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THE EFFECTS OF OCEAN ACIDIFICATION AND TEMPERATURE ON THE PHYSIOLOGY OF JUVENILE SCLERACTINIAN CORALS

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By

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The Effects of Ocean Acidification and Temperature on the Physiology of Juvenile Scleractinian Corals

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Ocean acidification (OA) from the equilibration of atmospheric CO_2 with seawater is predicted to negatively affect scleractinian reef corals over the 21st century. In adult corals, OA has been reported to reduce rates of coral calcification, induce coral bleaching, and affect coral respiration and the photosynthetic productivity of the coral's algal symbiont *Symbiodinium* spp. However, OA effects on the early life-history stages of corals (e.g., larvae, newly settled recruits, juveniles) are less understood. In this thesis research, I used juvenile *Seriatopora caliendrum* from southern Taiwan, and massive *Porites* spp. from Moorea, French Polynesia, to experimentally evaluate the effects of OA on three important physiological processes of scleractinians: coral bleaching, calcification, and metabolism inferred through respiration and excretion rates. In Chapter II, using *S. caliendrum*, results from my research indicate that OA (841 μatm pCO_2) does not affect juvenile *S. caliendrum* corals undergoing thermal bleaching at 30.53 °C, and OA alone does not result in coral bleaching under ambient temperatures (27.65 °C). Subsequently in Chapter III, I exposed juvenile massive *Porites* spp. to low-pH and elevated dissolved inorganic carbon (DIC) seawater within sealed respirometers incubated *in situ*. I determined that low-pH (pH_T 7.73, $\Omega_{arag} = 2.27$) at 976 μatm pCO_2

had no effect on calcification, respiration, or the energetic expenditure concurrent with calcification relative to control conditions. However, elevated [DIC] (~3 mM) stimulated calcification under low-pH and low- Ω_{arag} (pH_T 7.69, Ω_{arag} = 2.75), indicating calcification may be DIC limited under ambient [DIC] (~2 mM). These results agree with past studies identifying adult and juvenile massive *Porites* spp. as resistant to environmental stress, and emphasize the success and pragmatism of *in situ* designs for the future application of ecologically relevant OA experiments. Finally, in Chapter IV using *S. caliendrum*, I determined that OA (885 μatm pCO_2) affected the metabolism of intact juvenile corals by increasing nitrogen excretion, however OA did not affect rates of aerobic respiration or total protein content. Finally, I present preliminary results that suggest OA (885 μatm pCO_2) affects coral metabolism by increasing rates of nitrogen excretion, which has implications for amino acid and protein catabolism, as well as, the capacity for corals to regulate intracellular pH. In summation, findings presented here suggest: (1) OA does not affect or cause coral bleaching in juvenile corals; (2) certain coral taxa may be resistant to short-term exposure to OA conditions, and (3) OA may affect nitrogen metabolism in the coral-algal symbiosis.

Chapter 1

Introduction

Coral Reefs and Global Climate Change

Coral reefs are centers of biodiversity and species richness. These ecosystems, radiating 25° north and south from the equator (Veron 1986), are constructed primarily by reef-building scleractinian corals (Phylum: *Cnidaria*, Class: *Anthozoa*, Subclass: *Hexacorallia,* Order: *Scleractinia*). Through biogenic calcification, reef corals function as ecosystem engineers by contributing to the architectural complexity of the benthos, producing habitat for reef organisms (Wild et al. 2011). Tropical hermatypic reef-building corals exist in a symbiotic relationship with the dinoflagellate alga *Symbiodinium* spp. (Goreau 1959), which live within the oral endodermal tissue of corals. The symbiont translocates photosynthetic fixed carbon and other nutrients (e.g., amino acids, peptides) to the coral to fuel metabolism, growth, and calcification. In return, the alga receives respiratory CO_2 from the host, as well as, essential nutrients in the form of amino acids, ammonia, and phosphates derived from coral metabolism (Muscatine et al. 1981; Falkowski et al. 1984; Rahav et al. 1989). This unique symbiosis has allowed the scleractinians, and consequently coral reefs, to flourish in the oligotrophic tropical oceans for > 200 million years (Hoegh-Guldberg 1999).

Ecologically, coral reefs are indispensible ecosystems of the shallow tropical and subtropical oceans, and provide ~$30 billion a year in resources and ecosystem services, predominately in the form of fisheries, tourism, and coastal protection (Cesar et al. 2003).

However, between 50 and 70% of coral reefs worldwide are under direct pressure from human activities, resulting in marked declines in the health of these ecosystems (Gardner et al. 2003; Bruno and Selig 2007; Wilkinson 2008). The continued decline in the global abundance of coral reefs is an ominous harbinger for the future of these ecosystems and the millions of people who depend upon them for sustenance and livelihood (Reaser et al. 2000; Cesar et al. 2003).

Unprecedented losses of scleractinian corals and coral reef habitat have resulted from natural and anthropogenic factors (Wilkinson 2008). These perturbations include: overfishing and resource mismanagement, mass coral bleaching events, El Nino Southern Oscillation (ENSO) events, disease and crown-of-thorns seastar (COTS) outbreaks (Hoegh-Guldberg 1999; Hughes et al. 2003; Bellwood et al. 2004; Pandolfi et al. 2005; Toth et al. 2012). Additionally, global climate change is hypothesized to be a primary contributor to reef degradation by affecting abiotic (e.g., temperature, carbonate chemistry) and biotic interactions (e.g., disease outbreak, interspecific competition) (Buddemeier et al. 2004; Hoegh-Guldberg and Bruno 2010).

