic development was significantly delayed at a low Ω (Ω_{Ca} = 2.53, Ω_{Ar} = 1.65). This result is consistent with many ocean acidification studies where natural seawater was used to assess the effects of increased pCO_2 on echinoid early development (see Byrne 2011; Ross et al. 2011, for a review). The occurrence of embryonic abnormalities was highest in the extreme low Ω group, but surprisingly remained low throughout embryonic development relative to the number of normal embryos encountered. Although we did not quantify mortality rates, the lack of viable larvae (e.g., swimming in the water column) in the extreme low Ω group by day five was very apparent relative to the other treatment groups. Thus, it would appear that mortality rates were greater in the lowest carbonate saturation state group. In other larval studies of *L. variegatus*, echinoplutei only developed to the four-arm stage and survived until day 6 under starvation conditions (McEdward and Herrera 1999). *L. variegatus* are capable of feeding within 24-27 h of fertilization (Mazur and Miller 1971) and the presence and amount of food has been shown to increase growth rates (Boidron-Metairon 1988). Therefore, it is possible that

the combination of low carbonate saturation state and lack of food may have resulted in higher larval mortality. Very few studies that have investigated the impacts of reduced pH (and consequently, reduced carbonate saturation state) on echinoid early development have provided food for developing larvae, and it is possible that the presence of an energy source may alleviate some of the negative impacts of exposure to reduced carbonate saturation states. Low algae concentrations and high pCO_2 concentrations significantly decreased shell length growth in the blue mussel *Mytilus edulis* (Melzner et al. 2011). Future studies should incorporate food at relevant concentrations utilized by the aquaculture industry to determine if the presence of food alleviates the impacts observed in this study.

Reduced carbonate saturation state did not affect larval symmetry, but skeletal morphometric development was negatively impacted in terms of the time required for larvae to reach a given size. This delay was likely the result of energy (normally devoted to somatic growth) being redirected to deal with the stress of a reduced carbonate saturation state environment as suggested by studies examining the effects of reduced pH (Stumpp et al. 2011). The consequences of such a delay in larval development may include a delay in settlement or metamorphosis, decreased survival rates and potentially negative impacts to juvenile growth, growth to sexual maturity times, and gonad production. Juvenile *Lytechinus variegatus* that were raised at a pH of ≤ 7.8 from fertilization had significantly lower wet weights and displayed structural abnormalities in their spines (Albright et al. 2012) suggesting that even small drops in pH may be detrimental to aquaculture production.

Allometric relationships between skeletal BL and PL and BL and OLL were significantly different between the control group and those reared at an extreme low carbonate saturation state and the relationship between BL and OLL was significantly different in those reared at a low carbonate saturation state. This change in shape may reflect impacts from high amounts of CO_2 used to achieve our reduced carbonate saturation state conditions as hypercapnic effects include suppression of metabolism (see Pörtner 2008; Widdicombe and Spicer 2008, for a review). Our findings are in contrast to those of Catarino et al. (2012) and Stumpp et al. (2011), who found that the proportions of larvae, regardless of pH, were not significantly different. This difference may be due to the high amounts of CO_2 used in the current study to achieve the same pH levels. However, because dissolved oxygen levels were consistently > 90 % and significant impacts to the skeletal morphometrics were observed, it appears likely that the reduction in carbonate saturation state was a significant factor as carbonate saturation state can impact the process of calcification. Studies utilizing both natural and artificial seawater with varying alkalinities may provide additional knowledge on how the amount of CO_2, versus carbonate saturation state impact echinoid development.

The immediate consequence of changes in shape may be viewed in terms of larval function: the greater the length of the postoral arms, the greater the food-capturing efficiency of echinoplutei (Strathmann 1971). Larvae of the echinoids *Dendraster excentricus* and *Strongylocentrotus droebachiensis*, that possessed longer, ciliated bands (located on the post-oral arms), had greater maximum clearance rates (volume of water cleared of particles per unit time, Strathmann 1971). It should be noted that larvae reared at a reduced carbonate saturation state may eventually develop morphometric

relationships that more closely resemble those of the control group, though negative downstream impacts are also possible. In the present study, larvae were reared to day 5. There are very few studies that have evaluated the impact of reduced pH and carbonate saturation states over the entire life cycle of echinoids. Adult *Strongylocentrotus droebachiensis* exposed to high CO_2 concentrations had reduced feed intake and gonad growth (Siikavuopio et al. 2007) and reductions in somatic and reproductive growth due to changes in energy budgets and increased ammonium excretion (Stumpp et al. 2012). Juvenile sea urchins exposed to high pCO_2 conditions have also displayed reduced growth rates (Shirayama and Thornton 2005). Skeletal growth of adult *Paracentrotus lividus* was inhibited when urchins were exposed to pCO_2 conditions that were five to nine times as high as control groups (Grosjean et al. 1998). Fecundity and larval survival of gametes spawned from adult *S. droebachiensis* exposed to high pCO_2 conditions for four months prior to spawning was also negatively impacted (Dupont et al. 2012). However, when adults were exposed to higher pCO_2 conditions over a period of 16 months, female fecundity and larval survival of gametes spawned from those adults was not adversely affected (Dupont et al. 2012). Finally, juvenile *S. droebachiensis* that were raised under different pCO_2 conditions than they experienced as larvae displayed significantly lower growth rates (mm $month^{-1}$) than those that were maintained under the same pCO_2 regime throughout their life cycle (Dupont et al. 2012). Thus, research that evaluates the entire echinoid life cycle is needed to assess the impact of reduced carbonate saturation states on sea urchin aquaculture. As small fluctuations in pH throughout the life cycle of an echinoid may have significant impacts to its development (Dupont et al. 2012), the range in measurement error of pH may be important. Therefore,

to minimize the range in measurement error and for the greatest accuracy, we recommend that future studies measure total alkalinity and pH following the methods of Dickson et al. (2007) when logistically possible.

In an applied context, the results of the present study suggest that land-based aquaculture facilities need to be more vigilant about alterations to the carbonate chemistry of culture water (i.e., a pH of 7.5–7.8 may not result in ideal early echinoid development). Intensive culture situations where there is significant accumulation of CO_2 and therefore reduced carbonate saturation states may result in delayed growth and significant alterations to larval shape such that the ability to feed may be compromised. *Lytechinus variegatus* is an ideal species for aquaculture studies as it can be reared in commercial artificial seawater using artificial feeds and can achieve sexual maturity in a relatively small amount of time (one year, Moore et al. 1963). The findings of this study on the effects of reduced carbonate saturation states on early development have significant implications for the management and culture of *L. variegatus* and potentially other echinoid species used in aquaculture. It may be possible to mitigate some of these effects by raising the temperature of culturing conditions, as some research has indicated that a 2° - 3°C increase will decrease development time (Sheppard Brennand et al., 2010, Byrne et al., 2011). However, each echinoid species may react differently and therefore research on the specific species being raised in an aquaculture facility should be conducted. The use of separate biofilters and aeration of culture water to remove CO_2 should also be considered. However, aeration should take place before water is introduced to developing larvae as vigorous bubbling is likely to be detrimental to development. Studies are needed to investigate a range of biological loads (i.e., larval

and food densities) to determine the ideal scenario where maximum densities are achieved with minimal impacts to carbonate saturation states. In addition, the monitoring of major ions (i.e., calcium and magnesium) that can impact carbonate saturation states should be considered. Studies that investigate the influence of food concentration on early larval development are also needed to determine how food may alter carbonate chemistry and if delays in growth or alterations in shape may be less significant when abundant energy sources are available. Finally, research that examines the settlement of larvae and metamorphosis into competent juveniles is needed to determine what impacts reduced carbonate saturation state conditions may have on gonad production and quality so that aquaculture facilities can determine ideal conditions for maximum growth and efficiency.

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EFFECTS OF HYPERCAPNIA ON FEEDING, NUTRIENT ABSORPTION EFFICIENCY, NUTRIENT AND ENERGY ALLOCATION, AND GROWTH IN JUVENILES OF THE SEA URCHIN *LYTECHINUS VARIEGATUS* IN THE LABORATORY

by

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Abstract

Juvenile *L. variegatus* (horizontal diameter = 20 mm) were exposed to control (608 µatm pCO_2, pH 8.1) or hypercapnic conditions (1738 µatm pCO_2, pH 7.7) in synthetic seawater for 14 weeks. Fecal production rates were significantly higher and ash absorption efficiency (%) was significantly lower in individuals exposed to hypercapnic conditions, suggesting that the ability to process or retain dietary carbonates was affected. Sea urchins exposed to hypercapnic conditions had significantly reduced total dry matter production, contributed primarily by reduced test dry matter production. Significant increases in neutral lipid storage in the gut occurred under hypercapnic conditions. Gonads of individuals exposed to hypercapnic conditions had increased soluble protein storage. Conversely, organic production and energy allocation increased in the lantern of those individuals exposed to hypercapnic conditions. These results suggest chronic exposure to hypercapnic conditions alters allocation to organ systems and functions, leading to changes in somatic and reproductive production.

Introduction

The physiological impact(s) of carbon dioxide (CO_2) to aquatic animals are not well understood. Increases in CO_2 concentrations in seawater (pCO_2), whether from accumulation of metabolic waste under intensive laboratory conditions (e.g., high densities combined with low water exchange rates) or from increases in atmospheric CO_2, lead to decreases in seawater pH. It has been suggested that increases in pCO_2 affect the acid-base balance in extracellular and intracellular fluids (Melzner et al. 2009;

Hofmann and Todgham 2010). Those organisms that have low capacity for acid-base regulation may be physiologically more vulnerable when exposed to hypercapnic conditions (see Melzner et al. 2009, and Hofmann and Todgham 2010, for a discussion). Because of these reasons, echinoderms have been identified as a group of organisms likely to be sensitive to increased pCO_2 conditions (Pörtner et al. 2004; Melzner et al. 2009). The impacts of CO_2 on echinoids are of particular interest since they have important ecological roles and are important in fisheries and aquaculture.

The accumulation of CO_2 in aquaculture systems affects the health of sea urchins (Grosjean et al. 1998; Timmons et al. 2001; Siikavuopio et al. 2007). Echinoid feeding may be impacted by increases in pCO_2 as the process has been shown to be affected by other abiotic factors including temperature and phosphates (Sloan and Campbell 1982; Klinger et al. 1986; Lares and McClintock 1991; Böttger et al. 2001). At extremely high pCO_2 concentrations (8000 μatm, pH 6.98), feeding rates, feed intake, and feed conversion efficiency were significantly reduced in adult *Strongylocentrotus droebachiensis* (Siikavuopio et al. 2007). Adult *S. droebachiensis* exposed to high hypercapnic conditions (2800 – 3800 μatm, pH_{NBS} 7.25-7.19) also had reductions in ingestion and egestion, yet there was no difference in digestibility of organic material (absorption efficiency) (Stumpp et al. 2012).

In addition to feeding, chronic exposure to hypercapnic conditions may affect the processing of nutrients and allocation of energy. Changes in abiotic factors including temperature and phosphate concentrations affect these processes in echinoids (Klinger et al. 1986; Lares and McClintock 1991; Böttger et al. 2001; Watts et al. 2011). In adult *S. droebachiensis* exposed to high pCO_2 conditions (2800 -3800 μatm), organic (ash-free

dry mass) and inorganic (ash dry mass) matter content of body components were significantly reduced and less energy was allocated to the gonads and test (Stumpp et al. 2012). In addition to the acquisition and processing of nutrients, hypercapnic conditions affect the weight gain of echinoids. Adult *S. droebachiensis* exposed to high pCO_2 conditions have reduced somatic and gonad weight gain (Siikavuopio et al. 2007; Stumpp et al. 2012). In addition to adults, the few studies on juvenile sea urchins exposed to hypercapnic conditions have reported reductions in growth rates (Shirayama and Thornton 2005; Albright et al. 2012). Juvenile *Lytechinus variegatus* (initial body wet weight 30.4 ± 3.5 mg) exposed to high pCO_2 conditions (800 μatm) had reduced growth rates and structurally degraded spines (Albright et al. 2012). Juvenile (5 days post settlement) *Tripneustes gratilla* reared from fertilization in hypercapnic conditions (pH 7.6 -7.8) had significantly fewer spines and a significantly greater percentage of abnormalities (arrested embryonic or larval development, mortality, or irregular juvenile profile) (Byrne et al. 2011). Juveniles raised in high pCO_2 conditions (1200 μatm) from fertilization and whose parents had been exposed for a period of 4 months to the same high pCO_2 conditions experienced significant mortality (95%) (Dupont et al. 2012). Yet those surviving juveniles whose pCO_2 regime remained unchanged throughout early development and metamorphosis, regardless of whether it was a control (400 μatm) or high (1200 μatm) regime, had significantly better growth rates than those that experienced a change in pCO_2 regime during their lifetime (Dupont et al. 2012). Therefore, exposure to hypercapnic conditions can have significant impacts to the processing of nutrients and ultimately the growth (weight gain or production of new tissue) of sea urchins.

Echinoderms have discrete life stages with extensive physical and physiological differences. Several studies have indicated that subtle reductions (0.4 units) in pH due to increased pCO_2 can have significant negative effects on echinoid early development including changes in gene expression and slower larval growth rates (reviewed by Dupont et al. 2010, Byrne 2011, and Sewell and Hofmann 2013). However, the impact of increased pCO_2 exposure on juvenile and adult echinoids has not been studied extensively. The echinoid juvenile stage is the period of maximal weight gain. Therefore, the vulnerability of this stage to hypercapnic conditions may vary substantially from that of the embryonic and larval stages. We are unaware of any studies that have specifically examined aspects of feeding, absorption and allocation of nutrients, and energy allocation in juvenile urchins. Therefore, there is a need to further determine how exposure to hypercapnia affects juvenile sea urchin growth and physiology.

The basic and applied biology of *Lytechinus variegatus* has been well studied in the laboratory (e.g., Mazur and Miller 1971; Lawrence 1975; Klinger et al. 1986; Bishop et al. 1994; Beddingfield and McClintock 1998; Böttger and McClintock 2002; Hammer et al. 2004; Powell et al. 2004; Nelson et al. 2010; Heflin et al. 2012) and there has been a significant amount of research into its nutritional biology (e.g., Lowe and Lawrence 1976; Bishop and Watts 1992; Klinger et al. 1994; Böttger et al. 2001; Lawrence et al. 2003; Powell et al. 2004; Hammer et al. 2006a; Hammer et al. 2006b; Gibbs et al. 2009; Watts et al. 2011). These studies commonly use commercial synthetic sea salts and employ intensive culture such that accumulation of metabolic wastes such as CO_2 can occur. The objective of the present study was to evaluate the effects of hypercapnic

conditions on the feeding, nutrient absorption, energy and nutrient allocation, and growth in juveniles of the common sea urchin *Lytechinus variegatus* under laboratory conditions.

Methods

Collection and characterization of juveniles

In May, 2010, small *Lytechinus variegatus*, approximately 20 mm in diameter, were hand collected from Eagle Harbor at Saint Joseph Bay, FL, (29^0N, 85^0W) and transported to the University of Alabama at Birmingham, Birmingham, AL. Sea urchins were held for one week prior to the start of the experiment in synthetic seawater (Instant Ocean®, 32 ± 1 ‰ salinity) at constant photoperiod and temperature (12 h light; 12 h dark; 24 ± 1 $^{\circ}$C) and were not fed during this time. Twenty-four individuals were randomly selected to determine initial size and gonadal state. Individuals were placed on paper towels for 30 seconds to remove excess water and weighed. Diameter was measured using calipers (average of two measurements made perpendicular to each other at the ambitus). To obtain body component weights and indices, urchins were dissected and wet weights were obtained for the test (with spines and peristomial membrane), gut (esophagus, stomach and intestine; rinsed thoroughly in deionized water to remove gut contents, and Aristotle's lantern. Visible gonadal tissues were not observed. All components were rinsed briefly in deionized water to remove salt and were placed on paper towels for 30 seconds prior to weighing. Body components were then frozen and, at a later time, thawed and dried in an oven for 3 days at 50°C to a constant weight prior to biochemical analysis.

Seawater chemistry

Synthetic seawater was produced using Instant Ocean® (Spectrum Brands) commercial sea salts and purified water (tap water filtered through a Four-Stage Barracuda RO/DI three-part filter system, AQUAfx, Aqua Engineering and Equipment, Winter Park, FL). Experimental hypercapnic conditions were produced by bubbling pure CO_2 gas into each tank and maintained by measuring pH. pH was checked daily using a Fisher Scientific Accumet© basic model AB15 pH meter with an ACCUTU pH electrode (Cat. # 13-620-183) calibrated with NBS standard buffers. A pH monitoring system (Aqua-Medic) was used to ensure that the pH_{NBS} did not change more than 0.04 units. pCO_2 was calculated using pH and total alkalinity. Total potentiometric pH was determined weekly following the methods of Dickson et al. (2007). Total alkalinity was measured by potentiometric titration every third day for each tank using the methods of the American Public Health Association (APHA 1992) modified for use with a Hach digital titrator (Hach Company, Loveland, USA). This method was tested for accuracy against certified reference materials provided by the laboratory of Andrew G. Dickson (University of California, San Diego, Scripps Institute of Oceanography) and was found to have an SEM of ± 66 μmol kg^{-1} (n = 6). Calcite and aragonite saturation states and pCO_2 were determined using the Microsoft Excel spreadsheet 'co2sys.xls' based on work by Lewis and Wallace (1998) and provided by Pelletier et al. (2007) with the CO_2 constants of Millero et al. (2006) and the $KHSO_4$ constant of Dickson (1990). Room air was used as the control treatment and was supplied via air stones plumbed to a CORALIFE® SL-65 Super-Luft pump. As significant water exchanges (ca. 50%) were made every 24 -48 hours, we assumed that the concentration of calcium and magnesium

ions did not fluctuate significantly and therefore were within normal seawater ranges as reported by Instant Ocean® (400mg L^{-1} calcium ion and 1320 mg L^{-1} magnesium ion, http://www.instantocean.com). Water in the experimental group was replaced with previously acidified water to maintain pH levels and hypercapnic conditions. Total ammonia, nitrite, and nitrate levels were initially measured every 24 -48 hours using saltwater test kits from the Hach Company until levels were stable and then were measured weekly. Water quality parameters were maintained within safe levels for nitrogen (no negative effect on growth) according to Basuyaux and Mathieu (1999): total ammonia nitrogen and nitrite < 0.5 mg L^{-1} and nitrate < 10 mg L^{-1}. Dissolved oxygen and salinity were monitored weekly using an YSI 85 meter. Temperature was measured daily by using the thermistor on the pH meter mentioned above.

Experimental design

Juvenile sea urchins (average 23.4 mm ± 0.19 SEM diameter, 5.71g ± 0.11 SEM wet weight) were randomly selected from the collection and placed individually into plastic, cylindrical cages (25.4 x 10.2 cm, H x D) with two sea urchins per 19 L glass aquarium (n = 12 aquaria (24 urchins) per treatment). Each aquarium had its own filtration pump (Aqueon Quiet Flow 10) containing activated charcoal. The experimental treatment was held at pCO_2 of 1738 ± 25 μatm, pH = 7.7 and the control held at pCO_2 of 608 ± 12 μatm, pH = 8.1. A t-test confirmed that there was no significant difference in wet weights or diameters of individuals between treatments (t_{46} = -0.797, p 0.435 for weight; t_{46} = -0.911, p = 0.367 for diameter). Survivorship was 100% after 6 weeks and one urchin was removed from each aquarium to reduce biomass. Cages and aquaria were cleaned every three weeks to remove potential algal and bacterial growth. Diameter and

wet weight of each sea urchin was recorded at each three week time period Each individual was fed a formulated feed (Addison Lawrence, Texas A & M University) at a subsatiation level determined previously for *L. variegatus* (1.5 % of wet weight, Gibbs 2011) every 24 hours. Specifically, the amount of food fed was adjusted every three weeks (when new wet weights were measured) by taking the average of the wet weights of all individuals in a treatment and multiplying by 0.015. Subsatiation feeding allows accurate assessment of feed intake and production efficiencies (Watts et al. 2013). Prior to the start of the experiment, autogenic controls were placed in each aquarium for 24 hours to determine whether hypercapnic conditions affected wet weight of the food. No significant difference was found (t-test, $t_{22} = 1.282$, $p = 0.213$). Uneaten food and feces were removed every two days by siphon. Throughout the course of the experiment, salinity was maintained at 32.4 ‰ ± 0.02 SEM, temperature was 25°C ± 0.02 SEM, and photoperiod was held constant at 12 h light:12 h dark.

Apparent feed intake and digestibility

At the end of the 12 week experiment, a consumption and fecal production trial was performed on the remaining twelve individuals in each treatment. Pre-weighed agar blocks containing the same diet used in the study were fed to each individual (Hammer et al. 2004). Controls for each tank were evaluated (average change in wet weight after 24 hours exposure to tank water was 3.17 % ± 0.31 SEM and was not statistically significantly different across treatment groups using a t-test; $t_{22} = -0.039$, $p = 0.969$). Fresh agar feed was made every four days for two weeks. Each day prior to feeding, uneaten food was removed from the cage by hand and feces were removed by siphon and collected on a 50 μm filter. Collected uneaten feed and feces were rinsed with deionized

water to remove salt. Feces were visually inspected and any spines or other non-fecal material were removed. For each individual, uneaten food and fecal material were collected and placed in separate, pre-weighed aluminum pans. Each day pans were placed in a drying oven at 50°C to remove moisture. As the trial progressed, collected uneaten food and fecal material were pooled for each individual. On day 14, sea urchins were re-weighed. Dry matter and moisture content was determined for each batch of agar feed. Dry weight of the collected uneaten feed and feces was used to determine dry matter intake and egestion.