Increased atmospheric pCO_2 is resulting in two threats to coral reefs in the 21st century: ocean warming and ocean acidification (OA) (Hoegh-Guldberg et al. 2007). While there exists a broad and robust collection of field and laboratory research investigating the effects of temperature on the coral-algal symbiosis, the potential for increased atmospheric pCO_2 to affect marine organisms has not been addressed until relatively recently (Gattuso et al. 1998; Kleypas et al. 1999). The response of corals to

stochastic and seasonal increases in sea surface temperature, superimposed against progressive perturbations in seawater carbonate chemistry from OA, will shape the reefs of the future (Silverman et al. 2009). Whether tropical coral reefs and scleractinians are capable of acclimatization and adaptation to such marked environmental change remains to be determined (Edmunds and Gates 2004; Raven 2005).

Ocean Acidification and Global Warming

The industrial era (1750 – present) and the burning of fossil fuels has caused an increase in carbon dioxide (CO_2) in the atmosphere, of which about 33% has been sequestered by the ocean (Sabine et al., 2004; Le Quere et al. 2009). Present global atmospheric pCO_2 (~390 μatm or 39.5 Pa) is at an 800,000 year high (Lüthi et al. 2008) and continues to increase at a magnitude unparalleled in the last 22,000 years (Joos and Spahni 2008; Allison et al. 2009). Elevated concentrations of atmospheric CO_2 function as a greenhouse gas to increase global atmospheric and sea-surface temperatures (SST) (Hansen et al. 2006), and also reduce the pH of surface seawater through dissolution and equilibration of pCO_2 between the ocean and atmosphere in a process termed ocean acidification (Kleypas et al. 1999).

The oceans perform an important role in shaping the global climate. The heat capacity of the ocean is 1000-times greater than that of the atmosphere, and this increased heat capacity has allowed the ocean to absorb ~84% of excess heat from the Earth system (e.g., atmosphere, continents, cryosphere) since 1960 (Levitus et al. 2001; IPCC 2007). Mean global surface temperatures have increased by 0.74 °C (IPCC 2007) since the

beginning of the 20th century and have been increasing at 0.2 °C decade^{-1} since 1960 (Hansen et al. 2006), a rate two-fold greater than the previous century (IPCC 2007). Similarly, ocean warming has been occurring since the late 19th century (Roemmich et al. 2012) resulting in a ~0.67 °C increase in global SST from 1901–2004 (Rayner et al. 2006) accompanied by warming of the equatorial Pacific Ocean (Soloman and Newman 2012). The sharp increase in the rate of oceanic warming from 1979–2005 (0.13 °C per decade; IPCC 2007) suggests a strong effect of anthropogenic forcing driving the increased heat content of the world's oceans (Barnett et al. 2005). Continued emissions of greenhouse gases and CO_2 emissions are projected to contribute to substantial ocean warming and ocean acidification during the 21st century (IPCC 2007). This increase in ocean temperature, combined with OA, are predicted to reduce the resilience of coral reef ecosystems and diminish the capacity of coral reefs to remain in coral dominated states (Anthony et al. 2011).

OA acts to alter the carbonate chemistry of seawater by increasing the concentration of bicarbonate ions ($[HCO_3^-]$) and hydrogen ions ($[H^+]$) while reducing the concentration of carbonate ions ($[CO_3^{2-}]$) and the saturation state of aragonite (Ω_{arag}) (Gatusso and Hansson 2011) following the equation below:

Equation 1:

$$H_2O + CO_2 \leftrightarrow [H_2CO_3] \leftrightarrow H^+ + HCO_3^- \leftrightarrow 2H^+ + CO_3^{2-}$$

The majority of experimental studies on marine calcifiers and noncalcifiers show a negative trend in organism performance under elevated pCO_2 and reduced pH (Kroeker et

al. 2010). However, taxonomic variability in the response of marine organisms to OA has revealed that the physiological responses to OA may be more dynamic than once thought (Fine and Tchernov 2007; de Putron et al. 2011; Pandolfi et al. 2011).

Anthropogenic OA represents an unprecedented change to the carbonate chemistry of the ocean, proceeding at a magnitude and timescale unparalleled to natural variations in the carbonate chemistry of seawater determined from the geological record (Kerr 2010; Zeebe and Ridgwell 2011). OA and increased seawater temperature may jeopardize the existence of coral reefs as calcifying ecosystems (Silverman et al. 2009). The current rate of carbon input into the biosphere is greater than any previous identified event in the history of planet (Zeebe 2012). In the absence of drastic reductions in CO_2 emissions, global climate models predict atmospheric pCO_2 to increase to 490 – 850 μatm (~49.6 – 86.1 Pa) CO_2 by the end of the 21st century under the representative concentration pathway (RCP) 2.6 and RCP 6.0 (van Vuuren et al. 2011; Moss et al. 2012). Over the same period, increased atmospheric pCO_2 is predicted to lead to a 0.3 unit reduction in ocean pH from ~8.1 to 7.8 (Feely et al. 2009; Gattuso and Lavigne 2009) and a 1.8 °C – 4.0 °C increase in mean global surface temperature (IPCC 2007; Sokolov et al. 2009). An environmental change to this degree is hypothesized to drastically affect marine fauna and marine ecosystem function (Kleypas et al. 1999; Feely et al. 2009), with particularly adverse effects predicted for coral reefs and the scleractinian corals (Doney et al. 2009; Kleypas and Yates 2009).