Total dry feed intake (g individual^{-1}) was calculated by subtracting total dry feed recovered from total dry feed fed. Feeding rate (% body weight consumed individual^{-1} day^{-1}) was calculated by total dry feed fed (g) – total dry feed recovered (g) / average total weight of sea urchin (g) / number of days fed x 100. Fecal production rate (% body weight produced individual^{-1} day^{-1}) was calculated by total dry feces recovered (g) / average total weight of sea urchin (g) / number of days of feces recovery x 100. Apparent dry matter digestibility (%) was calculated by total dry feed intake (g) – total dry feces (g) / total dry feed intake (g) x 100. Nutrient intake was calculated by concentration of nutrient in the food (decimal %) x total dry feed intake (g). Apparent nutrient digestibility was calculated by the amount of nutrient consumed (g) – nutrient content of feces (g) / nutrient consumed (g) x 100. Apparent digestible energy (%) was calculated by energy content of total dry feed consumed (kcal) – energy content of feces (kcal)/ total energy content of dry feed consumed (kcal) x 100.

Growth and production

At time zero and every three weeks thereafter, wet weight and diameter were measured for each individual urchin. Diameter was measured via image analysis (Image J®) using digital photos of urchins recorded with a ruler for size reference. At the end of the experiment the diameter and height of the individuals were determined using calipers. Body component weights and indices were measured by dissecting the gut, gonad, Aristotle's lantern and test with spines (including peristomial membrane) as described above for the baseline individuals. To obtain body component indices, wet weights of body components were divided by the whole wet weight of the animal and multiplied by 100 to obtain percents. Body components were frozen, thawed at a later time, and dried in an oven for 3 days at 50°C to a constant weight. Dry weight and moisture content of each component were measured prior to biochemical analysis. Total dry matter was calculated as dry weight of all organs summed together. To eliminate moisture content as a variable, dry organ indices were also calculated by as the dry weight of the organ (g) / (dry weight of test with spines (g) + dry weight of Aristotle's lantern (g) + dry weight of gut (g) + dry weight of gonad (g)) x 100. To eliminate organ allometry as a statistical variable, dry organ indices relative to the test (with spines and peristomial membrane) were calculated by as the dry weight of the organ (g) / dry weight of the test (g) x 100.

Total production was calculated by subtracting the initial average dry weight of baseline individuals collected from the field from the total dry weight of urchins at the end of the experiment. This calculation was repeated to obtain total organic production and total inorganic production for each individual and each organ with the exception of the gonads, as there were none visible in the initial baseline group. The gut of the initial

urchins was too small for biochemical analyses. Therefore an average initial inorganic (ash) concentration was obtained from a previous study on *Lytechinus variegatus* collected from the same area ranging in horizontal diameter from 30 – 50 mm (11.55 %, Böttger et al. 2001). Initial gut inorganic dry matter was calculated as 0.1155 x initial dry weight of each baseline individual.

Prior to biochemical analysis, a small portion of each test with spines was removed using fine scissors. Spines were removed from these portions and an ossicle from the ambitus of the test of each individual was analyzed for estimated strength using a penetrometer. A steel pin attached to a circular acrylic base was placed in the middle of an ossicle and lead shot was slowly added to a beaker that rested on the acrylic base until the pin punched through the ossicle. The weight required to penetrate the test (g) was determined by combining the weight of the lead shot and beaker. The force required to penetrate the test (N) was calculated as weight required to penetrate the test (g) / 101.972 (g/N).

Biochemical analysis

Dried test with spines and peristomial membrane, Aristotle's lantern, food, and feces were ground in a Wiley Mill. Dried gut and gonad were ground using a mortar and pestle. Because of the small amount of tissue, each of the organs from two individuals were pooled randomly within the two treatments (n = 6 pooled replicates). When possible (i.e., enough tissue was available) analysis was performed in triplicate. Total soluble protein (soluble in 1 N sodium hydroxide, NaOH) was determined spectrophotometrically using the methods of Lowry et al. (1951) and total carbohydrate

(soluble in reagent-grade sulfuric acid) was determined spectrophotometrically using the methods of DuBois et al. (1956). Total lipid was determined gravimetrically using the methods of Folch et al. (1957) modified by Gibbs et al. (2009) by washing crude extracts with a 0.9 % solution of sodium chloride prior to isolation to increase the efficiency of phase separation. Total nonpolar lipid concentrations (% dry weight) were determined by separating total lipids into nonpolar fractions gravimetrically following the methods of Juaneda and Rocquelin (1985) using Sep Pak Vac 12cc (2 G) silica cartridges (Waters Corporation, Milford, MA, USA). Samples of dried body components, food and feces were ashed in a muffle furnace at 500°C for 5 hours to obtain ash concentrations. Insoluble protein was assumed to be the portion not soluble in 1 N NaOH and was obtained by subtraction [1 – (lipid + ash + soluble protein + carbohydrate concentrations)]. Organic concentrations were determined by 1 –ash decimal %. Nutrient content (mg dry weight) was determined by multiplying the nutrient concentration (decimal %) by the original dry weight of the organ (mg). When organs had been combined prior to biochemical analysis, content was determined by [(nutrient concentration (decimal %) x (combined dry weight of the 2 organs)] / 2.

Energetic analysis

Energy (cal g dry weight^{-1}) of dry feed fed (triplicate subsamples) and feces (n = 12 individuals per treatment) collected from the digestibility trial was determined by micro-bomb calorimetry (Model 1261, Parr Instruments, Moline, IL, USA). These values were used to calculate total energy ingested (kcal) and total energy of feces (kcal). To determine the average amount of caloric energy allocated to various body components, energetic equivalents were used (5.64, 9.44, and 4.11 kcal g^{-1} for proteins, lipids and

carbohydrates, respectively; Blaxter 1989) and multiplied by the nutrient content (g) of each organ. Total energy was calculated by summing the energy content of the organs of each individual.

Statistical analysis

Analyses were made on various parameters measured for the individuals that were present throughout the entire experiment. For all analyses $p < 0.05$ was considered statistically significant. Normality and equal variances were tested using the Shapiro Wilk's test for normality and the Levene's test for equal variances in Sigma Plot version 11.2.

Apparent feed and nutrient intake, apparent dry matter and nutrient digestibility, feeding rates and fecal production rates, total dry matter, ossicle strength, proximate biochemical concentrations, content, and energy were evaluated using a t-test. When the data did not meet the assumptions for parametric testing the Mann-Whitney U rank sum test was used. Because organs were combined in order to perform biochemical analyses, an ANCOVA on biochemical and energy content was not possible. All percentage data were arcsine transformed prior to statistical testing and all tests were performed using SigmaPlot 11.2. Total energy consumed (kcal) and apparent digestible energy (%) were analyzed using a t-test, or the Mann-Whitney U rank sum test when data did not meet the assumptions of parametric testing.

Mean whole wet weights and diameters between the two treatments were evaluated first by testing for sphericity. In all cases data were log transformed prior to analysis and the Huynh-Feldt Epsilon values were > 0.7. Therefore, departures from

sphericity were not great. Treatments were then tested using a repeated measures analysis of variance (RMANOVA) using SYSTAT version 11.0. Final volume, organ wet and dry weights, and organ indices were compared between treatments using a t-test in SigmaPlot 11.2. Body component indices were arcsine transformed prior to testing. When data failed the requirements of parametric testing, the Mann-Whitney U test was performed.

Results

Seawater chemistry

Over the course of the experiment pH was used to indicate hypercapnic condition and was stable in both the control and experimental treatments (Table 1). Average carbonate saturation states and pCO_2 (calculated using temperature, salinity, pH_{NBS} and total alkalinity) over the course of the experiment were also stable for the control (n = 40) and the experimental treatment (n = 54). Dissolved oxygen was never below 90% saturation in any aquarium.

Feed intake and apparent digestibility

The apparent total amount of dry feed intake and the apparent dry matter digestibility did not vary significantly with treatment (Table 2). Feeding rates were similar between different treatments; however, the fecal production rates of urchins exposed to hypercapnic conditions were significantly higher than those of the control. Intake for each nutrient (lipid, nonpolar lipid, soluble protein, carbohydrate and ash) was

Table 1. Measured and calculated parameters of seawater carbonate chemistry for the control (n = 40) and high pCO_2 treatments (n = 54) over the course of the experiment. Values are means and values in parentheses are standard error of the mean. pCO_2 and saturation states calculated using CO2SYS version 14 (Pelletier et al. 2007). Ω Ca = saturation state of calcite, Ω Ar = saturation state of aragonite.

Parameter	Control	Experimental
pH_{NBS}	8.10 (0.004)	7.70 (0.002)
$pH_{Total\ H}$	8.06 (0.006)	7.69 (0.003)
pCO_2 (µatm)	608.47 (11.77)	1738.64 (25.01)
Temperature (°C)	25.00 (0.03)	25.04 (0.03)
Dissolved oxygen (%)	91.20 (0.05)	91.02 (0.08)
Salinity (‰)	32.40 (0.02)	32.42 (0.03)
Total alkalinity (µmol kg^{-1})	2934.86 (22.71)	2730.88 (33.51)
Ω Ca	5.86 (0.09)	2.27 (0.04)
Ω Ar	3.84 (0.06)	1.49 (0.03)

Table 2. *Lytechinus variegatus.* Summary of feed intake, feeding and fecal production rates, and apparent digestibility for juvenile urchins maintained under control (608 µatm) and high pCO_2 (1738 µatm) conditions. Values represent means ± standard error of the mean. Asterick indicates significantly different values ($p < 0.05$), n = 12 per treatment. Test statistic U = Mann-Whitney U rank sum test; t = t-test.

Parameter measured	Control	High pCO_2	Test statistic	df	p
Total dry feed intake (g)	5.87 ± 0.08	5.50 ± 0.17	U = 45.000		0.126
Apparent dry matter digestibility (%)	77.21 ± 0.67	75.01 ± 0.84	t = 2.014	22	0.056
Feeding rate (% body weight day^{-1})	1.21 ± 0.05	1.33 ± 0.06	t = -1.660	22	0.111
Fecal production rate (% body weight day^{-1})*	0.27 ± 0.01	0.34 ± 0.02	t = -2.334	22	0.029
Total lipid intake (mg)	289.10 ± 3.94	270.65 ± 8.59	U = 45.000		0.126
Apparent total lipid digestibility(%)	87.83 ± 0.37	88.28 ± 0.77	t = -0.635	22	0.532
Nonpolar lipid intake (mg)	52.41 ± 0.71	49.06 ± 0.56	U = 45.000		0.126
Apparent nonpolar lipid digestibility (%)	88.76 ± 1.03	86.22 ± 1.19	t = 1.627	22	0.118
Total soluble protein intake (mg)	376.53 ± 5.13	352.50 ± 11.19	U = 45.000		0.126
Apparent total soluble protein digestibility (%)	33.33 ± 2.58	24.66 ± 3.67	t = 2.053	22	0.052
Total carbohydrate intake (mg)	1085.36 ± 14.80	1016.09 ± 32.26	U = 45.000		0.126
Apparent total carbohydrate digestibility (%)	96.65 ± 0.22	96.22 ± 0.25	t = 1.312	22	0.203
Total ash intake (mg)	2331.04 ± 31.78	2182.26 ± 69.28	U = 45.000		0.126
Apparent ash digestibility (%)*	77.02 ± 0.83	73.77 ± 0.96	t = 2.547	22	0.018
Total energy intake (kcal)	15.68 ± 0.21	14.68 ± 0.47	U = 45.000		0.126
Apparent digestible energy (%)	78.40 ± 0.52	77.13 ± 0.76	t = 1.330	22	0.197

not significantly different between treatments. Of all the nutrients, only the apparent digestibility of unknown elements within ash was significantly reduced in the hypercapnic conditions. Total energy intake and apparent digestible energy were not significantly different between treatments.

Growth

Over the course of the 14 week experiment there were significant increases in mean whole wet body weight within each treatment (RMANOVA, $F_{5, 110} = 1.34 \times 10^3$, $p < 0.0001$, n =12 per treatment). Individuals in the control had a final mean wet body weight of 40.50g ± 1.62 and those in hypercapnic conditions had a mean final wet body weight of 34.50g ± 1.85, representing a 16 % difference in final average wet weight (Fig. 1A). In addition, sea urchins in the hypercapnic conditions had a slower growth rate (i.e., time x treatment effect, RMANOVA, $F_{5, 110} = 6.89346$, $p < 0.0001$). The diameters of sea urchins increased significantly over time in both treatments (RMANOVA, $F_{5, 110} = 7.30 \times 10^2$, $p < 0.0001$, n = 12 per treatment) but were significantly smaller in individuals exposed to hypercapnic conditions (Fig. 1B). Individuals exposed to hypercapnic conditions had a slower growth rate in terms of change in diameter (RMANOVA, $F_{5, 110} = 4.08529$, $p = 0.00193$). Final coelomic volume, calculated using the volume for a spheroid ($(\pi/6)*(A^2C)$, where A = final average diameter and C = final height), was significantly higher in the control (31342.75 mm^3± 1052 SEM) than those held in hypercapnic conditions (27692.78 mm^3± 1344.05 SEM) (Mann-Whitney rank sum test, U = 37.000, p = 0.046, n = 12 per treatment).

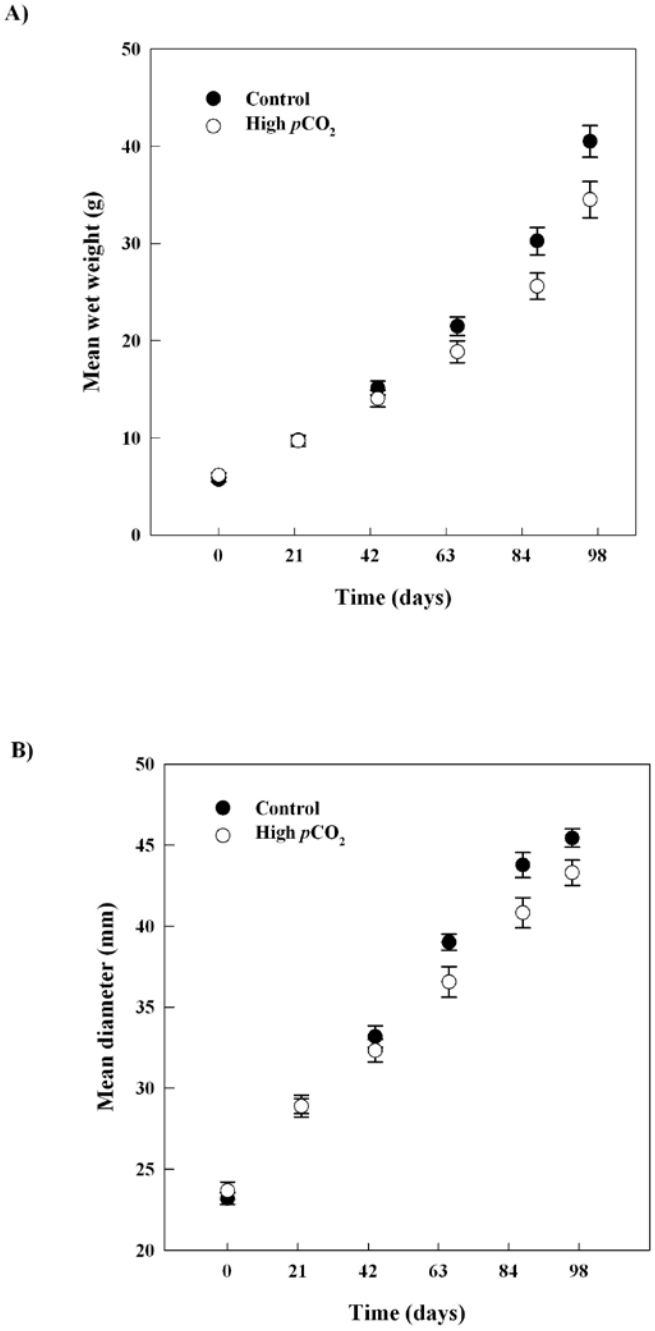

Figure 1. *Lytechinus variegatus*. Organismal growth. (A) Mean whole wet weights (g) and (B) mean test diameters (mm) of small sea urchins exposed to control versus high pCO_2 conditions over time. Values are means of each treatment ± standard error of the mean (n = 12 per treatment). It should be noted that the final weight and diameter measurements were determined after the feeding and digestibility trial, where urchins were fed ad libitum agar blocks (containing the same feed type) for two weeks. Growth rates (both wet weights and diameters) of the two treatments were significantly different by RMANOVA ($p < 0.05$).

For each body component, moisture content (%) was not significantly different between the two treatments (t- test or Mann-Whitney U rank sum test, data not shown). Final wet and dry test (with spines and peristomial membrane) weights were significantly smaller in sea urchins exposed to hypercapnic conditions (Table 3). Conversely, the wet and dry test indices were not significantly different. Final dry weights of the gut were not significantly different between treatments. However, the wet and dry gut indices and dry test:gut indices were significantly higher in individuals exposed to hypercapnic conditions. Final gonad wet weights were significantly lower in sea urchins exposed to hypercapnic conditions. Final dry weights, wet indices, dry indices and dry test:gonad indices of the gonads of individuals exposed to hypercapnic conditions were not significantly different from those in the control treatment. Final wet weights, dry weights, and wet indices of the Aristotle's lantern were not significantly different among treatments. However, the dry lantern indices and dry test:lantern indices were significantly higher in hypercapnic conditions. Total dry matter was significantly different between treatments with less total dry matter observed in individuals maintained in hypercapnic conditions (Table 6B). Similarly, total organic and inorganic dry matter were lower in those individuals exposed to hypercapnic conditions.

Organ composition

Concentrations of soluble protein were significantly higher in the test (with spines and peristomial membrane) of individuals raised under hypercapnic conditions (Table 4). Content (mg dry weight) of ash, insoluble protein, and organic matter was significantly lower in the test of sea urchins exposed to hypercapnic conditions compared to controls (Table 5A). Concentration (Table 4) and content (Table 5A) of nonpolar lipid in the gut

Table 3. *Lytechinus variegatus*. Mean body component wet weight, dry weight, and organ index ± SEM of the gut, gonad, test (with spines and peristomial membrane), and Aristotle's lantern. Sea urchins dissected after 14 weeks' exposure to control (608 µatm pCO_2) or experimental (1738 µm pCO_2) conditions (n = 12 per treatment). Asterick indicates significantly different values ($p < 0.05$) by t-test (t statistic) or Mann-Whitney U rank sum test (U statistic).

Parameter tested	Control	High p CO_2	Test statistic	df	p
Wet test weight (g)*	**15.22 ± 0.58**	**12.38 ± 0.55**	**t = 3.568**	**22**	**0.002**
Dry test weight (g)*	**5.33 ± 0.19**	**4.27 ± 0.20**	**t = 3.871**	**22**	**<0.001**
Wet test index (%)	**37.02 ± 0.82**	**35.72 ± 0.67**	**t = 1.211**	**22**	**0.239**
Dry test index (%)	**77.65 ± 1.17**	**75.11 ± 0.94**	**t = 1.729**	**22**	**0.098**
Wet gut weight (g)	**0.89 ± 0.06**	**0.90 ± 0.06**	**t = -0.115**	**22**	**0.909**
Dry gut weight (g)	**0.21 ± 0.03**	**0.18 ± 0.01**	**t = -0.0897**	**22**	**0.929**
Wet gut index (%)*	**2.17 ± 0.1**	**2.58 ± 0.09**	**t = -3.240**	**22**	**0.004**
Dry gut index (%)*	**2.55 ± 0.12**	**3.08 ± 0.08**	**t = -3.813**	**22**	**< 0.001**
Dry gut: test index (%)*	**3.30 ± 0.17**	**4.11 ± 0.12**	**t = -4.011**	**22**	**< 0.001**
Wet gonad weight (g)*	**4.77 ± 0.40**	**3.64 ± 0.37**	**t = 2.096**	**22**	**0.048**
Dry gonad weight (g)	**0.83 ± 0.08**	**0.67 ± 0.08**	**U = 44.000**		**0.112**
Wet gonad index (%)	**11.72 ± 1.03**	**10.30 ± 0.70**	**t = 1.097**	**22**	**0.284**
Dry gonad index (%)	**12.19 ± 1.10**	**11.44 ± 0.92**	**t = 0.484**	**22**	**0.633**
Dry gonad: test index (%)	**15.97 ± 1.69**	**15.43 ± 1.46**	**t = 0.199**	**22**	**0.844**
Wet lantern weight (g)	**0.88 ± 0.03**	**0.98 ± 0.06**	**t = -1.512**	**22**	**0.145**
Dry lantern weight (g)	**0.52 ± 0.02**	**0.59 ± 0.03**	**U = 42.000**		**0.089**
Wet lantern index (%)*	**2.15 ± 0.07**	**2.81 ± 0.07**	**t = -6.708**	**22**	**< 0.001**
Dry lantern index (%)*	**7.61 ± 0.21**	**10.37 ± 0.79**	**t = -8.731**	**22**	**< 0.001**
Dry lantern: test index (%)*	**9.84 ± 0.35**	**13.84 ± 0.38**	**t = -7.637**	**22**	**< 0.001**

Table 4. *Lytechinus variegatus*. Proximate biochemical composition of body components in terms of concentration (% dry weight) of nutrients of individuals exposed to either control pCO_2 (608 µatm) or high pCO_2 (1738 µatm) conditions over 14 weeks. Values are means ± standard error of the mean. n = 6 for each treatment. Asterick indicates significantly different values ($p < 0.05$) by t-test (t statistic) or Mann-Whitney U rank sum test (U statistic).

	Control	**High *p* CO_2**	**Test Statistic**	**df**	**p**
Gut					
Ash	8.52 ± 0.17	8.80 ± 0.44	t = -0.548	10	0.595
Carbohydrate	3.57 ± 0.33	4.54 ± 0.44	t = -1.782	10	0.105
Total Lipid	25.83 ± 0.84	24.94 ± 0.54	t = 0.871	10	0.404
Nonpolar Lipid*	3.63 ± 0.19	4.25 ± 0.10	t = -2.810	10	0.018
Soluble Protein	28.17 ± 0.59	27.26 ± 0.50	t = 1.191	10	0.261
Insoluble Protein	33.91 ± 0.95	34.47 ± 0.67	t = -0.495	10	0.631
Organic	91.48 ± 0.17	91.2 ± 0.44	t = 0.548	10	0.595
Gonad					
Ash	8.48 ± 0.47	8.54 ± 0.63	U = 17.000		0.937
Carbohydrate	26.81 ± 2.91	25.52 ± 2.37	t = 0.318	10	0.757
Total Lipid	20.95 ± 0.52	20.75 ± 0.28	t = 0.311	10	0.762
Nonpolar Lipid	4.06 ± 0.29	3.49 ± 0.08	t = 1.892	10	0.088
Soluble Protein*	21.44 ± 0.74	25.00 ± 0.74	t = -3.397	10	0.007
Insoluble Protein	22.32 ± 2.33	20.20 ± 1.58	t = 0.707	10	0.496
Organic	91.52 ± 0.47	91.46 ± 0.63	U = 17.000		0.937
Test					
Ash	88.29 ± 0.31	88.39 ± 0.23	t = -0.253	10	0.806
Carbohydrate	0.5 ± 0.02	0.14 ± 0.04	U = 10.000		0.24
Total Lipid	1.44 ± 0.08	1.55 ± 0.07	t = -1.023	10	0.33
Nonpolar Lipid	0.35 ± 0.04	0.41 ± 0.04	U = 9.000		0.18
Soluble Protein*	3.74 ± 0.08	4.31 ± 0.14	t = -3.614	10	0.005
Insoluble Protein*	6.37 ± 0.29	5.60 ± 0.18	t = 2.298	10	0.044
Organic	11.71 ± 0.31	11.61 ± 0.23	t = 0.253	10	0.806
Lantern					
Ash	86.90 ± 0.32	87.00 ± 0.22	t = -0.231	10	0.822
Carbohydrate	0.47 ± 0.05	0.52 ± 0.13	t = -1.782	10	0.105
Total Lipid	1.52 ± 0.18	1.21 ± 0.13	t = 1.355	10	0.205
Nonpolar Lipid	0.42 ± 0.08	0.27 ± 0.08	t = 1.546	10	0.153
Soluble Protein	4.30 ± 0.23	4.24 ± 0.21	t = 0.207	10	0.84
Insoluble Protein	7.05 ± 0.48	7.30 ± 0.23	t = -0.527	10	0.61
Organic	13.10 ± 0.32	13.01 ± 0.22	t = 0.231	10	0.822

were significantly higher in sea urchins maintained under hypercapnic conditions than controls. Concentrations of total soluble protein were significantly higher in the gonads of those sea urchins exposed to hypercapnic conditions compared to controls (Table 4). Ash, nonpolar lipid, and organic content of gonads of individuals exposed to hypercapnic conditions were significantly lower than individuals in the control treatment (Table 5B). Organic content was significantly higher in the lantern of those individuals exposed to hypercapnic conditions (Table 5B).

Ossicle strength

Force required to penetrate dry test ossicles was significantly less in the control treatment (8.16N ± 0.68 SEM) than those exposed to hypercapnic conditions (11.46 N ± 0.89 SEM; t-test, $t_{20} = -2.989$, $p = 0.007$, $n = 12$ in the control, $n = 10$ in the experimental).

Production and energy content

Total dry matter production, including both organic and inorganic production, and total caloric energy content were significantly lower in individuals exposed to hypercapnic conditions (Table 6B). Test dry matter production, including both organic and inorganic production, and energy content were significantly lower in those individuals exposed to hypercapnic conditions (Table 5A). Gut dry matter production and energy content was not significantly different between treatments (Table 5A). Gonad dry matter production was not significantly different between treatments; however, inorganic and organic production and energy content were significantly reduced in gonads of individuals exposed to hypercapnic conditions (Table 5B). Conversely, lantern organic

production and energy content were significantly higher in individuals exposed to hypercapnic conditions (Table 5B).

Table 5. *Lytechinus variegatus*. Production (mg), energy content (cal) and proximate nutrient content (mg dry weight) of the A) test and gut and B) gonad and lantern of individuals exposed to either control pCO_2 (608 µatm) or high pCO_2 (1738 µatm) conditions over the course of 14 weeks. Values are the mean ± standard error of the mean. For dry matter production, n = 12 per treatment. For organic and inorganic production, energy content, and nutrient content, n = 6 per treatment because organs were combined for biochemical analyses. Asterick indicates significantly different values ($p < 0.05$) by t-test (t statistic) or Mann-Whitney U rank sum test (U statistic). ‡ = Inorganic level for initial gut values was obtained from Böttger et al. 2001.

Table 5A.

	Control	High p CO_2	Test Statistic	df	p
Test					
Test Dry Matter Production*	4326.15 ± 187.74	3271.14 ± 197.56	t = 3.871	22	<0.001
Test Organic Production*	528.59 ± 20.52	401.26 ± 20.54	t = 4.385	10	0.001
Test Inorganic Production*	3797.56 ± 198.93	2869.88 ± 197.62	t = 3.308	10	0.008
Test Energy Content (cal)*	3787.78 ± 126.41	3031.18 ± 124.80	t = 4.259	10	0.002
Organic Content*	621.88 ± 20.52	494.55 ± 20.54	t = 4.385	10	0.001
Ash*	4707.00 ± 198.93	3779.31 ± 197.62	t = 3.308	10	0.008
Carbohydrate	8.24 ± 1.26	6.19 ± 1.98	U = 8.000		0.132
Total Lipid	77.10 ± 6.05	66.15 ± 3.73	t = 1.542	10	0.154
Nonpolar Lipid	18.30 ± 2.31	17.34 ± 1.58	t = 0.345	10	0.737
Soluble Protein	198.79 ± 6.95	183.98 ± 9.73	t = 1.238	10	0.244
Insoluble Protein*	337.75 ± 13.27	238.23 ± 9.88	t = 6.016	10	<0.001
Gut					
Gut Dry Matter Production	148.27 ± 8.59	149.33 ± 8.21	t = -0.0897	22	0.929
Gut Organic Production‡	136.39 ± 6.26	136.79 ± 5.18	t = -0.0486	10	0.962
Gut Inorganic Production‡	11.87 ± 0.79	12.55 ± 1.32	t = -0.436	10	0.672
Gut Energy Content (cal)	1058.57 ± 40.75	1054.10 ± 38.15	t = 0.0800	10	0.938
Organic Content	159.14 ± 6.26	159.53 ± 5.18	t = -0.0486	10	0.962
Ash	14.84 ± 0.79	15.52 ± 1.32	t = -0.436	10	0.672
Carbohydrate	6.19 ± 0.54	7.89 ± 0.68	t = -1.957	10	0.079
Total Lipid	44.86 ± 1.97	43.79 ± 2.38	t = 0.348	10	0.735
Nonpolar Lipid*	6.26 ± 0.22	7.45 ± 0.37	t = -2.776	10	0.020
Soluble Protein	48.92 ± 1.71	47.63 ± 1.52	t = 0.563	10	0.586
Insoluble Protein	59.17 ± 3.49	60.22 ± 1.83	t = -0.267	10	0.795

Table 5B.

	Control	High p CO_2	Test Statistic	df	p
Gonad					
Gonad Dry Matter Production	832.99 ± 79.42	666.83 ± 79.36	U = 44.000		0.112
Gonad Organic Production*	762.69 ± 33.98	610.04 ± 37.70	U = 4.000		0.026
Gonad Inorganic Production*	70.30 ± 3.51	56.80 ± 4.88	t = 2.247	10	0.048
Gonad Energy Content (cal)*	4621.92 ± 200.52	3704.86 ± 222.84	U = 4.000		0.026
Organic Content*	762.69 ± 33.98	610.04 ± 37.70	U = 4.000		0.026
Ash*	70.30 ± 3.51	56.80 ± 4.88	t = 2.247	10	0.048
Carbohydrate	222.11 ± 21.94	171.06 ± 20.29	t = 1.708	10	0.118
Total Lipid*	173.73 ± 4.61	138.42 ± 8.64	U = 5.000		0.041
Nonpolar Lipid*	33.52 ± 1.86	23.32 ± 1.69	t = 4.049	10	0.002
Soluble Protein	179.53 ± 12.67	166.55 ± 10.72	t = 0.782	10	0.452
Insoluble Protein	187.32 ± 22.32	143.00 ± 11.38	t = 2.128	10	0.059
Lantern					
Lantern Dry Matter Production	379.89 ± 18.87	450.81 ± 33.29	U = 42.000	22	0.089
Lantern Organic Production*	46.83 ± 3.52	55.37 ± 2.97	U = 5.000		0.041
Lantern Inorganic Production	430.97 ± 20.66	493.36 ± 26.51	t = -1.856	10	0.093
Lantern Energy Content (cal)*	417.33 ± 19.27	463.91 ± 18.56	U = 5.000		0.041
Organic Content*	68.16 ± 3.52	76.70 ± 2.94	U = 5.000	10	0.041
Ash	452.30 ± 20.66	514.69 ± 26.51	t = -1.856	10	0.093
Carbohydrate	2.42 ± 0.20	2.96 ± 0.63	U = 17.000		0.937
Total Lipid	7.83 ± 0.90	7.24 ± 0.97	t = 0.448	10	0.663
Nonpolar Lipid	2.17 ± 0.36	1.62 ± 0.49	t = 0.911	10	0.384
Soluble Protein	22.25 ± 1.05	24.83 ± 0.84	t = -1.919	10	0.084
Insoluble Protein	36.87 ± 3.63	43.15 ± 2.42	t = -1.440	10	0.180

Table 6. *Lytechinus variegatus*. Total dry matter and total dry matter production, total organic dry matter and total organic production, and total inorganic dry matter and total inorganic production. A) Initial values for baseline group collected at the beginning of the study. Mean ± SEM. ‡ = gut inorganic level derived from Böttger et al. 2001 to obtain content. B) Final mean ± SEM obtained at the end of the study (after 14 weeks) of total dry matter and total dry matter production for individuals exposed to either control (608 µatm pCO_2) or experimental (1738 µm pCO_2) conditions. For total dry matter and production, n = 12 per treatment. For total organic and total inorganic dry matter and production, individuals were combined for biochemical analyses, thus n = 6 per treatment. Asterick indicates significantly different values ($p < 0.05$) by t-test.

Table 6A.

	Total dry matter (mg)	**Total organic dry matter (mg)**	**Total inorganic dry matter (mg)**
Gut‡	25.72 ± 1.99	22.75 ± 1.76	2.97 ± 0.23
Test	1002.73 ± 60.64	93.29 ± 8.23	909.44 ± 61.00
Lantern	140.58 ± 86.80	21.33 ± 1.08	119.24 ± 6.08
Total	1169.02 ± 69.96	137.37 ± 8.64	1031.65 ± 61.53

Table 6B.

	Control	**High p CO_2**	**Test Statistic**	**df**	**p**
Total dry matter (g)*	6.86 ± 0.20	5.71 ± 0.29	t = 3.255	22	0.004
Total dry matter production (g)*	5.69 ± 0.20	4.54 ± 0.29	t = 3.255	22	0.004
Total organic dry matter (g)*	1.67 ± 0.05	1.34 ± 0.04	t = 4.423	10	0.001
Total organic production (g)*	1.47 ± 0.46	1.20 ± 0.40	t = 4.426	10	0.001
Total inorganic dry matter (g)*	5.24 ± 0.22	4.37 ± 0.27	t = 2.799	10	0.019
Total inorganic dry matter production (g)*	4.21 ± 0.22	3.33 ± 0.23	t = 2.799	10	0.019

Discussion

Feeding and growth

The absence of differences in feeding rate, despite differences in fecal production rate, suggests that retention of food within the digestive system may have been altered at high pCO_2. The higher ash content of the feces indicated that the absorption of inorganic material, presumably carbonates, is reduced during exposure to hypercapnia. It is thought that echinoids primarily utilize dissolved carbonates as the source for skeletogenesis (Grosjean et al. 1998) as the pH of the gut is not low enough to effectively dissolve solid carbonates (pH 6.8 in *Lytechinus variegatus*, Prim and Lawrence 1975; pH 6 to 8 in various echinoid species, reviewed in Lawrence 1982). However, the contribution of ingested carbonates to urchin growth is not known. Mechanistically, the differences in fecal production rate and ash digestibility may be the result of changes in microfaunal populations or metabolic activity in the gut. Microbial mediated processes are affected by experimental increases in pCO_2 (e.g., Beman et al. 2011; for a review on potential impacts of increased pCO_2 to microbes see Liu et al. 2010). Other studies investigating the impacts of high pCO_2 concentrations on adult or juvenile echinoids have observed reductions in both feeding rate and fecal production rates (Siikavuopio et al. 2007; Stumpp et al. 2012). Because feeding rate determinations were performed at the end of this study, it is possible that individuals exposed to hypercapnic conditions acclimated to elevated pCO_2 conditions. Ingestion, absorption efficiencies, and egestion rates of *L. variegatus* acclimated to altered temperature regimes in as little as 3 to 6 weeks (Klinger et al. 1986; Watts et al. 2011).

Although urchins were fed a subsatiation diet, individuals in both treatments received sufficient nutrients to support growth rates higher than those observed in field populations (Beddingfield and McClintock 2000). *Lytechinus variegatus* exposed to hypercapnic conditions had significantly smaller weights, diameters and volumes, resulting in smaller total dry matter production, organic matter content, and energy content. Reduced growth has been observed in other studies of juvenile and adult echinoids exposed to increased pCO_2 (Shirayama and Thornton 2005, Siikavuopio et al. 2007, Albright et al. 2012, Stumpp et al. 2012). As urchins were fed the same amount of energy per day in the present study, it is possible that reduced growth was a consequence of increased fecal production rates and/or reduced apparent ash digestibility (reduced efficiency), or that individuals in the high pCO_2 group diverted energy from somatic growth to stress-induced maintenance. Adult *Strongylocentrotus droebachiensis* exposed to high pCO_2 conditions excreted significantly higher amounts of ammonium, suggesting that a change in protein metabolism (i.e., increased protein catabolism) had occurred (Stumpp et al. 2012). We did not examine ammonium excretion and it is possible that this was a source of energy loss in individuals exposed to high CO_2 concentrations.

Organ production and composition

In this study, reduced total dry matter production under hypercapnic conditions was associated with reduced test dry matter production. These results are reflected in the smaller diameters and wet weights observed in these individuals. Exposure to high pCO_2 conditions did not result in reduced total dry matter production in the gut, gonad or lantern. Other studies have reported reduced growth in juveniles exposed to hypercapnic conditions but either did not investigate the impacts to growth of various body

components (800 μatm, Albright et al. 2012), or they did report significant reductions in other body components under more extreme hypercapnic conditions (2800-3800 μatm, Stumpp et al. 2012; 8000 μatm, Siikavuopio et al. 2007).

Mechanisms leading to increases in soluble protein concentration (or decreases in ash concentration) in the test (with spines) of individuals exposed to hypercapnic conditions are unclear. Whether changes are occurring to proteins within the stroma or the stereom of the test (with spines) is unknown. Several soluble and insoluble matrix proteins are essential for formation of calcium carbonate crystals (Feng 2011, refs within). Many proteins have been detected that are occluded within the calcium carbonate stereo of sea urchin spines (Mann et al. 2008a; Killian et al. 2009) and teeth (Mann et al. 2008b; Alvares et al. 2009; Killian et al. 2010). We hypothesize that changes in protein concentration observed in this study reflect changes in the quality or quantity of matrix protein in the test, spines and/or peristomial membrane.

Test ash and organic production was also significantly reduced in individuals exposed to hypercapnic conditions in this study. However, a significantly greater amount of force (40 %) was required to break the ossicles of tests of individuals exposed to high pCO_2 conditions when measured by coarse penetrometry. Altered spine morphology was observed in juvenile *Lytechinus variegatus* exposed to moderately high pCO_2 conditions (800 μatm, 7.83 pH_T) for three months, yet no structural changes were seen in the tests of those individuals (Albright et al. 2012). In general, increases in the pCO_2 result in an increase in overall dissolved inorganic carbon in seawater. Therefore, it is possible that inorganic carbon was limiting for calcification and calcification rates increased in those individuals exposed to hypercapnic conditions. Such a result has been found in other

studies. Net calcification rates (% of initial buoyant weight over 60 days exposure) of *Eucidaris tribuloides* increased under moderately high pCO_2 conditions (903 μatm, 7.7 pH) (Ries et al. 2009). Therefore, we suggest that an increase in calcification, or changes in quality or quantity of the matrix protein occurred in the ossicles of the tests of sea urchins exposed to high pCO_2 conditions, resulting in greater structural strength of the ossicles of the test rather than an increase in size. The echinoid test is a highly plastic body component that is affected by various factors including habitat (e.g., Moore 1935; Lewis and Storey 1984; Hernández and Russell 2010), the presence of predator cues (Selden et al. 2009), food availability (Dix 1970; Levitan 1991; Fernández and Boudouresque 1997), and the quality of food (Heflin et al. 2012).

The wet and dry gut indices were significantly higher in individuals exposed to high pCO_2 conditions, suggesting a change in allocation occurred in those individuals exposed to hypercapnic conditions. As a result of reduced apparent ash digestibility and increased fecal production rates, an increase in gut tissue may indicate compensation to increase absorption and retention efficiency. The echinoid gut has been identified as a nutrient storage organ (Giese 1961; Lawrence et al. 1966; Klinger et al. 1988; Bishop and Watts 1992) and it is possible that hypertrophic (increase in cell size) or hyperplastic (increase in cell number) processes resulted in a larger gut in those individuals exposed to high pCO_2 conditions. Increased storage of neutral lipids in the gut under hypercapnic conditions has not been reported. Whether this increase in neutral lipids is due to enhanced absorption or decreased ability to catabolize or translocate lipids to other organs is unknown. In vertebrates, abnormal retention of lipids within cells, or steatosis, has been associated with multiple pathologies and is most commonly observed in the

liver where lipid metabolism takes place. Decreases in pH are associated with an increased uptake of free fatty acids in isolated mammalian tumor cells (Spector 1969). Reductions in pH can facilitate lipid emulsification, digestion and absorption in the human intestine (Carey et al. 1983). Histological analyses of gut tissue would provide valuable insight into whether lipid uptake is enhanced.

In the present study, increased concentration of soluble proteins in the gonad of individuals exposed to hypercapnic conditions suggest alterations in intermediary metabolic pathways leading to changes in nutrient storage. The reduction in organic content of the gonad with hypercapnic conditions indicates an overall reduced investment in gonad nutrient storage. Whether these changes are associated with specific cell populations (nutritive phagocytes vs gametes) is not known. The reproductive stage of testes and ovaries in this study was not identified. Histological analysis of gonads may provide further information to determine whether the quantitative differences in protein and lipid allocation observed in this study reflect qualitative differences in histological state of the gonad. In a trans-life-cycle study, neither female fecundity (number of eggs per female) nor larval survival of *Strongylocentrotus droebachiensis* exposed to a long-term high pCO_2 regime (16 months at 1200 μatm) was impacted (Dupont et al. 2012). In contrast, fecundity and larval settlement were negatively impacted when *S. droebachiensis* were pre-exposed for only 4 months to similar hypercapnic conditions (Dupont et al. 2012).

Despite a reduced growth rate in sea urchins held at hypercapnic conditions, the size and nutrient allocation to the Aristotle's lantern were not reduced. In addition, lantern indices and organic production was significantly higher in individuals exposed to

high pCO_2 conditions. In a previous study, size of the Aristotle's lantern was unaffected by high pCO_2 conditions in adult *Strongylocentrotus droebachiensis* (2800 -3800 μatm, Stumpp et al. 2012). In response to varying food regimes, the lantern has a high degree of phenotypic plasticity (Heflin et al. 2012). The results of the current study suggest that the regulatory controls of lantern growth differ from those affecting other organs.

Energy

Exposure to hypercapnic conditions significantly affected the allocation of energy to both somatic and reproductive organs in *Lytechinus variegatus*. In the current study, reduced total organic matter production represents a reduction in the storage of metabolizable energy. We hypothesize that individuals exposed to hypercapnic conditions were inefficient at the processes related to production. It is interesting to note that energy allocation to the gut or the lantern was not reduced in individuals exposed to hypercapnic conditions. Energy allocation may have been maintained or even increased in these organs to compensate for reduced digestive efficiency and/or to improve food acquisition. The metabolic cost of maintenance under hypercapnic conditions in individuals of this study is not known. Adult *Strongylocentrotus droebachiensis* exposed to hypercapnic conditions (2800 -3800 μatm) also displayed significantly less energy allocation to both somatic and reproductive growth (Stumpp et al. 2012). Furthermore, O:N ratios of adult *S. droebachiensis* decreased at high pCO_2 conditions, suggesting increased costs related to protein catabolic rates (Stumpp et al. 2012).

The present study utilized commercial sea salts commonly employed in both basic and applied studies of echinoids (e.g.,Mazur and Miller 1971; Lawrence 1975; Klinger et

al. 1994; Gibbs et al. 2009; Taylor et al. 2009; Watts et al. 2011; Heflin et al. 2012). Commercial sea salts often contain additional bicarbonate to increase alkalinity above that of natural seawater. Therefore, the present study is not directly applicable to research on the near-future impacts of ocean acidification. However, the results of the present study are directly applicable to basic and applied (e.g., aquaculture) research with synthetic seawater where variations in CO_2 concentrations are common.