Scleractinian Reef Corals and Ocean Acidification

Biogenic calcification in reef corals and marine calcifiers is controlled by prevailing physical and chemical conditions (e.g., light, water motion, temperature, the saturation state of calcium carbonate in seawater) and the biology of the organism (Kleypass et al. 1999; Tambutte et al. 2011). The saturation state (Ω) of calcium carbonate ($CaCO_3$) in the form of aragonite (Ω_{arag}), the predominant form of $CaCO_3$ used in reef coral calcification, is defined as:

Equation 2:

$$\Omega = [Ca^{2+}]_{sw} \times [CO_3^{2-}]_{sw} / K'_{sp}$$

where K'_{sp} is the apparent stoichiometric solubility constant for the aragonite mineral phase of $CaCO_3$ (Smith and Buddemeier 1992; Kleypass et al. 1999, Gattuso et al. 1998). Saturation states (Ω) > 1 thermodynamically favors calcification, whereas $\Omega < 1$ favor mineral dissolution (Atkinson and Cuet 2008). Ω_{arag} is controlled largely by $[CO_3^{2-}]$ as $[Ca^{+2}]$ is conserved across oceans, however spatial variability in Ω_{arag} exists due to changes in pressure, temperature, and salinity of seawater (Kleypas et al. 1999). Surface seawater in the temperate and tropical oceans is supersaturated ($\Omega > 1$) with respected to aragonite ($\Omega_{arag} = 3.0 – 3.9$). Conversely, surface seawater at high latitudes experience reduced saturation ($\Omega_{arag} = 2.4 – 3.1$) due to the higher solubility of CO_2 in cold water (Feely et al. 2009). Climate models predict Ω_{arag} to decrease 20 – 40% in the tropical oceans by the year 2100 ($\Omega_{arag} = \sim 2.3 – 3.0$; Feely et al. 2009). Reductions in Ω_{arag} reduce calcification and reef accretion (Broecker and Takahashi 1966; Orr et al. 2005; Kleypas and Yates 2009) by making biogenic calcification thermodynamically

unfavorable and perhaps energetically expensive (Atkinson and Cuet 2008; Erez et al. 2011). Therefore, perturbations to the carbonate chemistry of across the global oceans are hypothesized to reduce calcification by reducing Ω_{arag} and reducing the pH of seawater favoring dissolution of $CaCO_3$ (Rodolpho-Metalpa et al. 2011). However, the mechanism by which corals calcify remains elusive and cannot be explained fully in geochemical terms due to the strong influence of biology on calcification (Erez et al. 2011; Tambutte et al. 2011).

Physiological Responses to Ocean Acidification

Manipulative laboratory and field (e.g., CO_2 seeps) CO_2-enrichment experiments have shown OA to reduce coral calcification (Gattuso et al. 1998; Langdon et al. 2000; Langdon and Atkinson 2005), cause coral bleaching (Anthony et al. 2008), disrupt coral recruitment and larval settlement (Albright et al. 2010; Doropoulos et al. 2012), and reduce coral diversity and abundance (Hall-Spencer et al. 2008; Fabricius et al. 2011). However, the effects of OA on the metabolism (e.g., photosynthesis, respiration) of corals and their algal symbionts are more equivocal. For example, elevated pCO_2 reduced protein-standardized net photosynthesis but not respiration in *Stylophora pistillata* (Reynaud et al. 2003), and reduced area-normalized photosynthesis but not dark respiration in *Acropora intermedia* and *Porites lobata* (Anthony et al. 2008). Dark respiration in *Acropora formosa* was unaffected by pCO_2 enrichment, however photosynthetic capacity per chlorophyll increased under medium-CO_2 (600 – 790 ppm) but not high-CO_2 enrichment (1160 – 1500 ppm) (Crawley et al. 2010). Conversely, net productivity in an artificial *Porites compressa/Montipora capitata* reef assemblage was

increased by elevated pCO_2 (Langdon and Atkinson 2005), whereas Schneider and Erez (2006) found area-normalized net photosynthesis and respiration in *Acropora eurystoma* to be unresponsive to increases in pCO_2. Whether incongruent OA effects across species indicate species-specific responses or are artifacts of methodological disparities between studies requires further research (Pandolfi et al. 2011).

Examples in the literature from non-coral marine invertebrates suggest OA may increase metabolic costs of intracellular pH regulation, leading to increased protein metabolism and ammonia excretion (Thomsen and Melzner 2010) while affecting respiratory costs (Comeau et al. 2010; Thomsen and Melzner 2010). Increased metabolic rates may manifest as a general physiological response to environmental stress (Sibly and Calow 1989). Alternatively, periods of stress may elicit metabolic depression (Hand 1996). A variety of stressors (e.g., low pO_2, extreme temperatures) can lead to depressed rates of metabolism and aerobic energy turnover (Guppy and Withers 1999), hypothesized to be an adaptive strategy for conserving energy under environmental stress (Hand and Hardewig 1996; Pörtner et al. 2000). High pCO_2 (i.e., hypercapnia) reduces metabolic rates and functions as an anesthetizing agent in animals tolerant to oscillations of CO_2 in the environment (Pörtner et al. 2004). Indeed, low extracellular pH (pHe) reduced rates of oxygen consumption and metabolism in the marine protosome invertebrate *Sipunculus nudus* (Reipschläger and Pörtner 1996), whereas extreme hypercapnia (~500 Pa) increased rates of ammonia excretion and reduced respiration in the mussel *Mytilus galloprovincialis* (Michaelidis et al. 2005). However, moderate hypercapnia (39 – 250 Pa) led to increased metabolic rates and ammonia excretion in the

blue mussel *Mytlius edulis,* potentially in an effort to compensate for increased costs of intracellular pH (pHi) regulation (Thomsen and Melzner 2010).