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EXPOSURE TO EXTREME HYPERCAPNIA UNDER LABORATORY CONDITIONS DOES NOT IMPACT RIGHTING AND COVERING BEHAVIOR OF JUVENILES OF THE COMMON SEA URCHIN *LYTECHINUS VARIEGATUS*

by

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Abstract

Changes in the carbonate chemistry (increased pCO_2, decreased pH, and decreased carbonate saturation state) of seawater can impact the growth and physiology of echinoids and therefore, it is possible that their behavior may also be negatively affected. We investigated the impact of extreme hypercapnia on righting activity and covering response in juvenile *Lytechinus variegatus* (avg. diameter = 20 mm) maintained in artificial seawater. Sea urchins collected from Eagle Harbor in Saint Joseph Bay, Florida (29°N, 85°W), were exposed to high pCO_2 conditions, (pCO_2 = 1738 ± 25.00 µatm, pH_{NBS} = 7.7 ± 0.002) for three months under subsatiation conditions. Righting activity (time to right to 90° position from inversion) was evaluated every three weeks and was not significantly different between treatments (repeated measures ANOVA, F = 0.84896, df = 1, 22, and p = 0.36684). At the end of the study, covering behavior (% surface area of the test covered with acrylic beads) was also not significantly different from those individuals raised under control conditions (pCO_2 = 608 ± 12.00 µatm, pH_{NBS} = 8.1 ± 0.004, and nonparametric repeated measures ANOVA, $\chi 2$ = 1.2831, df = 1, and p = 0.25732). These results suggest that juvenile and young adult *L. variegatus* behavior is not altered under conditions of extreme hypercapnia. These findings are particularly relevant to future studies on the basic and applied biology of sea urchins that employ the use of artificial sea salts.

Introduction

Increases in carbon dioxide (CO_2) concentrations in seawater (pCO_2) lead to decreases in seawater pH and corresponding decreases in carbonate saturation states (Ω),

also denoted as the calcite saturation state (Ω_{Ca}) or aragonite saturation state (Ω_{Ar}), depending on whether the organism secretes calcite or aragonite. It has been suggested that under conditions of increased pCO_2 / reduced carbonate saturation state or reduced pH, marine organisms may be increasingly challenged to calcify due to the inability to regulate their acid-base status (Hofmann & Todgham 2010, Refs. within). Several studies have indicated that increased pCO_2 can have significant negative effects on the early development of echinoids including changes in gene expression and slower larval growth rates (e.g., see Byrne 2011, for a review; Sewell & Hofmann 2013). Of the few juvenile and adult studies, most have primarily focused on impacts of alterations to seawater carbonate chemistry on growth and physiology. For example, adult *Strongylocentrotus droebachiensis* sea urchins exposed to high pCO_2 conditions displayed reductions in somatic and reproductive growth along with changes in energy budgets and increased ammonium excretion (pCO_2 = 1007–3800 μatm, pH = 7.25-7.19, Ω_{Ar} = 0.79–0.32, Stumpp et al. 2012). Moreover, juvenile *Lytechinus variegatus* sea urchins exposed to high pCO_2 displayed reduced growth rates and alterations to spine morphology (pCO_2 = 542–765 μatm, pH_T = 7.96–7.83, Ω_{Ca} = 5.2 – 4.1, Albright et al. 2012). It is possible that in addition to impacts to growth and physiology, exposure to increased pCO_2 conditions may affect organismal behavior. Yet to our knowledge, the impacts of hypercapnic conditions on echinoid behavior are unknown.

Organismal behavior has been used as an indication of stress due to environmental changes (Eisler 1979). Righting from inversion is a well-known behavior of echinoderms (Hyman 1955; Reese 1966) and the righting response, or the time to right from inversion, has been used in relation to changes in environmental parameters to

indicate the degree of physiological stress in many echinoderms (e.g., Lawrence 1973; Forcucci & Lawrence 1986; Watts & Lawrence 1986; Lawrence & Cowell 1996). In particular, righting has been used as a stress indicator in *L. variegatus* (Lawrence 1975; Böettger et al. 2001; Santos et al. 2013). Covering behavior, where regular urchins cover themselves with debris from the surrounding environment, has been well-characterized in *L. variegatus* (Millott 1955; Millott 1956; Sharp & Gray 1962; Amato et al. 2008). The function of the covering response may be to hold on to food before consumption (Péquigant 1966; Dix 1970; Douglas 1976) or for protection from sunlight/UV radiation (Lindahl & Runnström 1929; Mortensen 1943; Millott 1956; Lewis 1958; Refs. within Millott 1975; Adams 2001).

L. variegatus is an excellent model organism for this study because its basic and applied biology have been studied exhaustively in the laboratory (e.g., Hammer et al. 2004; Powell et al. 2004; Hammer et al. 2006; Gibbs et al. 2009; Nelson et al. 2010; Heflin et al. 2012). Such studies often employ high urchin densities where CO_2 can become elevated and also utilize artificial sea salts. The objective of this study was to evaluate two stereotypic behavioral responses (righting and covering) of juvenile *L. variegatus* exposed to high pCO_2 conditions.

Methods

Small, pregonadal (no obvious, visible gonads) *L. variegatus* were hand collected in May, 2010, from Eagle Harbor at Saint Joseph Bay, FL, (29°N, 85°W) and transported to the University of Alabama at Birmingham, Birmingham, AL. Prior to the start of the

experiment, sea urchins were held for one week in 19 L tanks with synthetic seawater (Instant Ocean®, 32 ± 1 ‰) at constant photoperiod and temperature (12 h light; 12 h dark; 24 ± 1°C). Food was not provided during this pre-exposure period.

Artificial seawater (ASW) was made from Instant Ocean® (Spectrum Brands) salts and purified water (tap water filtered through a Four-Stage Barracuda RO/DI three-part filter system, AQUAfx). The control treatment consisted of ambient air supplied via a CORALIFE® SL-65 Super-Luft pump and delivered through an air stone to each tank. Experimental hypercapnic conditions were obtained by bubbling pure CO_2 gas and maintained using a pH-monitoring system (Aqua-Medic) such that the pH_{NBS} did not change more than 0.04 units. The Aqua-Medic system was ground-truthed daily using a Fisher Scientific Accumet© basic model AB15 pH meter with an ACCUTU pH electrode (Cat. # 13-620-183) calibrated with NBS standard buffers. As pCO_2 must be calculated with 2 parameters of the carbonate system in addition to salinity and temperature, we chose to use pH and total alkalinity. Total potentiometric pH was determined weekly following the methods of Dickson et al. (2007). Total alkalinity was measured by potentiometric titration every third day for each tank using the methods of the American Public Health Association (APHA 1992) modified for use with a Hach digital titrator (Hach Company, Loveland, USA). This method was tested for accuracy against certified reference materials provided by the laboratory of Andrew G. Dickson (University of California, San Diego, Scripps Institute of Oceanography) and was found to have an error of ± 66 μmol kg^{-1} (standard error of the mean; n = 6). Calcite and aragonite saturation states and pCO_2 (N = 40 for the control replicates and N = 54 for the experimental replicates) were determined from total alkalinity, pH_{NBS}, temperature and

salinity using the Microsoft Excel spreadsheet ‘co2sys.xls’ based on work by Lewis and Wallace (1998) and provided by Pelletier et al. (2007) with the CO_2 constants of Millero et al. (2006) and the $KHSO_4$ constant of Dickson (1990). Total ammonia, nitrite, and nitrate levels were checked every other day using saltwater test kits from the Hach Company until levels remained consistently stable. Weekly measurements of dissolved oxygen and salinity were performed using an YSI 85 meter. Daily temperature measurements were made using the accompanying thermistor on the pH meter mentioned above. Water exchanges of 30-50 % were performed every other day. Experimental acidified water was replaced with previously acidified water in order to maintain pH levels.

After one week of pre-exposure, juveniles (average 23.40 mm ± 0.19 SEM diameter, 5.71g ± 0.11 SEM wet weight) were randomly placed into plastic, cylindrical cages (25.4 x 10.2 cm, H x D) with two urchins per 19 L tank. Each tank contained its own filtration pump (Aqueon Quiet Flow 10) and air supplied via an air stone plumbed to a CORALIFE® Super LuftSL-65 high pressure aquarium air pump. There was no significant difference in wet weights or diameters of individuals in each treatment group (t- test, $t = -0.797$, $df = 46$, $p = 0.435$ for weight; $t = -0.911$, $df = 46$, $p = 0.367$ for diameter). There were 12 tanks for each treatment (control pH = 8.1; experimental pH = 7.7) with a total of 24 urchins per treatment. One urchin from each tank was removed after 6 weeks to ensure water quality. Thus from the middle of the experiment until the end of the experiment there were a total of 12 urchins per treatment and the righting response times measured throughout the experiment correspond to these individuals that remained. Every three weeks, cages and tanks were cleaned to remove algal and bacterial

growth. Every 24 h each individual was fed a formulated feed (provided by Addison Lawrence, Texas A & M) at a subsatiation level previously determined (1.5 % of wet weight, Gibbs 2011). Specifically, the average wet weights of all individuals in a given treatment group was multiplied by 0.015; this was repeated every three weeks when new wet weights were obtained. Prior to the start of the experiment, autogenic controls were placed in each tank for 24 h to determine if pH impacted the integrity (wet weight of the food) and no significant difference was found (t-test, $t = 1.282$, $df = 22$, $p = 0.213$). Every two days uneaten food and feces were removed by siphon. Average salinity over the course of 14 weeks was 32.40 ‰ ± 0.02 SEM, whereas average temperature was 25.02^{0}C ± 0.02 SEM. Photoperiod was held constant at 12 h light: 12 h dark.

Righting responses were measured every three weeks. Individuals were placed in a glass petri dish and submerged under ASW, and placed on their aboral side. Time until the urchin reached an angle of 90° relative to the surface was measured in seconds. Urchins from the experimental treatment group were placed in previously acidified ASW for the test. Righting response times between the two treatments were evaluated by testing for sphericity. Data were log-transformed prior to analysis and the Huynh-Feldt Epsilon values were > 0.7. Therefore, departures from sphericity were not severe. Treatment groups were then tested using a repeated measures analysis of variance (RMANOVA) using SYSTAT version 11.0.

After 14 weeks of exposure to treatment conditions, covering response experiments were conducted over a 24-h period (12 h light: 12 h dark). Prior to the experiments, light measurements were done using a LI-COR cosine quantum light sensor connected to a LI-250 light meter to ensure that all aquaria received approximately the

same amount of light exposure (~2.31 μmol s^{-1} m^{-1}) during the light exposure portion of the 24-h experiment. Urchins were removed from their cages and permitted to freely roam the tank 24 h prior to the start of the experiment. At the start of the experiment, each sea urchin was provided a total of 15 acrylic beads (20 mm diameter) which were placed equidistant from each individual in a circle. We choose to use beads over shell pieces as the beads were of uniform size and would be oriented in the same fashion such that % surface area of the urchin's test could be calculated. Measurements of the number of beads held on the test were determined every four hours over a 24-h period such that three observations were made in the light and three in the dark. Observations in the dark were made using a light equipped with a red filter. Exposed surface area of the sea urchin test (mm^2) was calculated as half the surface area of an oblate spheroid (A_{os}):

$$A_{os} = \left[H^2 + \left(\frac{V^2}{\sin(\alpha)}\right) \ell n \left(\frac{1+\sin(\alpha)}{\cos(\alpha)}\right)\right],$$

where H = the height of the sea urchin in mm (determined by calipers), V = the radius of the sea urchin in mm (determined by calipers), and α = the angular eccentricity of the sea urchin, or:

$\alpha = \arccos\left(\frac{V}{H}\right)$. Bead surface area ($mm^2$) was determined by calculating the area of a circle ($A = \pi r^2$), where r is the radius of the bead. We did not calculate the surface area of a sphere as it is the greatest dimension (i.e. the diameter) of the bead that would provide shading or covering. Covering response (% sea urchin surface covered) was calculated by estimating the total bead surface area covering exposed sea urchin surface area and expressed as a percent: (number of beads found on an individual x area of the bead) / (exposed surface area of sea urchin test) x 100. The numbers of beads on the test of each sea urchin in the control group were compared to that of sea urchins in the

experimental group using a nonparametric repeated measures ANOVA using SAS (Brunner et al. 2001).

Results

Average pCO_2 values for the treatments were 608 ± 12.00 μatm (pH_{NBS} = 8.1 ± 0.004) and 1738 ± 25.00 μatm (pH_{NBS} = 7.7 ± 0.002) (Table 1). The interaction of time and treatment for righting responses was not significant (RMANOVA, F = 0.53339, df = 4, p = 0.71151). As the interaction was not significant, we evaluated righting response times between treatments and found that they also were not significantly different (RMANOVA, F = 0.84896, df = 1, 22, p = 0.36684). However, both groups did significantly increase in righting time over the course of the experiment (Figure 1, RMANOVA, F = 3.52947, df = 4, 88, p = 0.01016). Similarly, covering responses were not significantly different between treatments (RMANOVA, $\chi 2$ = 1.2831, df = 1, p = 0.25732) but both treatments covered with significantly more beads during the dark portion of the experiment (Figure 2, RMANOVA, $\chi 2$ = 2.8950, df = 2.3172, p = 0.04729). The interaction of time and treatment was also not significantly different (RMANOVA, $\chi 2$ = 0.60594, df = 2.3172, p = 0.56919).

Table 1. Seawater chemistry parameters measured throughout the study. Mean with the standard error of the mean in the parentheses.

Parameter	Control	Experimental
pH_{NBS}	8.10 (0.004)	7.70 (0.002)
$pH_{Total\ H}$	8.06 (0.006)	7.69 (0.003)
pCO_2 (µatm)	608.47 (11.77)	1738.64 (25.01)
Temperature (^{0}C)	25.00 (0.03)	25.04 (0.03)
Dissolved oxygen (%)	91.20 (0.05)	91.02 (0.08)
Salinity (‰)	32.40 (0.02)	32.42 (0.03)
Total alkalinity (µmol kg^{-1})	2934.86 (22.71)	2730.88 (33.51)
Ω Ca	5.86 (0.09)	2.27 (0.04)
Ω Ar	3.84 (0.06)	1.49 (0.03)

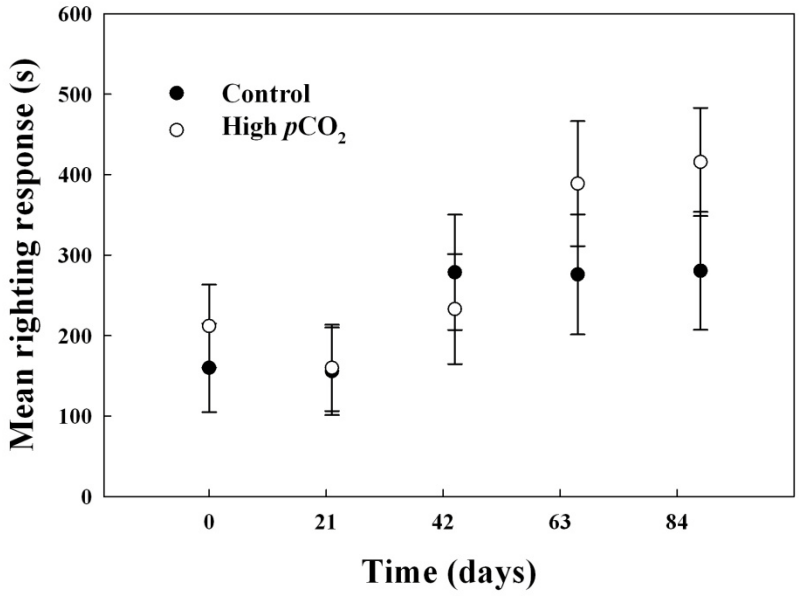

Figure 1. *L. variegatus*. Righting response times in seconds (mean ± standard error of the mean) of sea urchins exposed to either a control or high pCO_2 environment over time (N = 12 per treatment). Treatments were not significantly different by RMANOVA (p = 0.36684). Both groups significantly increased righting time over the course of the experiment (RMANOVA, p = 0.01016).

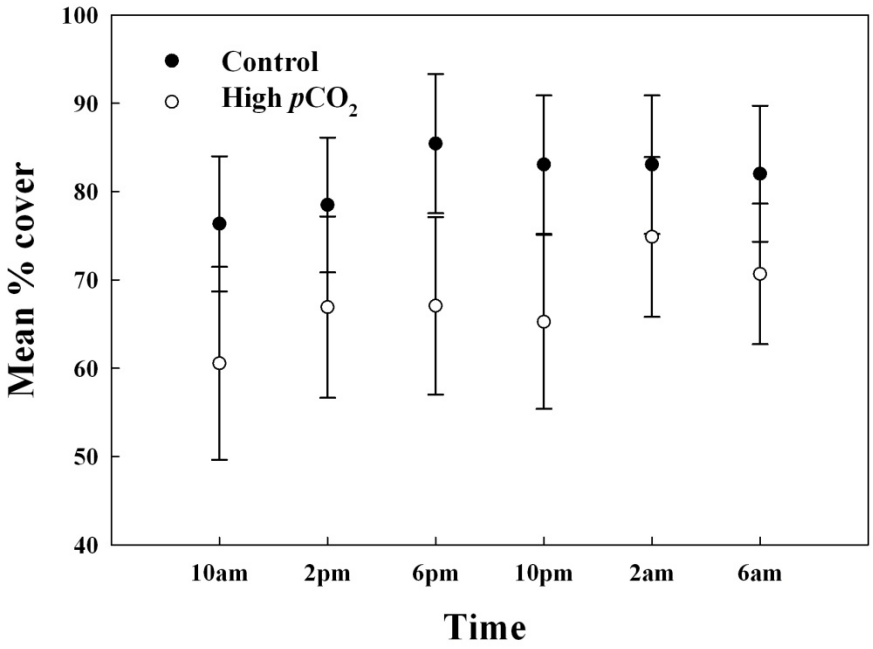

Figure 2. *L. variegatus*. Covering behavior (mean ± standard error of the mean) as a percent of the surface area covered by acrylic beads at four-hour intervals over a 24-h period in sea urchins exposed to control (pCO_2 = 608 µatm) versus high pCO_2 (1738 µatm) treatments. Treatments were not significantly different (nonparametric RMANOVA, p = 0.25732). Both significantly increased the percentage of surface area covered during the dark hours (nonparametric RMANOVA, p = 0.04729).

Discussion

The results of this study indicate that the righting and covering behavior of juvenile and young adult *L. variegatus* is not affected by long-term (three month) exposure to extreme hypercapnic conditions. Righting behavior, especially in echinoderms, has been utilized as an indication of the overall health of an organism exposed to environmental changes. For example, the righting times of the sea star *Luidia clathrata* decreased in individuals that were exposed to low temperature and low salinity over 30 days (Watts & Lawrence 1990). Adult *L. variegatus* displayed slower righting

responses under combinations of temperature and salinity outside the normal range typically experienced in the field (Lawrence 1975). *L. variegatus* exposed to both inorganic and organic phosphates displayed significantly lower righting coefficients (righting coefficient = 1000 [1/righting time (s)] (Böettger et al. 2001). In comparison to these examples, the results of the present study indicate that exposure to extreme hypercapnia does not affect righting behavior. It has been hypothesized that poor acid-base regulation capacity may result in metabolic depression under hypercapnic conditions (Pörtner et al. 2004). Although acid-base status and respiration rates were not measured in this study, adult *Strongylocentrotus droebachiensis* individuals exposed to high pCO_2 conditions for 45 days displayed full (pCO_2 1007–1431 μatm) or partial (2800–3800 μatm) extracellular pH compensation and did not exhibit significantly reduced routine metabolic rates in any of the experimental treatments (Stumpp et al. 2012). These results were attributed to partial or full acclimation and pre-adaptation. Thus, the results of this study may be a reflection of the current carbonate chemistry that *L. variegatus* is exposed to. *L. variegatus* are located throughout the Gulf of Mexico (Watts et al. 2007). Although we do not have information on the carbonate chemistry of the location from which the individuals in this study were taken, other Florida bays have exhibited significant fluctuations in their carbonate chemistry. For example, surface seawater samples from Florida Bay have been found to have a large range in pCO_2 values (325 – 725 μatm, Millero et al. 2001). Surface seawater samples from Tampa Bay have been found to have a maximum pCO_2 value of 580 μtm (Yates et al. 2007). Thus, the individuals in this study may possess mechanisms for dealing with hypercapnic conditions such that their righting behavior was not affected. Righting responses of

individuals of the sea star *L. clathrata*, taken from Tampa Bay were also not affected by hypercapnic conditions created in the lab using artificial seawater (780 μatm, Schram et al. 2011). It is of note that, in this study, larger individuals in all treatments displayed significantly longer response times, perhaps due to the biomechanics of greater body mass.

The covering behavior of *Lytechinus variegatus* exposed to hypercapnic conditions was not significantly different from that of individuals raised under control conditions. Covering behavior is well characterized in *L. variegatus* (Millott 1956; Sharp & Gray 1962; Amato et al. 2008). When exposed to a narrow band of light, *L. variegatus* coordinates its covering behavior such that covering materials are moved to shade itself (Millott 1956). When exposed to ultraviolet light, *L. variegatus* exhibits immediate, negative phototaxis and covers with shells (Sharp & Gray 1962). In the present study, the numbers of beads held as cover did not differ significantly between the treatment groups. Interestingly, both groups significantly increased the number of beads used as cover during dark hours. The reason(s) for this are unclear, as *L. variegatus* have been observed to reduce the amount of covering material at night in both field and laboratory investigations (Millott 1956; Sharp and Gray 1962).

The present study, similar to past studies that have focused on the basic and applied biology of this species and other sea urchins (e.g., Hammer et al. 2004, 2006; Gibbs et al. 2009; Taylor et al. 2009; Watts et al. 2011; Heflin et al. 2012), employed artificial sea salts. Commercially prepared artificial sea salts tend to contain higher amounts of bicarbonate than natural seawater so as to boost total alkalinity values in order to avoid decreases in pH. The amount of CO_2 (avg. pCO_2 = 1738 μatm) we

employed in our extreme hypercapnia treatment group was higher than that predicted to occur under near-future scenarios of ocean acidification (IPCC 2007). As a result, while our study is not directly applicable to studies on the impacts of ocean acidification, it does suggest that *L. variegatus* may have a robust behavioral capacity in the face of rising anthropogenic CO_2 concentrations.

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VARIABILITY OF THE CARBONATE CHEMISTRY IN A SHALLOW, SEAGRASS-DOMINATED ECOSYSTEM: IMPLICATIONS FOR OCEAN ACIDIFICATION EXPERIMENTS

by

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Abstract

Open ocean observations have shown that increasing levels of anthropogenically-derived atmospheric CO_2 are causing acidification of the world's oceans. Yet little is known about coastal acidification and studies are just beginning to characterize the carbonate chemistry of shallow, nearshore zones where many ecologically and economically important organisms occur. We characterized the carbonate chemistry of seawater at four sites located within an area dominated by seagrass beds (Eagle Harbor in Saint Joseph Bay, Florida), to determine the extent of variation in pH and pCO_2 that local organisms are currently experiencing over monthly, daily, and hourly timescales. Extensive variation was observed over both space and time with distinct diurnal fluctuations observed at all timescales, indicating the influence of photosynthetic and respiratory processes on the local carbonate chemistry. Over the course of a year, values of pH ranged from 7.36 – 8.28 and had an average diurnal range of 0.27 units. When sampled on a daily basis over the course of a week and every two hours over the course of the day, the range in pH (7.70 – 8.06 and 7.57 – 7.63, respectively) was smaller and considerably different. Calculated pCO_2 values of samples taken on a monthly basis (194.63 – 2536.80 μatm), daily basis (378.31 – 1018.53 μatm) and every two hours (1227.66 – 1451.87 μatm) also exhibited significant range and variation between timescales. The results of this study have significant implications for the design of ocean acidification experiments where nearshore species are utilized. Many coastal species are experiencing far greater fluctuations in carbonate chemistry than previously thought. However, whether these findings indicate the potential for organismal acclimatization and resilience to near-future ocean acidification conditions is not well understood.

Introduction

The field of ocean acidification (OA) has grown immensely over the past decade in an effort to predict the potential consequences of increasing atmospheric carbon dioxide (CO_2) concentrations on marine ecosystems (e.g., Orr et al. 2005; Fabry et al.2008; Doney et al. 2009; Hofmann et al. 2010). There has been a rapid increase in laboratory experiments investigating the impact of altered seawater carbonate chemistry due to increases in atmospheric CO_2 (e.g., increased partial pressure of CO_2 (pCO_2) and dissolved inorganic carbon (DIC), reduced pH and calcium carbonate polymorph saturation states) on marine organisms (see Dupont et al. 2010; Hendriks et al. 2010; Kroeker et al. 2010; Byrne 2011, for reviews and data meta analyses). These experiments are largely designed around the use of experimental pCO_2 and pH values predicted by the Intergovernmental Panel on Climate Change emission scenarios (IPCC 2007) rather than values that more closely mimic coastal environments from which organisms are collected. This is partially due to the lack of information on nearshore carbonate chemistry and the focus on coastal species with important ecological and economic roles. Therefore, there is a need for monitoring studies that investigate the local variability in the seawater carbonate chemistry of ecosystems where targeted model study organisms occur. Such data will not only allow researchers to establish relevant target experimental pH and pCO_2 values, but also put into context the results that have been generated on previous OA studies in terms of the organism's current physiological tolerance for pH and pCO_2 variability and potential ability to acclimatize and/or adapt to changes in seawater carbonate chemistry.

Significant fluctuations in seawater carbonate chemistry have been documented for many marine ecosystems (e.g., DeGrandpre et al. 1995; Ohde et al. 1999; Hales et al. 2005; Wootton et al. 2008; Hofmann et al. 2011). Coastal data sets have primarily consisted of large-scale observations covering extensive regions of the open coastal ocean (e.g., Cai et al. 2011; Wang et al. 2013). Yet many study organisms for OA research are collected from nearshore ecosystems. These areas are often shallow and contain dense beds of macroalgae or seagrasses and therefore have the potential to display significantly wider ranges in carbonate parameters on diurnal and seasonal timescales due to the influence of photosynthetic and respiratory processes.

There are a variety of biological processes that can influence the carbonate chemistry of a body of water. Photosynthesis of macroalgae and seagrasses increases the pH of the surrounding water while decreasing DIC (mainly through uptake of CO_2 and HCO_3^-) (e.g., Invers et al. 1997; Beer et al. 2006; Middelboe and Hansen 2007; Semesi et al. 2009). Conversely, respiration of marine organisms decreases the pH but increases DIC due to the increase in CO_2. Diurnal fluctuations > 1 pH unit have been observed in seagrass meadows (Semesi et al. 2009) and increases in pH of up to 0.38 units have been associated with the presence of seagrass meadows (Unsworth et al. 2012). In Florida Bay (located between the Florida Keys and the U.S. mainland) where seagrass beds are common, extensive variation in pH (7.85 – 8.1) and pCO_2 (325 – 725 μatm) has been observed (samples taken every two months, Millero et al. 2001). An additional study in Florida Bay and Tampa Bay (located on the west coast of central Florida) found average diurnal differences (over a 3 day period) of 0.22 pH units and 218 μatm pCO_2 (Yates et

al. 2007). Therefore, it is likely that there is significant diurnal variability in the carbonate chemistry of other Florida bays where seagrass beds are dominant.

Saint Joseph Bay is a shallow, subtropical lagoon located on the northwest coast of Florida (29.8^0N, 85.3^0W) that is semi-enclosed by the Saint Joseph Peninsula (Fig. 1). Other than freshwater runoff, the bay does not have any significant sources of freshwater such as those from major rivers, and has been characterized as having pristine waters with no elevated concentrations of nutrients or chlorophyll (FDEP 2012). Approximately 15 miles long and six miles wide, the average water depth in the bay is 6.4 m, with the deepest area (9 – 18 m) located in the northwest region of the bay. The bay contains large areas of dense seagrass beds primarily comprised of *Thalassia testudium* with scattered patches of well-sorted, siliceous sand (Valentine and Heck 1993, Beddingfield and McClintock 2000). Several invertebrate and vertebrate species have been observed to occur in high densities associated with the seagrass beds within the bay including amphipods, mussels, crabs, scallops, gastropods, sea urchins, sand dollars, sea turtles and a variety of juvenile fish (Valentine and Heck 1993; Heck et al. 2000; Heck et al. 2003).

Interpreting coastal seawater chemistry can be challenging due to the many factors involved including the influence of nearby riverine inputs and pollution. Therefore, because of the lack of large freshwater sources and its pristine state, Saint Joseph Bay is an excellent model site to obtain information on the extent of variability in carbonate chemistry of nearshore, subtropical seagrass ecosystems. Moreover, in shallow seagrass zones, the degree of fluctuation in carbonate parameters will likely depend on the intensity of wave action, low tidal flushing, and water exchange with the open ocean. As Saint Joseph Bay is a lagoon characterized by low-energy current regimes (Heck et al.

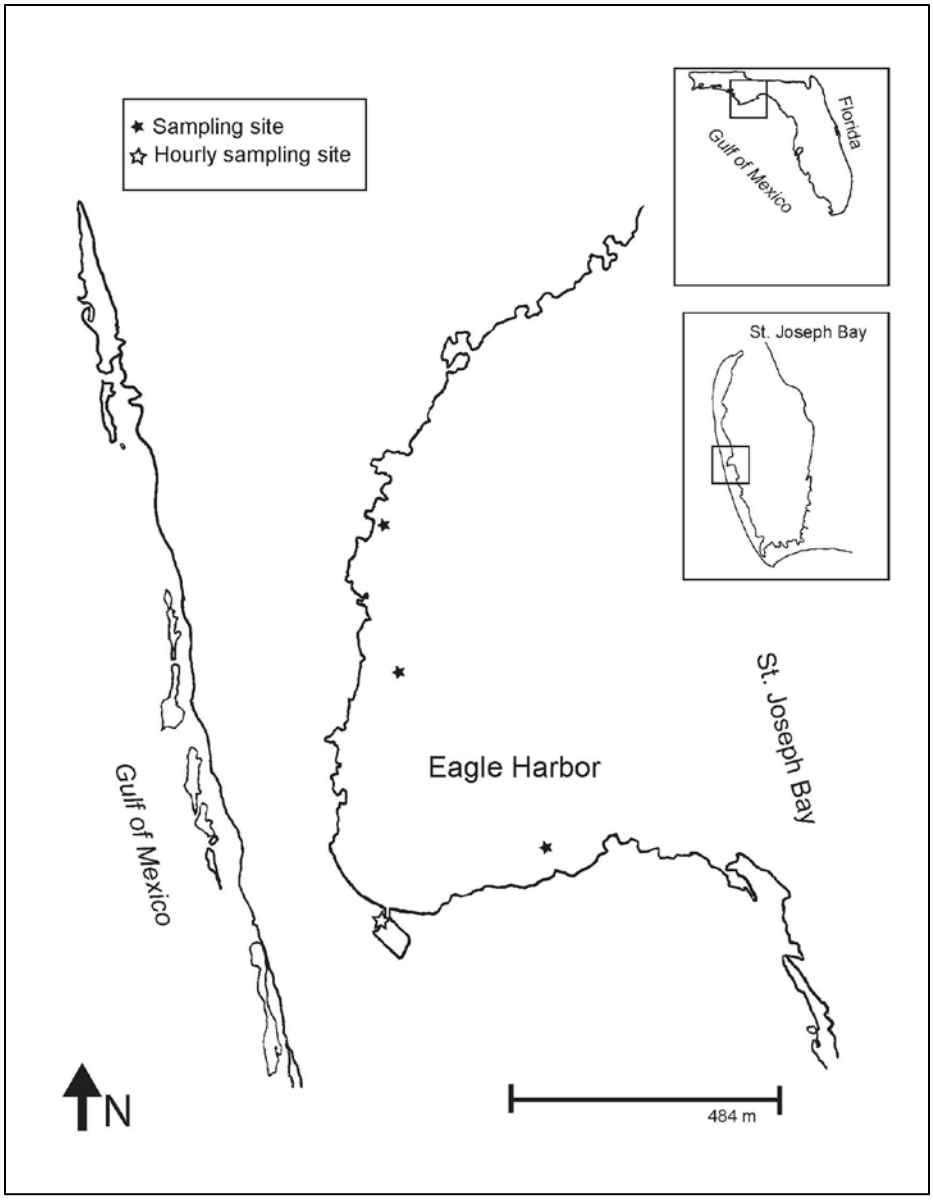

Figure 1. Map of sites sampled on a monthly, daily and hourly timescales within Eagle Harbor, an area located within Saint Joseph Bay along the northwest coast of Florida. Figure illustrated by Jessica Reynolds and used with permission.

2000), greater variation in carbonate parameters, such as pH and pCO_2, are expected. Seagrasses contribute significantly to the health of coastal habitats and have experienced a global decline over the past several decades (Short and Wyllie-Echeverria 1996; Hemminga and Duarte 2000; Duarte 2002; Orth et al. 2006). Characterizing the natural variation in the carbonate system of Saint Joseph Bay will identify conditions that marine organisms are experiencing in the field and may indicate the potential for organismal preadaptation and/or acclimatization to near-future ocean acidification conditions in nearshore seagrass ecosystems.

The objective of the current study was to characterize the carbonate chemistry of a nearshore seagrass ecosystem on multiple timescales (monthly, daily, and hourly) to ascertain the extent of variation in pH and pCO_2 that marine organisms are experiencing currently.

Methods

Sample Collection

Three sampling sites were chosen within Eagle Harbor, an area that is located within the Saint Joseph Peninsula State Park (Fig. 1). Samples were collected monthly throughout the year 2012, daily during the week of July 28 – August 4th, 2012, and every two hours during the day on July 28, 2012. To collect samples, we modeled our sampling device after the dissolved oxygen sampling device of Daniel and Boyden (1975) as it was designed to minimize gas exchange (and therefore avoid potential perturbation of seawater chemistry). We chose to use this device to ensure consistency in

sampling method as traditional sampling methods (i.e., Niskin bottles) would be logistically challenging and potentially disrupt sediments in the extremely shallow waters (< 1m) that occur at low tide in Eagle Harbor. The device consisted of a 300 ml borosilicate glass bottle with a rubber stopper plumbed with silicone tubing similarly to that of Daniel and Boyden (1975): a short section of tubing to allow water to enter from the surrounding seafloor and a longer piece that extended to the surface to allow air to escape when opened. This device was attached to a 2 m length of PVC pipe in order to allow the device to be quickly moved to the seafloor with minimal gas exchange.

Once the device was securely on the seafloor, the tubing at the air-water interface was unplugged and air in the bottle was allowed to escape to the surface, ensuring that water that entered the bottle was from near the bottom of the seafloor. Triplicate samples were taken from each of 3 locations over seagrass beds in Eagle Harbor within 1 hour of dawn in an effort to capture maximum pCO_2 values and again later in the day in order to capture minimum pCO_2 values. A 'control' site lacking seagrass beds within the boat launch area was sampled every two hours (Fig 1.). At the time of each sampling at all sites, depth was recorded and temperature and dissolved oxygen (D.O.) were measured using a YSI-85 meter. Upon collection, samples were immediately fixed with 100 µL of mercuric chloride and stored according to the methods of Dickson et al. (2007) until analysis could take place at the University of Alabama at Birmingham.

Carbonate Chemistry Analysis

Upon opening, samples were immediately tested for pH using a Honeywell DL421 sensor module equipped with a DuraFET III pH electrode (Seelaus Instrument Co., Miamisburg, OH) previously calibrated with Tris buffers provided by Andrew G. Dickson (University of California, San Diego, Scripps Institute of Oceanography). This equipment was chosen over spectrophotometric measurement to ensure consistency, as we expected some pH values to be outside the range detected using m-cresol purple. Samples were then analyzed for total alkalinity following the methods of Dickson et al. (2007) using a Mettler-Toledo T50 open cell titrator with pH probe (Model DGi115-SC) and HCl of known concentration and density from the laboratory of Andrew Dickson. Measurements were recorded in the computer program LabX®, downloaded into a Microsoft Excel spreadsheet, and total alkalinity calculated following the methods of Dickson et al. (2007). Certified reference materials (CRM) for total alkalinity (TA) were also obtained from the laboratory of Andrew Dickson and the titration method found to have an average error of 6.80 μmol kg^{-1} ± 1.19 SEM (n = 29). Internal testing on the precision of the pH probe was performed periodically on samples of Tris buffer (average error 0.0095 ± 0.011 SEM, n = 21). Salinity was measured using an Orion 3 Star conductivity meter with an Orion 013005MD conductivity probe (Thermo Fisher Scientific) and values are based on the Practical Salinity Scale of 1978 (PSS 78). Carbonate saturation states, dissolved inorganic carbon (DIC), and pCO_2 were calculated using pH, temperature, total alkalinity, and salinity using CO2SYS version 14 (Lewis and Wallace 1998; Pelletier et al. 2007) with the K1 and K2 constants of Millero et al. (2006) and the $KHSO_4$ constant of Dickson (1990).

Cloud cover averages were determined as daily averages for monthly and daily afternoon samples, and hourly averages for samples taken every two hours (National Oceanographic and Atmospheric Association weather station at Apalachicola Municipal Airport, station ID 39698 located 30 miles east from Saint Joe Bay and directly on the coast).

Differences in means between sites for carbonate parameters and D.O. were tested for significance using a two-way analysis of variance (ANOVA) with either month or day as blocks. The southernmost site was labeled "A", the northernmost site labeled "C" and the middle site was labeled "B" (see Fig. 1 for locations). Analyses were done using SAS 9.2. As relationships between parameters can assist with determining the factors controlling the carbonate system, several linear regressions were performed.

Results

Seasonal seawater chemistry

All of the carbonate parameters exhibited clear diurnal trends on each month sampled. Values of pH and carbonate saturation states (Ω_{Ca} and Ω_{Ar}) were lower in the morning relative to afternoon values with the highest afternoon values observed in the months of May and June, 2012 (Fig. 2). Conversely, pCO_2, DIC, and total alkalinity values were higher in the morning relative to afternoon values with the lowest afternoon values of pCO_2 and DIC observed in the months of May and June (Fig. 3). D.O. and temperature values were consistently lower in the morning, yet the degree of variation

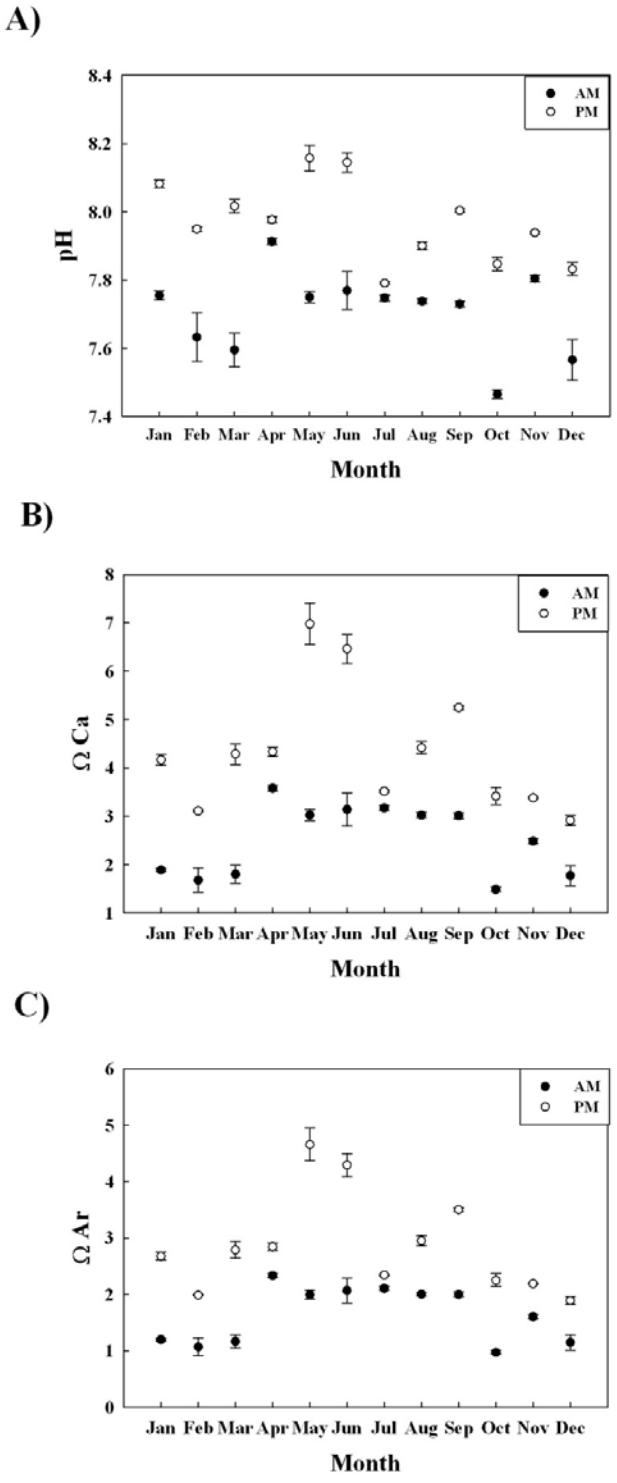

Figure 2. Mean ± SEM (n = 9 per sampling time) for monthly A) pH, B) calcite saturation state (Ω_{Ca}), and C) aragonite saturation state (Ω_{Ar}) measured during 2012.

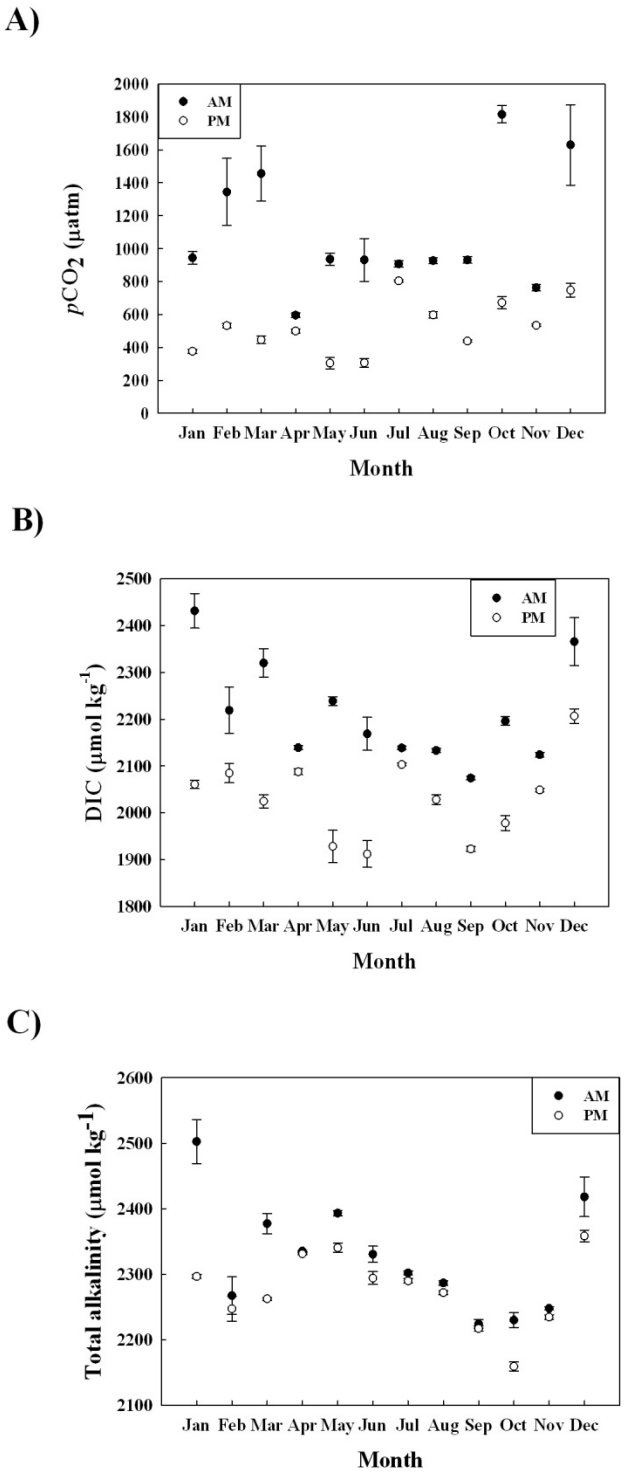

Figure 3. Mean ± SEM (n = 9 per sampling time) for monthly A) pCO_2, B) dissolved inorganic carbon (DIC), and C) total alkalinity measured during 2012.