Sessile marine invertebrates, including hydrozoans and cnidarians, are hypothesized to have a reduced capacity for regulating pHi relative to the external environment (e.g., acid-base regulation), and as a consequence, a high sensitivity of metabolism to perturbations of pHe (Pörtner 2008). Therefore, these organisms may be particularly sensitive to changes in pHi and pHe and vulnerable to metabolic depression under hypercapnia. However, regulation of pHi has been observed in the symbiotic cnidarian *Anemonia viridis* and the scleractinian coral *Stylophora pistillata* (Venn et al. 2009; 2011). Light- and dark-incubated cells of *A. viridis* and *S. pistillata* containing *Symbiodinium* displayed regions of host cytoplasm with reduced pHi adjacent to the symbionts (Venn et al. 2009). Further, in *S. pistillata*, pHe at the site of calcification—below the calicoblastic epithelium—was elevated ~0.2 and ~0.5 units above ambient seawater in light and dark incubations, respectively (Venn et al. 2011). In contrast, pHi of the calicoblastic epithelium remained stable in the presence or absence of light (Venn et al. 2011). The maintenance of pH gradients between the external environment and internal cellular compartments in scleractinians and other symbiotic cnidarians potentially plays an important role in the cnidarian-algal symbiosis by contributing to the functionality of key physiological processes, including calcification and photosynthesis (Venn et. 2011).

Identification of the mechanisms underlying OA-mediated metabolic depression remain elusive, in part due to difficulties in resolving effects under short-term experiments and the need for very-high pCO_2 concentrations (e.g., > 250 Pa) to observe acute metabolic effects (see, Pörtner 2008). Additionally, threshold pCO_2 concentrations may exist, where the physiological responses of organisms to hypercapnia may be disparate above and below the threshold concentration (Thomsen and Melzner 2010). Edmunds (2012) determined that elevated pCO_2 (87.2 Pa or 861 μatm) likely caused metabolic depression in massive *Porites* spp. by reducing area-normalized respiration and the photochemical efficiency of *Symbiodinium.* However, respiration and photochemical efficiency in intermediate pCO_2 conditions (76.6 Pa or 756 μatm) were not significantly different from control treatments (42.9 Pa or 423 μatm). The difference in response between high pCO_2 and intermediate pCO_2 treatments suggests a potential threshold pCO_2 value between 76.6 and 87.2 Pa driving reduced aerobic turnover and impairing symbiont function at 87.2 Pa. While massive *Porites* spp. has been reported to be tolerant to OA (Edmunds 2011), this species may be affected deleteriously by OA above a pCO_2 threshold, or may show reduced physiological performance after prolonged exposure to OA conditions (Fabricius et al. 2011). Whether pCO_2 threshold concentrations for physiological performance exist for other coral taxa remains to be determined. Reconciling the physiological effects of OA on the coral holobiont across coral taxa is an important step in determining the ecological implications of OA at the organisms and ecosystem level.

Coral Bleaching and Elevated Sea Surface Temperatures

Coral bleaching describes the loss of coral pigmentation from the expulsion of their *Symbiodinium* endosymbionts and associated photopigments (e.g., chlorophylls, carotenoids) occurring under times of heightened environmental stress. Coral bleaching has been suggested to be an adaptive response allowing for shuffling of the algal symbiont community *in hospite* (see, Buddemeier and Fautin 1993), however this hypothesis remains contentious. Bleaching responses in corals, originally reported by Vaughn (1914) as incidences of reduced coral pigmentation, have been documented in response to numerous environmental factors including, but not limited to, increased temperatures (reviewed in, Jokiel and Coles 1990), low temperatures (Gates et al. 1992; Lirman et al. 2011) ultraviolet radiation (Gleason and Wellington et al. 1993; Brown et al. 1994), sedimentation (Dollar and Grigg 1981), elevated salinity and prolonged darkness (Vaughn 1914). Recently, coral bleaching has been shown to occur under OA conditions (Anthony et al. 2008). Of the aforementioned bleaching factors, elevated temperature has proven to be a principal cause of reef decline by facilitating mass bleaching events (Hoegh-Guldberg 1999).

Elevated seawater temperatures cause oxidative damage to the reaction centers of photosystem II (RCIIs) within the chloroplasts of *Symbiodinium*, thereby disrupting carbon fixation and photosynthesis (Lesser 1997; Warner et al. 1999) and leading to algal cell expulsion from the coral. Corals experiencing low-to-moderate environmental stress can recover from bleaching caused by these conditions, and in some cases, utilize heterotrophy or tissue energy reserves to fuel bleaching recovery (Grottoli-Everett et al.