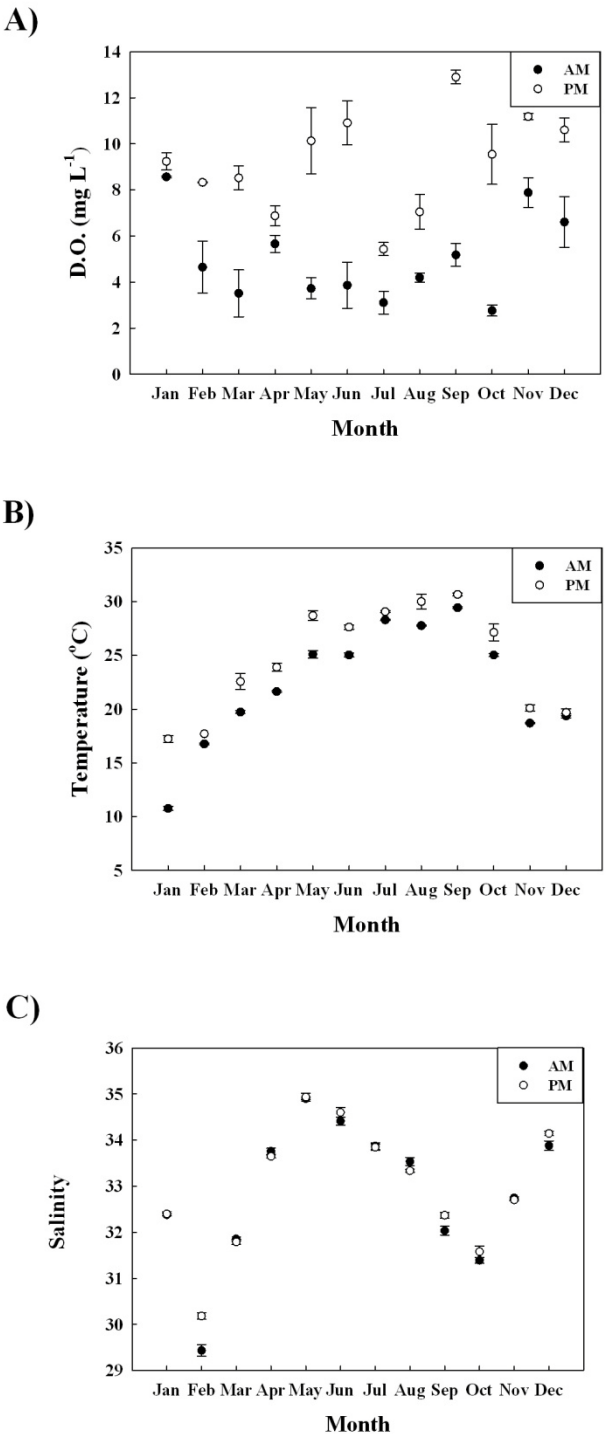

Figure 4. Mean ± SEM for monthly A) dissolved oxygen (D.O.) (n = 3 per sampling time), B) temperature (n = 3 per sampling time), and C) salinity (psu, n = 9 per sampling time) measured during 2012.

between morning and afternoon samples varied (Fig. 4). Similar to pH and carbonate saturation states, D.O. values were highest in the afternoon samples taken in the months of May and June. Salinity varied on a monthly basis, but did not exhibit significant diurnal fluctuations (Fig. 4). Water depth at sampling varied over the course of the year both between months and within days (Fig. 5). Average cloud cover (%) was below 20 % for a majority of the months sampled with the exception of February, August, October and December (Fig. 5).

A summary of several descriptive statistics including the range (maximum value – minimum value in the complete data set for the year), and average Δ diurnal values (average difference in values measured each month) for all parameters for the entire year can be found in Table 1. The annual average pH was 7.84 ± 0.01. Carbonate saturation states did reach values below saturation (minimum of 0.65 Ω_{Ar} and 1.01 Ω_{Ca}) but mean values were well above saturation (maximum 5.63 Ω_{Ar} and 8.42 Ω_{Ca}). Average pCO_2 was 814 ± 32 μatm. The average range in pH, Ω_{Ar} and pCO_2 between morning and afternoon samples (average Δ diurnal) were extensive (0.27, 1.23, and 576 μatm respectively).

Ignoring the monthly variation, parameters varied depending on the location of sampling with the middle site ('B') being significantly different from sites A and C in both morning and afternoon samples (Table 2). Water depth and temperature were not significantly different between sites at any time sampling (data not shown).

Correlations between parameters measured and calculated on a monthly basis (Table 3) indicated that TA was not significantly correlated with salinity, pCO_2, pH,

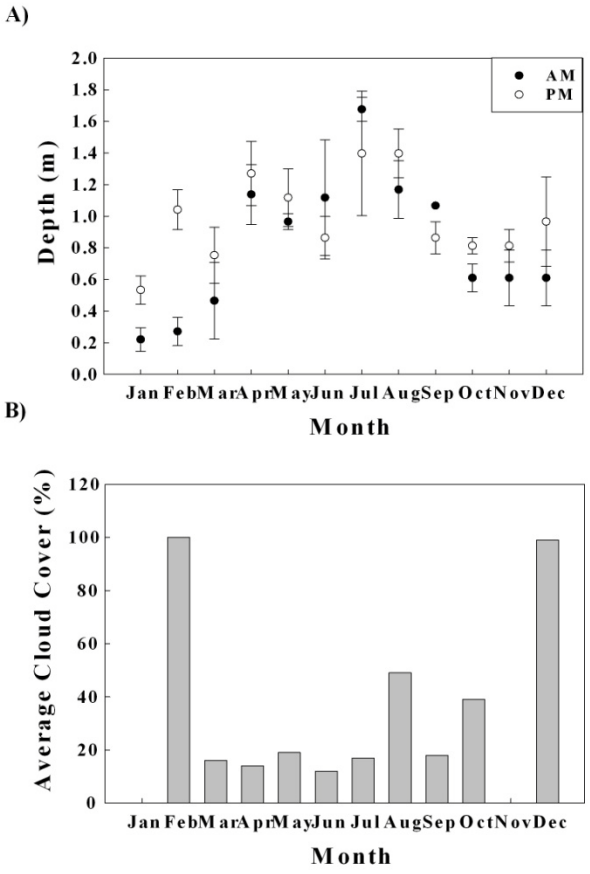

Figure 5. A) Mean ± SEM (n = 3 per sampling time) for water depth recorded during monthly sampling events over the course of 2012. B) Average cloud cover for the day sampled.

Table 1. Descriptive statistics on Eagle Harbor parameters sampled on a monthly basis in 2012. SEM = standard error of the mean. Salinity in psu. Avg. Δ diurnal = average difference in monthly minimum and maximum values.

	pH	Ω Ca	Ω Ar	$p\,CO_2$ (µatm)	DIC (µmol kg^{-1})	TA (umol/kg)	D.O. (mg L^{-1})	Temp (°C)	Salinity
Mean	7.84	3.43	2.25	813.83	2122.86	2301.48	7.08	23.5	32.9
SEM	0.01	0.10	0.07	31.76	10.01	5.63	0.36	0.6	0.1
Median	7.83	3.23	2.13	692.41	2108.56	2293.95	6.63	24.7	33.2
Std. Dev.	0.19	1.46	0.98	463.55	146.12	82.19	3.07	5.2	1.4
Min.	7.36	1.01	0.65	194.63	1806.88	2135.84	1.62	10.4	28.7
Max.	8.28	8.42	5.63	2536.80	2549.10	2604.62	13.34	31.4	35.3
Range	0.92	7.40	4.98	2342.17	742.19	468.78	11.72	21.0	6.6
Avg. Δ Diurnal	0.27	1.85	1.23	576.88	180.28	50.95	4.25	2.2	0.2

Table 2. Statistical results on site variability of monthly samples as determined by two-way ANOVA blocking for monthly variation. Mean ± SEM. Superscript letters denote statistical significance as determined by pair wise comparisons ($p < 0.05$).

	A_{AM}	B_{AM}	C_{AM}	A_{PM}	B_{AM}	C_{AM}
pH	7.70 ± 0.03^{A}	7.76 ± 0.03^{B}	7.66 ± 0.02^{A}	7.99 ± 0.02^{A}	7.93 ± 0.02^{B}	8.00 ± 0.02^{A}
Ω Ca	2.47 ± 0.83^{A}	2.73 ± 0.85^{B}	2.31 ± 0.79^{A}	4.51 ± 0.25^{A}	4.00 ± 0.16^{B}	4.68 ± 0.25^{A}
Ω Ar	1.62 ± 0.09^{A}	1.79 ± 0.09^{B}	1.51 ± 0.09^{A}	2.97 ± 0.17^{A}	2.62 ± 0.11^{B}	3.08 ± 0.17^{A}
$p\,CO_2$ (µatm)	1099.30 ± 79.26^{AB}	953.87 ± 70.57^{B}	1241.92 ± 84.90^{A}	498.47 ± 26.51^{A}	571.64 ± 28.52^{B}	490.90 ± 30.79^{A}
DIC (µmol kg^{-1})	2193.95 ± 14.49^{A}	2178.20 ± 20.75^{A}	2265.25 ± 25.91^{B}	2017.40 ± 17.42^{A}	2063.27 ± 13.23^{B}	2009.65 ± 16.77^{A}
TA (umol/kg)	2310.95 ± 8.71^{A}	2307.81 ± 14.28^{A}	2345.55 ± 16.42^{B}	2280.87 ± 5.13	2286.05 ± 7.74	2279.52 ± 5.94
D.O. (mg L^{-1})	4.78 ± 0.70	5.53 ± 0.58	4.62 ± 0.52	9.76 ± 0.62^{A}	8.45 ± 0.63^{B}	9.57 ± 0.79^{AB}

Table 3. Summary of regressions on parameter relationships measured and calculated on a monthly basis (n = 213).

Monthly	R^2	Equation
TA (µmol kg^{-1}) and Salinity	0.135	TA = 1585.364 + (21.738 * Salinity)
TA (µmol kg^{-1}) and Temperature (°C)	0.185	TA = 2462.637 - (6.858 * Temp)
TA (µmol kg^{-1}) and Depth (m)	0.040	TA = 2335.279 - (37.386 * Depth)
TA (µmol kg^{-1}) and DIC (µmol kg^{-1})	0.555	TA = 1411.791 + (0.419 * DIC)
TA (µmol kg^{-1}) and $p\,CO_2$ (µmol kg^{-1})	0.110	TA = 2253.569 + (0.0589 * $p\,CO_2$)
TA (µmol kg^{-1}) and pH	0.068	TA = 3171.531 - (111.030 * pH)
TA (µmol kg^{-1}) and D.O. (mg L^{-1})	0.045	TA = 2341.919 - (5.710 * D.O.)
DIC (µmol kg^{-1}) and Temperature (°C)	0.345	DIC = 2514.396 - (16.662 * Temp)
DIC (µmol kg^{-1}) and D.O. (mg L^{-1})	0.299	DIC = 2308.324 - (26.183 * D.O.)
pH and D.O. (mg L^{-1})	0.534	pH = 7.508 + (0.0463 * D.O.)
$p\,CO_2$ (µmol kg^{-1}) and Temperature (°C)	0.041	$p\,CO_2$ = 1242.418 - (18.239 * Temp)
$p\,CO_2$ (µmol kg^{-1}) and D.O. (mg L^{-1})	0.447	$p\,CO_2$ = 1532.582 - (101.470 * D.O.)

D.O., depth, or temperature. Temperature did not correlate with pCO_2 but was weakly correlated with DIC. DIC was also weakly correlated with D.O. However, TA did correlate more strongly with DIC (Fig. 6A). D.O. and pH (Fig. 6B) and D.O. and pCO_2 were also correlated (Fig. 6C).

Daily seawater chemistry

Distinct diurnal trends in the carbonate parameters were observed on each day sampled during the week of July 28 – August 2, 2012. Similar to the monthly pattern, pH, Ω_{Ca} and Ω_{Ar} were lower in the morning relative to afternoon values on a daily timescale (Fig. 7) and pCO_2, DIC, and total alkalinity were higher in the morning relative to afternoon values (Fig. 8). D.O. and temperature were consistently lower in the morning each day relative to afternoon values whereas salinity was consistently higher in the morning relative to afternoon samples (Fig. 9). Sampling on day four did not take place due to inclement weather and D.O. data was not available on day six due to equipment malfunction. Water depth at sampling was higher in the morning on days one and six but tended to decrease as the week progressed (Fig. 10A). Average cloud cover (%) was above 10% each day and was almost 50% on day five (Fig 10B).

Summary statistics including the range (maximum value – minimum value in the complete data set for the week) and average Δ diurnal (average difference in values measured each day) for all parameters for the entire week can be found in Table 4. Average diurnal ranges for the week were not as large as those for the year, yet the average ranges in pH, Ω_{Ar}, and pCO_2 were still extensive: 0.27 units, 0.96 units and 577 μatm respectively.

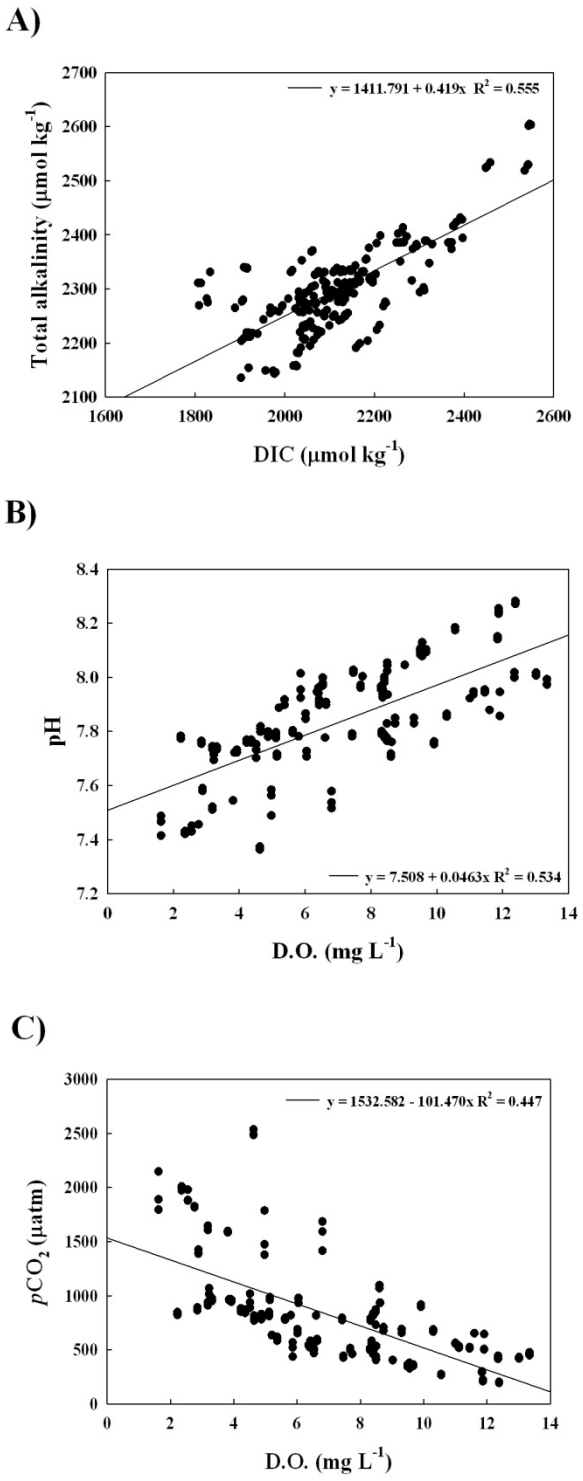

Figure 6. Relationships between A) total alkalinity and dissolved inorganic carbon (DIC), B) pH and dissolved oxygen (D.O.) and C) pCO_2 and D.O. on morning and afternoon samples obtained on a monthly basis for 2012.

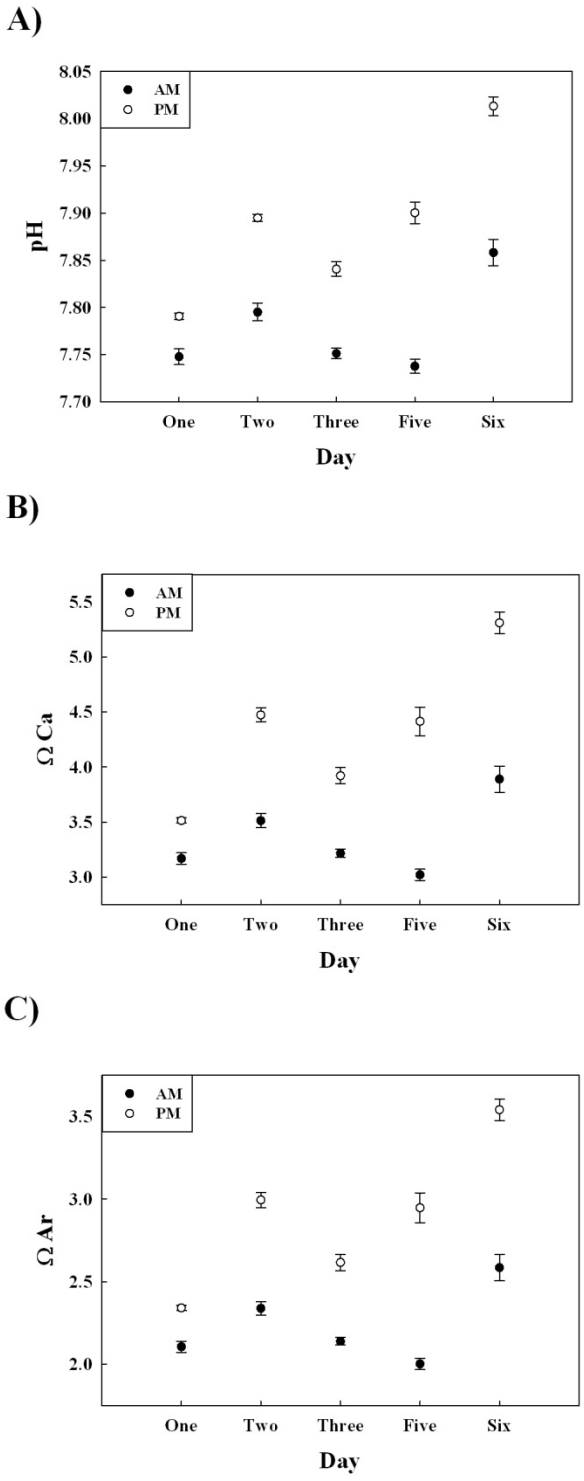

Figure 7. Mean ± SEM (n = 9 per sampling time) for daily A) pH, B) calcite saturation sate (Ω_{Ca}), and C) aragonite saturation state (Ω_{Ar}) measured during the week of July 28 – August 2, 2012.

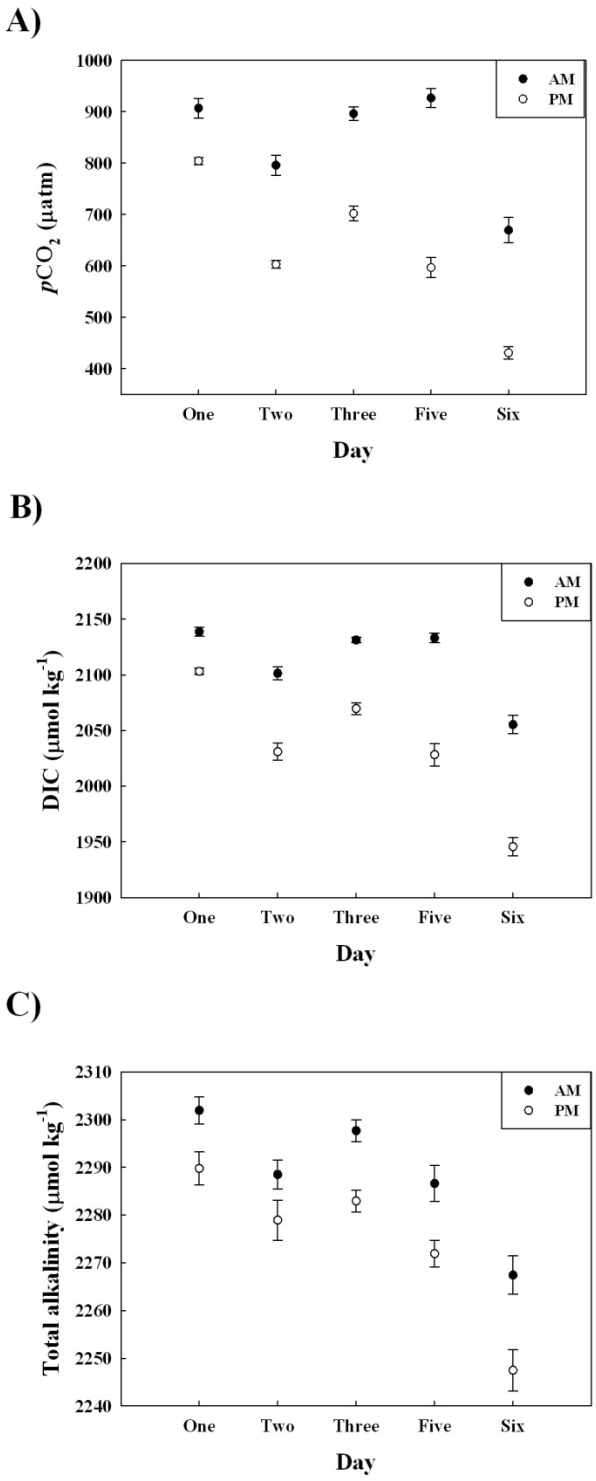

Figure 8. Mean ± SEM (n = 9 per sampling time) for daily A) pCO_2, B) dissolved inorganic carbon (DIC), and C) total alkalinity measured during the week of July 28 – August 2, 2012.