2006). However, severe coral bleaching can result in coral mortality, as is evident through extensive field observations of stochastic bleaching episodes (Loya et al. 2001).

Coral Bleaching: 1900 – Present

Coral bleaching events have been reported across the world since the 1870s. However, these events were viewed as largely benign (Glynn 1993; Wilkinson et al. 1999). Indeed, seasonal bleaching and loss of pigmentation is a natural occurrence characteristic of reef ecosystems (Fitt et al. 2000) and may play an important part in the dynamics of coral reef populations and communities. The first report of coral bleaching in response to elevated temperature was by Yonge and Nichols (1931) on the Great Barrier Reef. In the seminal study by Yonge and Nichols (1931), the authors determined corals could survive and recover from short-term exposures to elevated temperature and bleaching. However, prolonged exposure to high temperatures results in substantial bleaching and mortality (Jokiel and Coles 1990). Yet, the mechanism behind coral bleaching and the cascade of processes occurring in corals undergoing bleaching remained uncertain. In the early 1970s, interest in the effects of temperature on corals largely centered upon the effects of thermal pollution arising from coastal power plants (Jokiel and Coles 1974; Coles 1975). These early studies determined that subtropical and tropical corals lived 1 – 2 °C from their upper thermal limit during summer months, identified seasonal and geographical differences in coral thermal tolerance, and determined that coral bleaching was correlated with reductions in photopigmentations (Jokiel and Coles 1974; Coles 1975; Coles et al. 1976).

During the 1980s, large-scale coral bleaching events began occurring with an unprecedented frequency and severity (see review by Glynn 1993), leading to concerns that increased frequency of bleaching was attributed to global warming (Glynn 1991). Mass coral bleaching episodes were observed on reefs across the world in 1982–83 with some reefs in the eastern Pacific exhibiting 50 – 99% live coral mortality (Glynn 1990) and 80–90% live coral mortality in the Thousand Islands, Indonesian (Brown and Suharsono 1990). The devastation of a number of contemporary reefs in the 1980s coincided with reefs experiencing prolonged exposure to unseasonably warm temperatures (Goreau and Hayes 1994) attributed to large El Niño-Southern Oscillation (ENSO), notably the ENSO event of 1982–83. At the time, the 1982–83 ENSO was described as the strongest ENSO event of the 20th century (Glynn 1985). The frequency of widespread thermal episodes on coral reefs remained high throughout the 1980s, with massive bleaching events again observed during the ENSO events in 1986–88 and 1991 (Williams and Bunkley-Williams 1990; Goreau and Hayes 1994).

Nineteen ninety-eight was the warmest year recorded since temperature data records began (~1850) and corresponded to a combined El Niño and La Niña events stronger than the previous record of 1982–83 (Wilkinson et al. 1999). The extent of coral bleaching and live coral mortality in the aftermath of the 1998–99 ENSO event was extremely large (Hoegh-Guldberg 1999; Loya et al. 2001), resulting in local extirpation of acroporid and pocilloporid corals from some reefs across the western Pacific and Indian Ocean (Wilkinson et al. 1999; Spencer et al. 2000; McClanahan 2000, Bruno et al. 2001; Loya et al. 2001). The 1998 bleaching event was most severe near “hot spot”

regions—areas experiencing extended periods of seawater temperature > 1 °C above the monthly mean temperatures—adding more evidence to the hypothesis that elevated temperatures were driving local and regional bleaching (Goreau and Hayes 1994; Hoegh-Guldberg 1999).

The increased incidence of large-scale bleaching events from 1979-present are expected to continue into the 21st century as the world's oceans continue to warm, bringing corals ever closer to their thermal maximum (Hoegh-Guldberg 1999). ENSO events are predicted to continue to increase in both strength and frequency with sustained ocean warming. Furthermore, recent evidence suggests that bleaching occurs under prolonged exposure to OA, and that OA and elevated temperature may act additively to exacerbate bleaching (Anthony et al. 2008). Global climate change may precipitate further degradation of already threatened coral reef ecosystems (Anthony et al. 2011) and impede the role of scleractinian corals as the primary builders of tropical reef substrate (Wild et al. 2011). Therefore, the combined impact of OA and ocean warming, along with synergistic impacts of non-climate change effects (e.g., coastal development, overfishing), may push coral reefs into an unsustainable state characterized by high coral mortality, slow reef accretion, and reduced ecosystem quality and function (Hoegh-Guldberg and Bruno 2010).

Thesis Research Objectives

The overarching purpose of this thesis research is to test the hypotheses that OA negatively influences juvenile (≤4.0 cm diameter) reef corals by affecting key

physiological and metabolic processes (e.g., calcification, respiration, photosynthesis), and that OA exacerbates coral bleaching under elevated temperatures. Juvenile corals were chosen due to the importance of this life history stage in the lifecycle of reef corals (Bak and Engel 1979), and the strong potential for environmental change to differentially affect small corals relative to adult conspecifics (Nakamura and van Woesik 2001). Field and laboratory work was performed in two locations in the Western and Southern Pacific, at the National Museum of Marine Biology and Aquarium in Checheng, Taiwan, and the Richard B. Gump South Pacific Research Station in Moorea, French Polynesia.