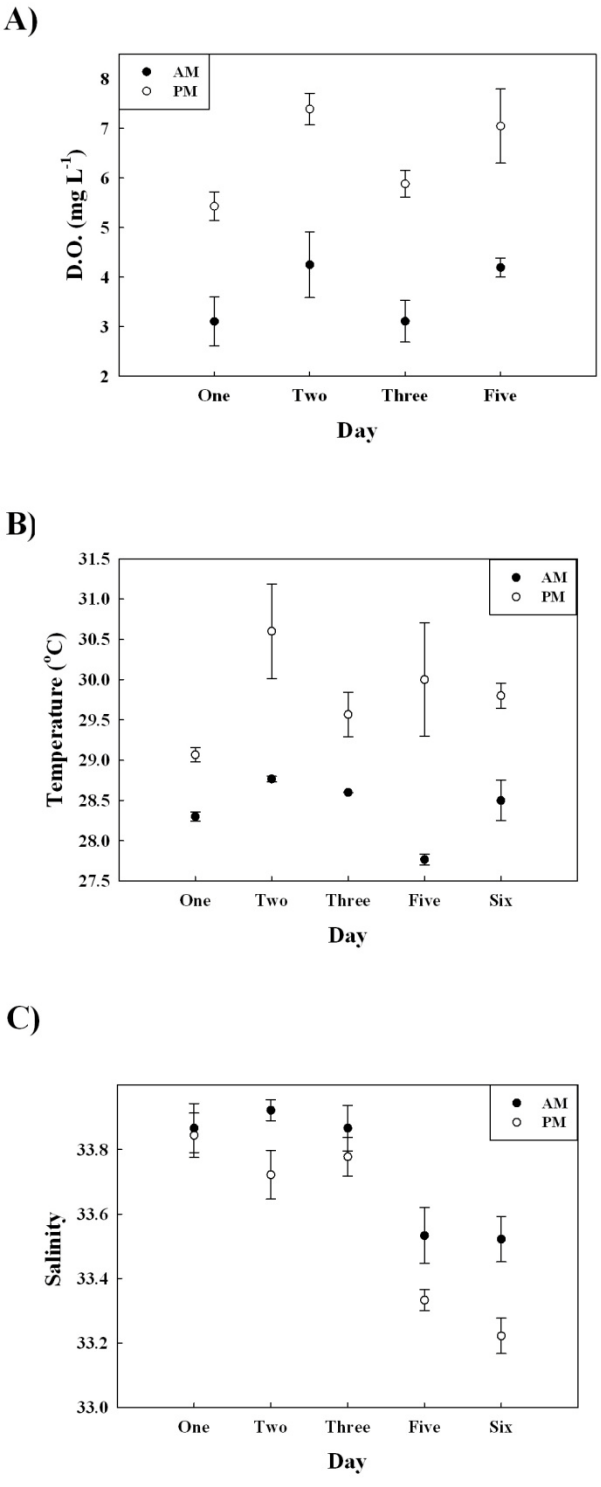

Figure 9. Mean ± SEM for daily A) dissolved oxygen (D.O.) (n = 3 per sampling event), B) temperature (n = 3 per sampling event), and C) salinity (psu, n = 9 per sampling event) measured during the week of July 28 – August 2, 2012.

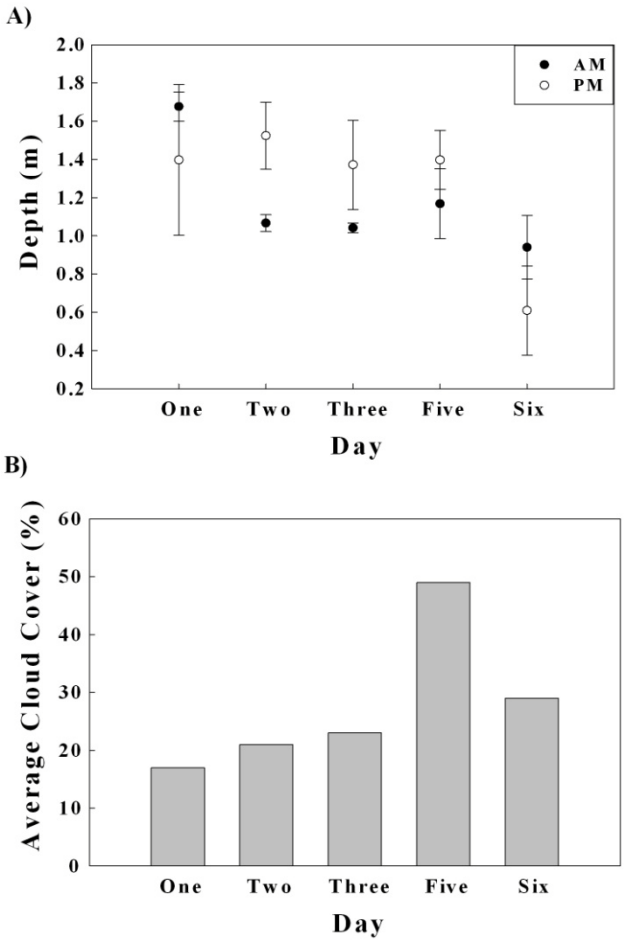

Figure 10. A) Mean ± SEM (n = 3 per sampling time) for water depth recorded during daily sampling events over the course of the week July 28 – August 2, 2012. B) Average cloud cover for the day sampled.

When variation due to day was ignored, parameters measured throughout the week on a daily basis did exhibit variation by site but the pattern was not as clear as for those parameters measured throughout the year (Table 5). In the morning, the two more northern sites ('B' and 'C') tended to be significantly different whereas in the afternoon the two more southern sites ('A' and' B') were different, depending on the parameter. Depth was not significant by site ($F = 1.51$, $df = 6, 8, 14$, $p = 0.2777$, $F = 1.04$, $df = 6, 8, 14$, $p = 0.3957$ for pm). Temperature was not significant by site in the morning, but was significantly different between sites A and B, $p = 0.0415$, temperatures were 30.44°C ± 0.49 for A, 29.38°C ± 0.13 for site B).

Various parameters on daily samples exhibited stronger correlations than that of monthly samples and regression results can be found in Table 6. TA was weakly correlated with salinity but not correlated with temperature or depth. TA was strongly correlated with DIC (Fig. 11A) and to a lesser extent correlated with pCO_2, pH, and D.O. D.O. and pCO_2 were strongly correlated (Fig. 11B) and pCO_2 was correlated with temperature, although not to the same degree. DIC was most strongly correlated with D.O. (Fig. 11C) and with temperature to a lesser extent. D.O. and pH were also strongly correlated (Fig. 11D).

Hourly seawater chemistry

Trends in carbonate parameters were less clear in samples taken over the course of the day on July 28, 2012. The values of pH, Ω_{Ca} and Ω_{Ar} fluctuated throughout the day but varied similarly with lowest values exhibited at noon and highest values observed at eight am and two pm (Fig. 12). Total alkalinity and DIC continued to increase over the

Table 4. Descriptive statistics on Eagle Harbor parameters sampled on a daily basis in the week of July 28 –August 2, 2012. SEM = standard error of the mean. Salinity in psu. Avg. Δ diurnal = average difference in daily minimum and maximum values.

	pH	Ω Ca	Ω Ar	$p\,CO_2$ (µatm)	DIC (µmol kg^{-1})	TA (umol/kg)	D.O. (mg L^{-1})	Temp (°C)	Salinity
Mean	7.83	3.84	2.56	733.30	2073.77	2281.32	5.05	29.1	33.7
SEM	0.01	0.08	0.05	16.99	6.41	1.89	0.35	0.2	0.0
Median	7.82	3.67	2.44	741.72	2084.58	2283.04	5.14	29.0	33.7
Std. Dev.	0.09	0.72	0.48	161.15	60.83	17.98	1.72	1.0	0.3
Min.	7.70	2.80	1.86	379.31	1908.46	2228.14	2.22	27.7	32.9
Max.	8.06	5.77	3.85	1018.53	2154.82	2312.44	8.50	31.7	34.1
Range	0.35	2.96	1.99	639.22	246.37	84.30	6.28	4.0	1.2
Avg. Δ Diurnal	0.11	0.96	0.65	211.71	76.41	14.23	2.78	1.4	0.2

Table 5. Site variability in parameters measured daily for the week of July 28 – August 2, 2012 based on a two-way ANOVA blocking for day variation. Mean ± SEM. Letters denote significant difference ($p < 0.05$) based on pair wise comparisons.

	A_{AM}	B_{AM}	C_{AM}	A_{PM}	B_{PM}	C_{PM}
pH	7.78 ± 0.01^{AB}	7.76 ± 0.01^{A}	7.80 ± 0.02^{B}	7.99 ± 0.02^{A}	7.87 ± 0.02^{B}	7.89 ± 0.02^{A}
Ω Ca	3.34 ± 0.06^{AB}	3.25 ± 0.07^{A}	3.50 ± 0.14^{B}	4.49 ± 0.16^{A}	4.15 ± 0.14^{B}	4.34 ± 0.20^{AB}
Ω Ar	2.22 ± 0.04^{AB}	2.16 ± 0.05^{A}	2.32 ± 0.09^{B}	3.00 ± 0.11^{A}	2.77 ± 0.09^{B}	2.90 ± 0.14^{AB}
$p\,CO_2$ (µatm)	838.04 ± 20.67^{AB}	874.11 ± 24.21^{A}	805.31 ± 37.84^{B}	607.86 ± 33.42^{A}	651.12 ± 29.80^{B}	623.35 ± 38.93^{AB}
DIC (µmol kg^{-1})	2114.54 ± 7.51^{AB}	2121.54 ± 7.07^{A}	2099.85 ± 11.59^{B}	2022.14 ± 13.07^{A}	2052.35 ± 13.44^{B}	2032.22 ± 17.30^{A}
TA (umol/kg)	2290.40 ± 4.00	2289.97 ± 4.27	2284.93 ± 3.63	2270.97 ± 3.17^{A}	2279.93 ± 5.28^{B}	2271.71 ± 5.01^{AB}
D.O. (mg L^{-1})	3.66 ± 0.25	3.79 ± 0.36	3.55 ± 0.75	7.17 ± 0.65^{A}	6.16 ± 0.32^{A}	5.99 ± 0.51^{B}

Table 6. Summary of regressions on parameter relationships measured and calculated on a daily basis (n = 90 with the exception of D.O. measurements where n = 72). Salinity in psu.

Weekly	R^2	Equation
TA (µmol kg^{-1}) and Salinity	0.364	TA = 1057.019 + (36.371 * Salinity)
TA (µmol kg^{-1}) and Temperature (°C)	0.194	TA = 2523.867 - (8.336 *Temp)
TA (µmol kg^{-1}) and Depth (m)	0.253	TA = 2253.219 + (22.514 * Depth)
TA (µmol kg^{-1}) and DIC (µmol kg^{-1})	0.732	TA = 1756.970 + (0.253 * DIC)
TA (µmol kg^{-1}) and $p\,CO_2$ (µmol kg^{-1})	0.565	TA = 2219.816 + (0.0839 * $p\,CO_2$)
TA (µmol kg^{-1}) and pH	0.580	TA = 3521.864 - (158.376 * pH)
TA (µmol kg^{-1}) and D.O. (mg L^{-1})	0.448	TA = 2312.833 - (5.058 * D.O.)
DIC (µmol kg^{-1}) and Temperature (°C)	0.523	DIC = 3420.642 - (46.289 *)
DIC (µmol kg^{-1}) and D.O. (mg L^{-1})	0.814	DIC = 2213.701 - (24.078 * D.O.)
pH and D.O. (mg L^{-1})	0.767	pH = 7.638 + (0.0335 * D.O.)
$p\,CO_2$ (µmol kg^{-1}) and Temperature (°C)	0.535	$p\,CO_2$ = 4340.909 - (123.987 * Temp)
$p\,CO_2$ (µmol kg^{-1}) and D.O. (mg L^{-1})	0.756	$p\,CO_2$ = 1122.792 - (68.057 * D.O.)

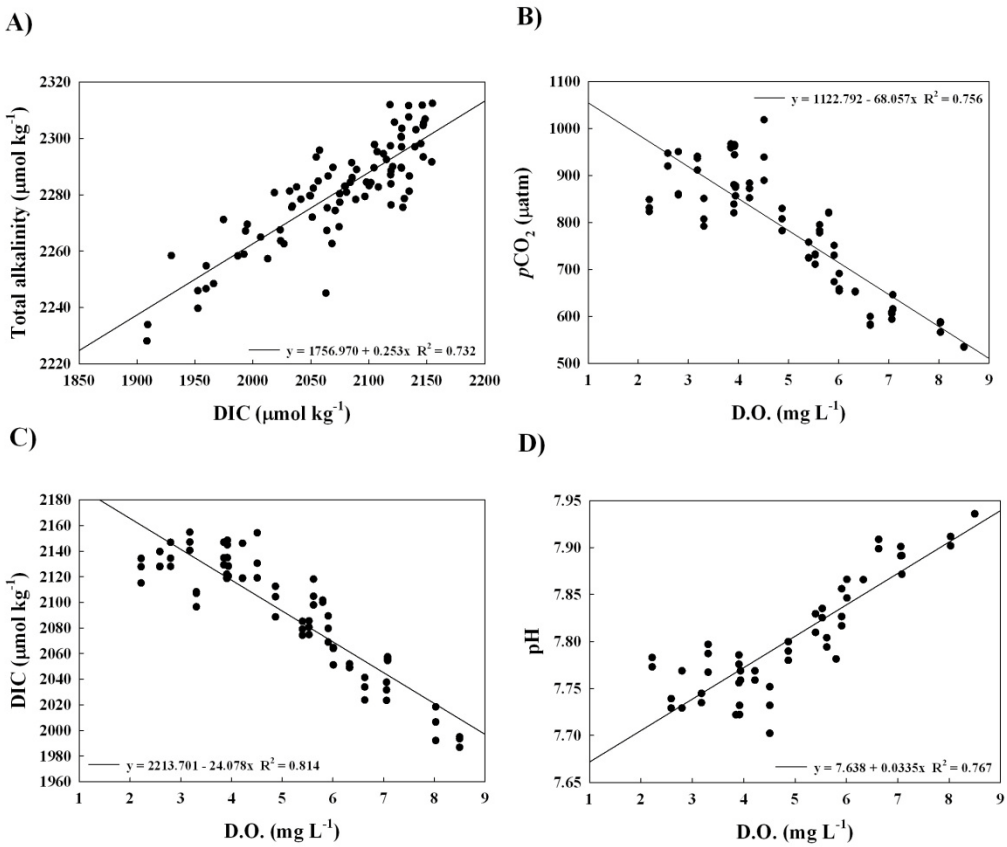

Figure 11. Relationships between A) total alkalinity and dissolved inorganic carbon (DIC), B) pCO_2 and dissolved oxygen (D.O.), C) DIC and D.O. and D) pH and D.O. on morning and afternoon samples obtained on a daily basis during the week July 28 – August 2, 2012.

course of the day whereas pCO_2 displayed a pattern opposite that of pH and carbonate saturation states with highest values observed at noon and lowest values at eight am and two pm (Fig. 13). Temperature and D.O. increased over the course of the day whereas salinity decreased (Fig. 14). Data on D.O. was not available at eight am. Water depth decreased throughout the day (Fig. 14D). Maximum cloud cover for the day was reported to be 100 %, yet at the times sampled, the highest average cloud cover was recorded at noon and was 88 %, with only 25 % reported for 10 am, and 0% cloud cover reported for the other time samplings (data not shown).

The difference over the course of the day of various parameters was less than that of monthly and daily samples, however average pH, Ω_{Ar}, and pCO_2 were significantly different from that of the monthly and daily samples (7.60 pH units, 1.58, and 1321 μatm respectively, Table 7).

Similar to daily samples, some parameters exhibited strong correlations. TA was weakly correlated with salinity but not correlated with pCO_2 or pH (Table 8). Yet TA was strongly correlated with DIC (Fig. 15) and D.O. and to a lesser extent with temperature and depth. DIC was correlated to some extent with temperature and D.O., and pCO_2 was correlated somewhat with temperature. D.O. was not correlated with pCO_2 or pH.

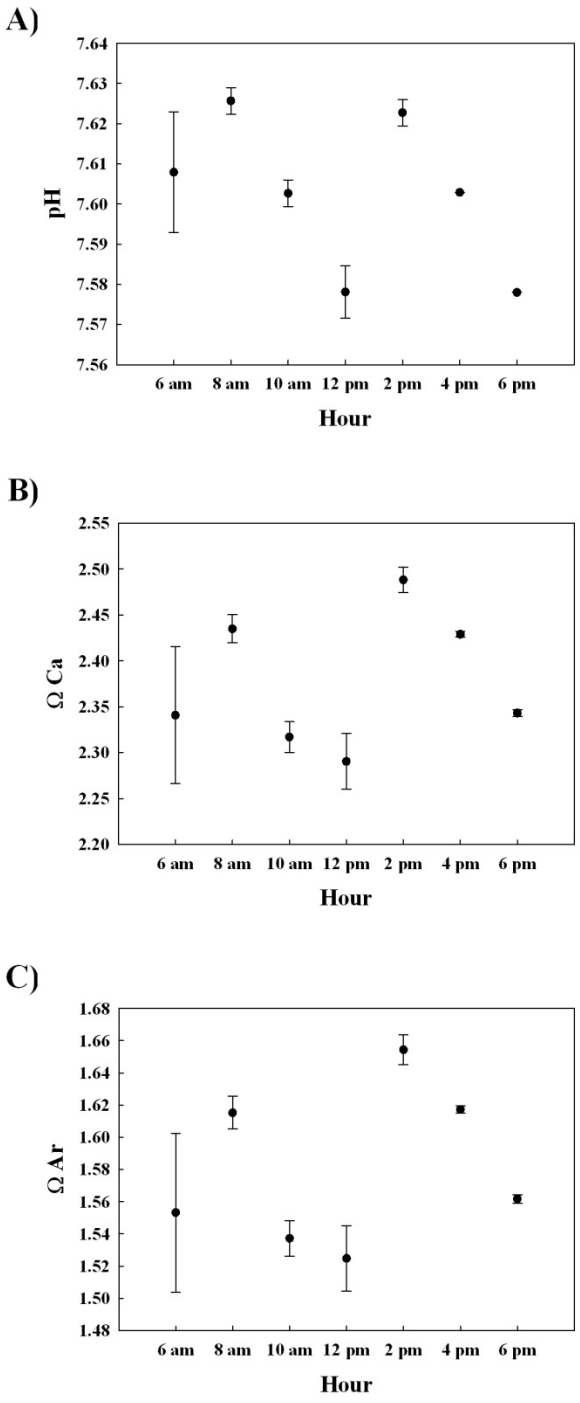

Figure 12. Mean ± SEM (n = 3 per sampling time) of A) pH, B) calcite saturation sate (Ω_{Ca}), and C) aragonite saturation state (Ω_{Ar}) measured every two hours over the course of July 28, 2012.

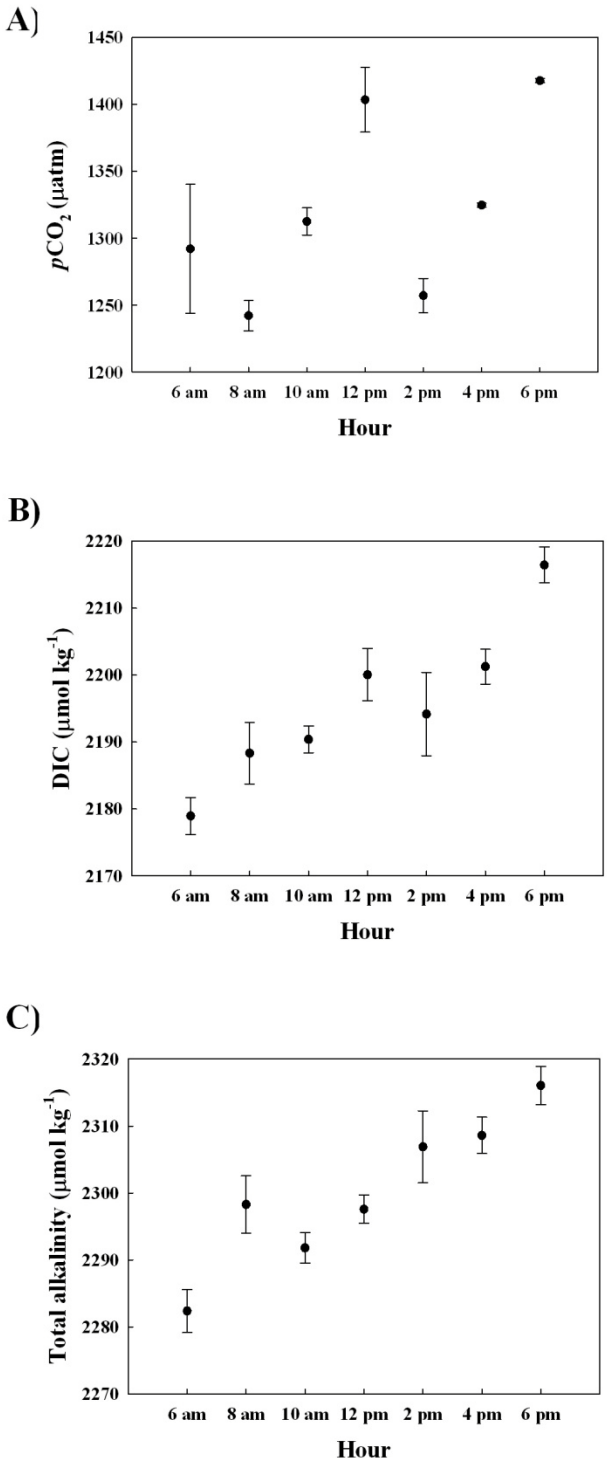

Figure 13. Mean ± SEM (n = 3 per sampling time) of A) pCO_2, B) dissolved inorganic carbon (DIC), and C) total alkalinity measured every two hours on July 28, 2012.