In Chapter 2, I present a test of the hypothesis that elevated pCO_2 (445 vs. 840 μatm) results in coral bleaching under ambient temperature conditions (27.65 °C), and that OA exacerbated thermal bleaching (30.53 °C) in the juvenile coral *Seriatopora caliendrum* from Nanwan Bay, Taiwan. The effects of OA and temperature on coral bleaching are quantified in three phases: (1) effect on the photophysiology of *Symbiodinium* (e.g., photochemical efficiency), (2) effect on the photosynthetic efficiency and photosynthetic capacity, and (3) the effects on algal cell and photopigment densities. In Chapter 3, using juvenile massive *Porites* spp. from Moorea, French Polynesia, I test the hypothesis that low-pH_T (8.04, 7.73 and 7.69) reduces rates of coral calcification by reducing rates of aerobic respiration or by increasing the energetic expenditure concurrent with calcification. Additionally, I quantified the effects of low-pH and elevated dissolved inorganic carbon (DIC) concentration (2008, 2177, 2938 μmol DIC kg^{-1}) on the rates of calcification in massive *Porites* spp. to test the hypothesis that increased [DIC] can increase calcification rates under OA conditions (e.g., low-pH, high-

pCO_2, low-Ω_{arag}), and that calcification is DIC-limited at ambient [DIC] of ~2 mM. Finally, in Chapter 4, using *S. caliendrum*, from Nanwan Bay, Taiwan, I test the hypothesis that elevated pCO_2 (465 vs. 891 μatm) reduces rates of aerobic respiration while increasing rates of ammonium excretion. Here, hypercapnia is hypothesized to reduce rates of oxygen consumption by a general response to environmental stress or by reducing costs of ion transport. Conversely, hypercapnia is predicted to increase ammonium excretion by increasing protein metabolism and deamination to compensate for extracellular acidosis and increased costs of acid-base regulation as an effect of OA. In the terminal chapter, the results of the individual chapters are amalgamated to discuss the physiological responses of juvenile corals to OA and temperature stress.

Chapter 2

Ocean acidification and temperature effects on bleaching in the juvenile reef coral *Seriatopora caliendrum*

Introduction

The industrial era has caused a massive contribution of carbon dioxide (CO_2) to the biosphere, of which about 33% has been sequestered by the ocean (Sabine et al., 2004; Le Quere et al. 2009). Elevated concentrations of atmospheric pCO_2 function as a greenhouse gas to increase global atmospheric and sea-surface temperatures (SST) (IPCC 2007; Hansen et al. 2006), and reduce the pH of surface seawater through dissolution and equilibration in a process termed ocean acidification (OA) (Kleypas et al. 1999). During the 20th century, rising atmospheric pCO_2 contributed to a 0.7 °C increase in mean sea surface temperature (SST), and a 0.1 unit decrease in pH (Caldiera et al 2003; Raven et al. 2005; IPCC 2007). Accompanying OA are changes to seawater carbon chemistry to increase bicarbonate concentration [HCO_3^-] and reduce carbonate concentration [CO_3^{2-}] as well as the saturation state of calcium carbonate (Ω) (Gattuso and Hansson 2011). These changes generally result in reduced calcification by marine calcifiers (Doney et al. 2009; Kleypas and Yates 2009).

While the physiological responses to OA varies among taxa (Kroeker et al. 2010), much of the current experimental work suggests OA will negatively affect scleractinians by reducing calcification in adult corals (Langdon and Atkinson 2005; Schneider and Erez 2006; de Putron et al. 2011), and impairing settlement success of coral planulae

(Albright and Langdon 2011; Doropoulos et al. 2012). The effect of OA on *Symbiodinium* photosynthesis, however, remains uncertain, with data suggesting OA reduces rates of net photosynthesis in corals (Reynaud et al. 2003; Anthony et al. 2008) and the maximum rate of photosynthesis (e.g., P_{max}) (Crawley et al. 2010); others studies show no response of coral photosynthesis to pCO_2 enrichment (Leclercq et al. 2002; Godinot et al. 2011). A doubling of preindustrial atmospheric pCO_2 (~280 μatm) to 560 μatm is projected to occur by 2050 (RCP6, van Vuuren et al. 2011) and is hypothesized to reduce coral growth by 10 – 50% (Kleypas and Langdon 2006). However, the rate at which corals calcify has shown signs of reduction for several decades since the 1980s (Bak et al. 2009; Manzello 2010).

Seawater warming is hypothesized to have contributed to a 14% decline in calcification of massive *Porites* spp. on the Great Barrier Reef from 1990 – 2005 (Cooper et al. 2008; De´ath et al. 2009) and reduced growth rates of coral juveniles in the Caribbean (Edmunds 2007), *Pocillopora damicornis* in the Eastern Pacific (Manzello et al. 2010), and *Acropora palmata* in the Western Atlantic (Bak et al. 2009). Additionally, studies suggest OA in combination with seawater warming has led to a global trend of reduced coral growth (Cooper et al. 2008; De´ath et al. 2009; Manzello et al. 2010). The potential for OA and seawater warming to act in synergy to negatively affect multiple life stages of corals (i.e., larvae, recruits, juvenile and adult colonies) has far-reaching ramifications for coral reefs, with some suggesting the long-term persistence of coral reefs as a calcified system is threatened (Kleypas et al. 1999; Hoegh-Guldberg 2005; Fabricius et al. 2011).