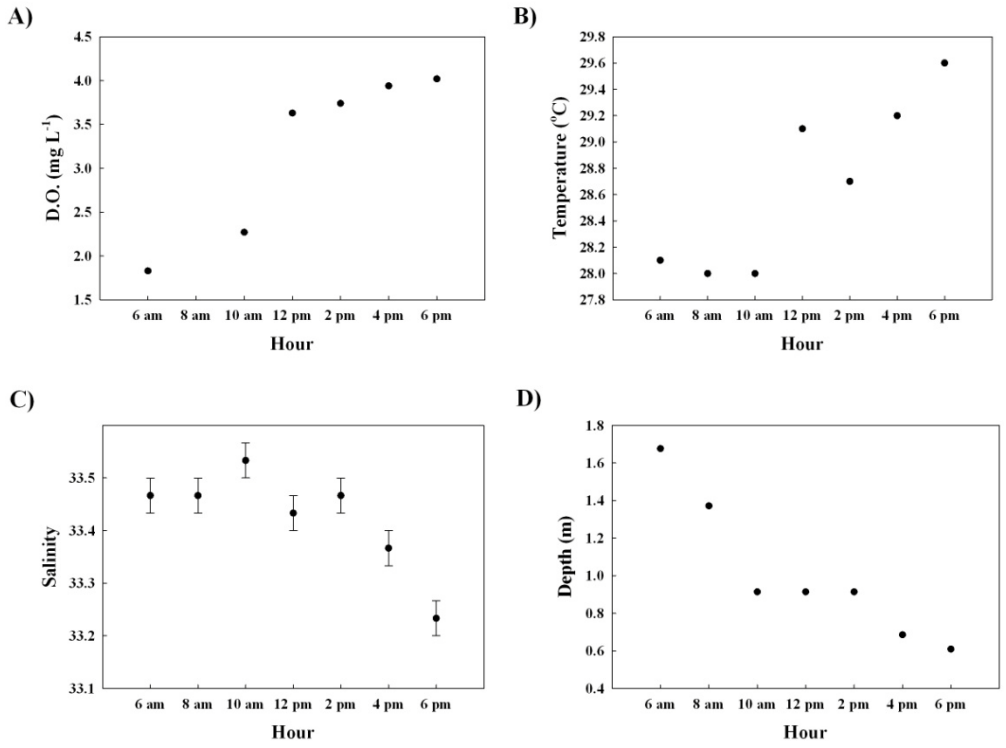

Figure 14. Values measured every two hours for A) dissolved oxygen (D.O.), B) temperature, C) salinity (psu, n = 3 per sampling time) and D) depth measured over the course of the day on July 28, 2012.

Table 7. Descriptive statistics on Eagle Harbor parameters sampled every two hours on July 28, 2012. SEM = standard error of the mean. Salinity in psu.

	pH	Ω Ca	Ω Ar	*p* CO_2 (µatm)	DIC (µmol kg^{-1})	TA (umol/kg)	D.O. (mg L^{-1})	Temp (°C)	Salinity
Mean	7.60	2.38	1.58	1321.42	2195.64	2300.23	3.24	28.7	33.4
SEM	0.00	0.02	0.01	15.57	2.73	2.58	0.38	0.3	0.0
Median	7.60	2.39	1.58	1322.26	2193.69	2299.91	3.69	28.7	33.4
Std. Dev.	0.02	0.08	0.06	71.36	12.53	11.83	0.94	0.7	0.1
Min.	7.57	2.19	1.46	1227.66	2175.15	2276.81	1.83	28.0	33.2
Max.	7.63	2.51	1.67	1451.87	2221.49	2321.55	4.02	29.6	33.6
Range	0.06	0.32	0.22	224.21	46.34	44.74	2.19	1.6	0.4

Table 8. Summary of regressions on parameter relationships measured and calculated on a daily basis (n = 21 with the exception of D.O. measurements where n = 18).

Hourly	R^2	**Equation**
TA (µmol kg^{-1}) and Salinity	0.387	TA = 4654.132 - (70.426 * Salinity)
TA (µmol kg^{-1}) and Temperature (°C)	0.525	TA = 1903.237 + (13.846 * Temp)
TA (µmol kg^{-1}) and Depth (m)	0.558	TA = 2325.011 - (24.481 * depth)
TA (µmol kg^{-1}) and DIC (µmol kg^{-1})	0.747	TA = 508.258 + (0.816 * DIC)
TA (µmol kg^{-1}) and *p* CO_2 (µmol kg^{-1})	0.079	TA = 2238.695 + (0.0466 * *p* CO_2)
TA (µmol kg^{-1}) and pH	0.029	TA = 3056.156 - (99.431 * pH)
TA (µmol kg^{-1}) and D.O. (mg L^{-1})	0.735	TA = 2261.157 + (12.165 * D.O.)
DIC (µmol kg^{-1}) and Temperature (°C)	0.663	DIC = 1723.206 + (16.477 * Temp)
DIC (µmol kg^{-1}) and D.O. (mg L^{-1})	0.594	DIC = 2160.477 + (11.235 * D.O.)
pH and D.O. (mg L^{-1})	0.072	pH = 7.618 - (0.00589 * D.O.)
***p* CO_2 (µmol kg^{-1}) and Temperature (°C)**	0.463	*p* CO_2 = -928.118 + (78.459 * Temp)
***p* CO_2 (µmol kg^{-1}) and D.O. (mg L^{-1})**	0.139	*p* CO_2 = 1241.489 + (28.762 * D.O.)

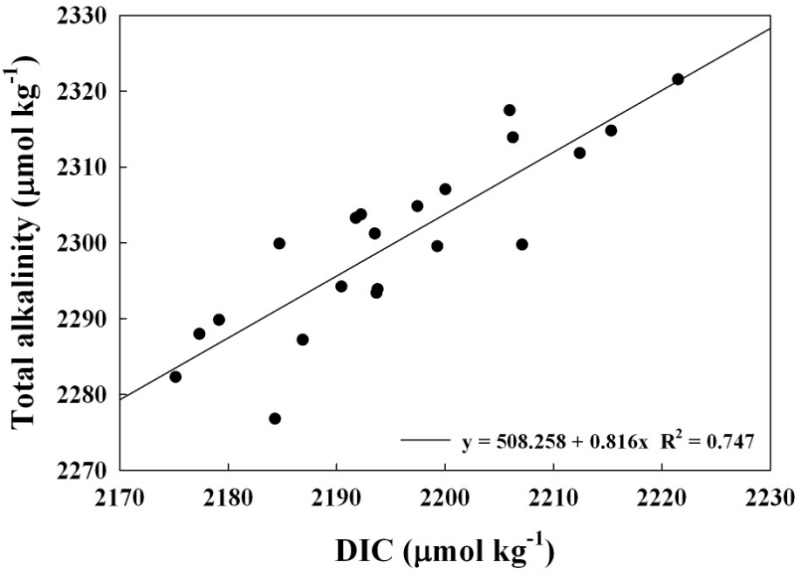

Figure 15. Relationship between total alkalinity and dissolved inorganic carbon (DIC) measured every two hours on July 28, 2012 off the dock at the boat launch area in Eagle Harbor.

Discussion

Factors influencing carbonate chemistry

To our knowledge, there are no published studies on the carbonate system of Saint Joseph Bay. However, other bays in Florida such as Tampa Bay have exhibited similar diurnal trends in carbonate parameters to those observed in the current study (Yates et al. 2007). The source of these diurnal trends is likely biological. As photosynthesis occurs, CO_2 is 'drawn down': pCO_2 and DIC decrease and pH, D.O., and carbonate saturation states increase. Respiration results in opposite trends. The fact that these parameters often correlated well with one another in the current study further supports the significance of the biological influence on the seawater carbonate chemistry of Saint

Joseph Bay. Temperature does affect the solubility of gases and was correlated to some extent with pCO_2 in daily and hourly samples. Yet the impact of temperature (e.g., for every 1°C increase, an increase in pCO_2 of 4.4 % occurs, Gordon and Jones 1973) was considerably less than differences observed between morning and afternoon temperature and pCO_2 values on the majority of days sampled. Salinity and nutrient concentrations can influence the buffering capacity of a body of water, otherwise known as total alkalinity (TA) (see Wolf-Gladrow et al. 2007, for a discussion). However, in the current study, TA and salinity were not strongly correlated on any time scale. Although data on seawater nutrient concentrations was not available for the current study, Saint Joseph Bay is classified as having oligotrophic waters and nutrient and chlorophyll-a concentrations within the bay have been consistently low and stable from 2001 – 2009 (FDEP 2012).

Tidal fluctuation can also be an important factor in carbonate chemistry (e.g., Borges and Frankignoulle 1999) however, water depth (as a function of tidal cycles) was not strongly correlated with TA except with respect to the samples taken every two hours ($R^2 = 0.558$). Further studies that involve hourly samples over 48 to 72 hours may better determine the influence of tides within the bay. Cloud cover (as a function of the degree of solar insolation) can exert control over photosynthetic processes, but given the diurnal trends in D.O. observed in the current study, cloud cover did not appear to have a large influence on the majority of days sampled. It is important to point out that we did not investigate changes in CO_2 flux at the air-sea interface or the influence of wind speeds. Wind speed can impact that rate of CO_2 flux (Wanninkhof 1992) and the difference between the partial pressure of CO_2 in seawater and that of the air (~ 400 μatm) can

significantly impact the flux of CO_2 either into (i.e., pCO_2 of seawater < 400 μatm) or out of (pCO_2 of seawater is > 400 μatm) the water.

The fact that TA was strongly, positively correlated with DIC at all time scales may indicate the influence of calcification in Saint Joseph Bay. Precipitation of calcium carbonate ($CaCO_3$) results in a decrease in DIC and TA, whereas dissolution of $CaCO_3$ results in increases in these parameters (for a discussion, see Wolf-Gladrow et al. 2007). Precipitation of $CaCO_3$ can be in the form of inorganic precipitation, due to the mixing of sediments, or formed biogenically by calcifying organisms. Given the low energy regimes (Heck et al. 2000) and abundance of calcifying epiphytes and marine invertebrates within the bay (FDEP 2012), biogenic calcification is likely the main source of $CaCO_3$ precipitation. According to Gattuso et al. (1999), the ratio of $\Delta TA/\Delta DIC$ can indicate whether net calcification or net photosynthesis is the dominant metabolic process occurring in a community. For every mole of $CaCO_3$ precipitation, DIC decreases 1 mole and TA decreases 2 moles. Therefore, as the ratio of $\Delta TA/\Delta DIC$ approaches 2.0, calcification is the sole process occurring. The addition or removal of CO_2 to the water column does not affect TA (Wolf-Gladrow et al. 2007), however, as explained above, photosynthesis can have a significant impact on DIC (i.e., remove CO_2 from the water column and decrease DIC). Therefore, when net photosynthesis is the sole process occurring, the ratio $\Delta TA/\Delta DIC$ approaches zero. As these processes are likely to be co-occurring in the field, $\Delta TA/\Delta DIC$ ratios observed often reflect this fact. For example, $\Delta TA/\Delta DIC$ ratios of coral reef flats increased from 0.47 to 0.54 as the percentage of live coral cover increased from 10 to 20 % (Yates and Halley 2006b, calculated by Yates et al. 2007). In the current study, $\Delta TA/\Delta DIC = 0.42$ (fig. 6A), 0.25 (Fig. 11A) and 0.816

(Fig. 15) for the monthly, daily, and hourly samples, suggesting that the Harbor may exhibit a dominant photosynthetic role depending on the time scale employed. The site where hourly samples were obtained was not a seagrass bed (Fig. 1), but rather a semi-enclosed boat launch area where various marine invertebrates including sea urchins and oysters were observed in close proximity during sampling. Thus, the high $\Delta TA/\Delta DIC$ ratio is supportive of the lack of seagrass in the area and may not be indicative of the carbonate chemistry dynamics of the bay. It is interesting to note that at this site, hourly DIC and TA both increased under an especially low pH profile (pH 7.57 – 7.63 with a mean of 7.60), suggesting that the high $\Delta TA/\Delta DIC$ ratio may actually be indicative of dissolution of $CaCO_3$ rather than calcification. Although less common, daytime net sediment dissolution has been observed in Florida Bay under conditions of high cloud cover, turbidity or salinity (Yates and Halley 2006a). In the current study, heavy cloud cover was observed on the day of hourly sampling (100 %). Although the threshold of pCO_2 values needed in order for dissolution of local sediments to occur was not determined in the current study, the average pCO_2 threshold value of various substrate types for a reef flat in Hawaii was 654 ± 195 μatm (Yates and Halley 2006b). Values of pCO_2 observed in this study were well above this threshold on the day of sampling (1227 – 1451 μatm). Therefore, it is likely that net dissolution of $CaCO_3$ was occurring.

Although mean values of carbonate parameters did vary significantly with site, this variation may not be biologically relevant as differences in diurnal fluctuations at a specific site were greater than mean differences between sites. For example, mean daily site pH values for am and pm samples varied by 0.04 and 0.1 pH units, whereas

differences in mean daily diurnal values at each site were 0.21, 0.11 and 0.09 pH units for site A, B, and C respectively (Table 5).

Implications for ocean acidification experiments

The results of the current study have important implications for the design of ocean acidification experiments that employ nearshore organisms both in terms of target carbonate chemistry values and fluctuations of those values. For example, current OA experiments on marine organisms living in nearshore waters often use target experimental values predicted for near-future conditions in the open ocean (e.g., pCO_2 values of 800 μatm, pH 7.7). The mean values reported in the present study (pH 7.84, pCO_2 813.83 μatm) suggests that the control values typically employed in OA experiments (pH 8.1, pCO_2 400 μatm) with nearshore organisms may not be representative of natural conditions in coastal waters such as bays and lagoons. Therefore, in some instances experimental values employed may be more ecologically relevant when used as control values. This observation has important implications for interpreting OA results. For example, several species of echinoids have displayed negative impacts such as delayed larval development when exposed to changes in pH for extended periods of time (e.g., 0.4 pH units, see Dupont et al. 2010, Byrne 2011; Sewell and Hofmann 2013, for a review). In Saint Joseph Bay, high gonadal indices have been reported during late July/early August for the sea urchin *Lytechinus variegatus* (Beddingfield and McClintock 2000), indicating that spawning and fertilization events likely take place during that time of year. The current study found daily pH values in the range of 8.06 – 7.70 and pCO_2 values of 1018.53 – 379.31 μatm (with a mean of 7.83 pH units and 733.30 μatm) during late July/early August, suggesting that the larvae of *L.*

variegatus that occur in the bay may experience reduced pH/high pCO_2 conditions. Laboratory studies on the early development of *L. variegatus* indicate that larval echinoplutei exhibit delayed development and altered larval shape if exposed to low pH and high pCO_2 values for an extended period of time (Challener et al. 2013). Yet the population of *L. variegatus* at Eagle Harbor has been relatively stable for over 25 years (Stephen Watts, pers. obs.). Therefore, the low pH/ high pCO_2 conditions of OA experiments may be more representative of the current natural conditions and thus, more indicative of the natural developmental rates of echinoids in the field.

The extensive diurnal fluctuations observed in the current study are also important aspects to consider in the design of OA experiments. Most OA studies maintain static pH and pCO_2 values over the course of an experiment and have not incorporated the daily fluctuations observed in the current study. These fluctuations may serve as critical periods of organismal recovery from hypercapnic conditions. For example, accretion rates of coral reef sites have been observed to be higher at sites that exhibit longer number of hours of high pH values (Price et al. 2012). Populations of marine organisms within Saint Joseph Bay have been characterized as highly productive and healthy (with the exception of sporadic red tide events, FDEP 2012, refs within), suggesting that these organisms have either acclimatized or adapted successfully to extensive variation in pH and pCO_2 values. The physiological mechanisms underlying the ability to cope with such dramatic fluctuations in both physical and chemical parameters are not well understood and the biological consequences of increasing ocean acidification superimposed on these natural fluctuations are unknown. If periods of high pH/low pCO_2 conditions are essential for organismal function, then the negative consequences of a high pCO_2 future

(i.e., where the 'high pH' is no greater than 7.8) may be significant. To date, the ability of echinoids to acclimate to hypercapnic conditions within 16 months has been observed in one species (Dupont et al. 2012), highlighting the need for additional studies on the capacity for acclimation and potentially acclimatization in the field. Long-term OA studies that incorporate the natural diurnal variation into both control and experimental treatments are needed to determine nearshore organismal resilience to near-future ocean acidification conditions.

Acknowledgments

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CONCLUSIONS

The common, edible sea urchin *Lytechinus variegatus* exhibited negative impacts to aspects of development, production, and physiology under chronic exposure to hypercapnic conditions ($pH \leq 7.8$, $pCO_2 \geq 1738$ μatm, $\Omega_{Ar} \leq 1.65$). Although the pCO_2 values employed in this dissertation were significantly higher than those predicted to occur in ocean surface waters by the end of this century (e.g., 800 μatm), the pCO_2 values observed in the nearshore habitat of *L. variegatus* suggest that the experimental values used in this dissertation are more ecologically relevant than previously thought. These results have both ecological and economic implications. The immediate consequences of delayed development and altered larval allometry (reduced arm length) (Chapter 1) in the field are likely increased predation rates (Hare and Cowen 1997; Allen 2008) and impaired feeding (Strathmann 1971) with downstream consequences on settlement and recruitment timing and/ or success. Reduced somatic and reproductive production in early adults due to changes in nutrient and energy allocation may impact gametogenesis and/ or the timing of gonad maturity and reproduction (Chapter 2) with subsequent impacts on population dynamics. *L. variegatus* plays a significant role in the community structure of seagrass beds (Goodbody 1970; Keller 1973; Valentine and Heck 1993; Valentine et al. 1997, 2000; Beddingfield and McClintock 1999, 2000; Macia 2000). Therefore, chronic hypercapnic conditions that impact populations of *L. variegatus* may ultimately influence the community structure of seagrass-dominated habitats where *L. variegatus* exists.

Slower growth rates and reduced production also have significant negative economic ramifications for aquaculture facilities and therefore, the maintenance of ideal seawater carbonate parameters is paramount. For example, oyster farms along the western coast of the U.S. (where upwelling of very low pH exacerbated by anthropogenic carbon dioxide now occurs regularly) have begun monitoring the pH of incoming seawater pH in order to avoid introducing low pH water into their culturing systems that causes catastrophic mortality among oyster spat (Barton et al. 2012). Long-term exposure of adult echinoids to hypercapnic conditions prior to spawning may facilitate the resilience of future generations to extended hypercapnic conditions (Dupont et al. 2012).

Extensive variation in the carbonate chemistry of the nearshore habitat of *L. variegatus* is primarily due to the influence of the seagrasses that dominate the area (Chapter 4). Whether exposure to periods of hypercapnic conditions encourages acclimatization to low pH/ high pCO_2 conditions in *L. variegatus* is unknown. Currently, larvae of *L. variegatus* that occur in St. Joseph Bay are potentially experiencing reduced pH/high pCO_2 conditions (e.g., mean values of 7.83 pH units and 733.30 μatm in late July/early August; Chapter 4). Therefore, the slower developmental rates and smaller larval sizes observed under hypercapnic conditions employed in this dissertation may be more indicative of the natural developmental rates and sizes of larval echinoids in Saint Joseph Bay. However, the fluctuations in carbonate parameters may serve as critical periods of organismal recovery from hypercapnic conditions. For example, accretion rates of coral reef sites have been observed to be higher at sites that exhibit longer number of hours of high pH values (Price et al. 2012). There is evidence that echinoid

larvae have the genetic capacity to respond quickly to rising CO_2 concentrations, as the greatest changes in allele frequencies under exposure to hypercapnic conditions have been associated with genes that improve larval fitness under high pCO_2 conditions (e.g., genes involved in lipid metabolism, ion homeostasis, cell signaling, and protein modification, Pespeni et al. 2013). Therefore, those larvae with the ability to develop normally under hypercapnic conditions are likely to be selected for under near-future ocean acidification conditions.

As the righting response, covering behavior and feeding behavior were not affected by chronic exposure to hypercapnic conditions (Chapter 2 and 3), *L. variegatus* may have some capacity to acclimatize when it comes to these specific behavioral parameters. Moreover, increased organic production and energy allocation to the lantern, and increased test ossicle strength (Chapter 2) may indicate that *L. variegatus* has some capability to compensate for the effects of exposure to hypercapnic conditions. However, longer-term studies that incorporate the natural diurnal variability in carbonate chemistry are needed to further understand whether *L. variegatus* will be resilient to near-future ocean acidification conditions.

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APPENDIX

APPROVAL OF INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: November 25, 2009

TO: McClintock, James B.
CH-368 1170
975-2525

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Effects of Ocean Acidification on the Activity, Physiology and Reproductive Condition of Lytechinus Variegatus
Sponsor: Internal
Animal Project Number: 091109002

On November 25, 2009, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Invertebrates	A	150

Animal use is scheduled for review one year from November 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 091109002 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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