Increased global SST has contributed to the worldwide decline in abundance of reef building corals (Glynn 1993; Hoegh-Guldberg 1999; Hughes et al. 2003) largely though the effects of mass coral bleaching in killing corals at local and regional scales (Edwards et al. 2001; Loya et al. 2001). Coral bleaching refers to the loss of chlorophyll and reduction in the population density of endosymbiotic *Symbiodinium* spp. algae in the coral tissue in response to a wide range of environmental stressors (Kleppel et al. 1989; Glynn 1996). Of these, prolonged exposure to UV-radiation and elevated temperature are most prominent in causing large-scale bleaching episodes (Loya et al. 2001; Gates et al. 1992; Gleason and Wellington 1993; Fitt et al. 1993; Glynn 1993; Brown 1997).

Preceding the expulsion of *Symbiodinium* from coral tissue, the combination of oxidative stress and photodamage causes reductions in the efficiency with which photosystem II (PSII) harvests light energy and generates high-energy electrons (hereafter, photochemical efficiency) and a reduced capacity for carbon fixation in the Calvin Cycle (hereafter photosynthetic capacity) (Iglesias-Prieto 1992; Warner et al. 1996; Brown 1997, Jones et al. 1998). While the mechanism by which corals bleach is dependent upon the bleaching stimulus (Douglas 2003), experimental evidence suggests the initiation of thermal bleaching begins with oxidative damage to the D1-protein associated with the photosystem II reaction center (RCIIs) of *Symbiodinium,* termed photoinhibition (Lesser 1997; Warner et al. 1999). Alternatively, Jones *et al.* (1998) found damage to PSII in heat-stressed *Symbiodinium* to be a secondary effect resulting from limitations in assimilatory electron flow and impairment of CO_2 fixation.

The susceptibility of corals to bleaching varies among coral species (Fitt and Warner 1995) and can also be affected by colony-level traits such as tissue thickness and corallum morphology (Loya et al. 2001; Nakamura and van Woesik 2001), genetic variants of *Symbiodinium* (Rowan et al. 1997; Abrego et al. 2008; Baird et al. 2009), and colony size or life-stage (Harriott 1985; Mumby 1999; Loya et al. 2001). The relationship between colony size and mortality is an important characteristic shaping community composition on coral reefs (Bak and Engel 1979; Connell 1973). Juvenile corals are an important component of the coral community (Bak and Engel 1979) and integral to coral population recovery following physical and biological disturbances, including bleaching (Connell 1978; McClanahan 2000).

Size-dependent mortality rates in corals results in higher rates of mortality in small corals (Connell 1973), however field and laboratory evidence suggests juvenile corals may be more resilient to changes in temperature that result in bleaching in adult conspecifics (Loya et al. 2001; Nakamura and van Woesik 2001). Potentially, increased rates of mass transfer in smaller colonies facilitate effective removal of reactive oxygen species (ROS), mitigating oxidative damage and preventing bleaching from occurring (Nakamura and van Woesik 2001). Observations from the Great Barrier Reef (GBR) (Loya et al. 2001), Caribbean (Mumby 1999), Atlantic (Lirman et al. 2012), Pacific (Hoeksema 1991), and Mediterranean (Shenkar et al. 2005) support the hypothesis of small, juvenile coral colonies exhibiting higher survival rates than larger colonies under warm- and cold-water bleaching stress. However, Harriot (1985) observed increased mortality in smaller corals across four taxonomic families during an irradiance-induced

local bleaching event on the GBR. Potentially, bleaching causes higher percentages of tissue loss in small versus large corals (e.g., relative vs. absolute), therefore precluding substantial regeneration of tissue and post-bleaching recovery (Harriot 1985).

Mass coral bleachings resulting from high-temperature are predicted to increase in frequency and duration due to climate change (Hoegh-Guldberg 1999; 2005; Hughes et al. 2003; Manzello et al. 2010). The persistent effects of high seawater temperatures combined with OA are predicted to reduce the resilience of coral reefs to perturbations (Bellwood et al. 2004; Anthony et al. 2010) and the ecological properties of the reef community (Hall-Spencer et al. 2008; Fabricius et al. 2011). Indeed, *in situ* evidence shows that the ecological impacts of OA may affect coral species and life-history stages differentially (Fabricius et al. 2011). At a shallow volcanic CO_2 vent in Papua New Guinea, Fabricius *et al.* (2011) found hard coral species richness and juvenile colony density to decline with proximity to low-pH (on total scale, pH_T) and high-pCO_2 conditions. However, the abundance of juvenile massive *Porites* spp. per area (e.g., density) was reduced more than fourfold, while the cover of adult massive *Porites* spp. increased twofold with declining pH_T (Fabricius et al. 2011). However, while studies of shallow CO_2 vents in coral reef habitats support experimental laboratory and modeling predictions of the effects of OA on coral reefs, CO_2 vents are subject to high temporal variability in pH_T, carbon chemistry of seawater and other covarying factors (e.g., currents, temperature). Thus, predictions of the physiological and ecological impacts of OA based on these systems remain problematic (Hall-Spencer et al. 2008).

OA may alter the health of coral ecosystems and reef corals by altering rates of symbiont photosynthesis (Reynaud et al. 2003), negatively impacting rates of coral growth and linear extension (De´ath et al. 2009; Manzello et al. 2010), and inducing coral bleaching, alone or in concert with ocean warming (Anthony et al. 2008). In an 8-week study, OA caused coral bleaching and reduced rates of net photosynthesis and calcification in *Porites lobata* and *Acropora intermedia* (Anthony et al. 2008). Colorimetric bleaching determination on the luminance scale (represents change in chlorophyll content; expressed as % color change relative to 'maximum luminance' controls) showed high pCO_2 (1100 – 1300 ppm) caused a 20% decrease in luminance (e.g., bleaching) in *P. lobata*, and 40 – 50% decrease in luminance in *A. intermedia*. Bleaching of *A. intermedia* corals in high-pCO_2 treatments caused a stronger bleaching effect than elevated temperature (25 – 26 °C vs. 28 – 29 °C) alone. Combinations of elevated pCO_2 and temperature increased the bleaching response in both *P. lobata* and *A. intermedia*. However, Anthony *et al.* (2008) categorized bleaching qualitatively by colorimetric analysis of photographs without a detailed quantitative analysis of *Symbiodinium* density or chlorophyll content. Therefore, in the absence of physiological data characteristic of bleaching studies, the existing evidence for OA-induced bleaching remains phenomenological. To date, no study has explicitly tested the hypothesis that OA causes coral bleaching, alone or in synergy with high temperature. While OA can affect growth in early life stages of corals (Albright et al. 2008; Anlauf et al. 2011), it is unknown whether OA induces bleaching in juvenile corals.

The mechanism of OA-induced bleaching was hypothesized by Anthony *et al.* (2008) to involve the disruption of carbon-concentration mechanisms used by *Symbiodinium* (Leggat et al. 1999), or through impairment of photoprotective mechanisms, including photorespiration (Crawley et al. 2010) and the dissipation of excess excitation energy away from PSII through nonphotochemical quenching (Hill et al. 2005). However, the effects of OA on the photochemistry and quantum efficiency of PSII of *Symbiodinium* remain unclear. Decreased pH_T (8.09, 7.78, and 7.46) had no effect on the maximum photochemical efficiency of open RCIIs following dark adaptation (F_v/F_m) *in Stylophora pistillata* (Godinot et al. 2011), while *P. australiensis* showed reductions in F_v/F_m with decreasing pH_T (8.0, 7.6, and 7.4) (Iguchi et al. 2011). However, in juvenile colonies of massive *Porites* spp., Edmunds (2012) found low-pH_T (7.80) decreased F_v/F_m relative to pH_T of 8.06 and 7.85, and reduced the photochemical efficiency of open RCIIS in actinic light ($\Delta F/F_m'$) compared to pH_T of 7.85. Further experimental testing is needed to determine the effects of OA on the photochemical efficiency of *Symbiodinium* and to evaluate whether reduced photochemical efficiency under OA leads to coral bleaching, as has been recorded in corals experiencing thermal stress (Warner et al. 1996; Fitt et al. 2001).

The objective of this study was to test whether elevated pCO_2 causes bleaching in juvenile scleractinians, either individually or synergistically with high temperature. Specifically, I tested the hypothesis that high pCO_2 reduces photochemical efficiency of RCIIs and photoprotective mechanisms and therefore may increase oxidative damage to RCIIs resulting in bleaching (after Crawley et al. 2010). A manipulative experiment was

conducted in which corals were exposed for 14 d to combinations of two temperatures and two pCO_2 regimes, with the effects assessed using dependent variables that detect the early onset of bleaching in three stages (after Fitt et al. 2001): (1) initial depression of photochemical efficiency, (2) subsequent declines in photosynthetic capacity, and (3) final reduction in *Symbiodinium* population size and chlorophyll *a* concentrations. I hypothesized that coral bleaching induced under OA is caused by a decrease in photochemical efficiency, photosynthetic capacity, and reductions in chlorophyll *a* and *Symbiodinium* densities, and that that these effects will be exacerbated with elevated temperature. The experiment was conducted with the branching pocilloporid coral *Seriatopora caliendrum* (Ehrenberg, 1834) that is common on shallow coral reefs in the Indo-Pacific (Veron 2000), including the reefs of southern Taiwan (Dai and Horng 2009) where this study was conducted. *Seriatopora caliendrum* has previously been reported to be susceptible to thermal bleaching (Loya et al. 2001).

Materials and Methods

Experimental Design

Four treatments were created to contrast ambient temperature-ambient pCO_2 ($AT-ACO_2$), ambient temperature-high pCO_2 ($AT-HCO_2$), high temperature-ambient pCO_2 ($HT-ACO_2$), and high temperature-high pCO_2 ($HT-HCO_2$). In these contrasts, ambient temperature referred to the seawater temperature on shallow reefs in Nanwan Bay when the experiment was conducted in July – August 2011 (28.02 ± 0.02 °C, ± SE, n = 2,965), which was maintained at 27.5 °C, and ambient pCO_2 refers to the pCO_2 in the building where the study was conducted (~440 μatm CO_2). The high temperature

treatment was close to the maximum recorded at 3-m depth on the study reef in summer (30.5 °C) (T-Y Fan personal communication), and the high-pCO_2 treatment represented conditions projected to occur by 2100 (~850 μatm) under the high emission representative concentration pathway RCP 8.5 of van Vuuren et al. (2011).

Four treatments were created in 8 tanks (77 x 77 x 30 cm) with n = 2 tanks treatment^{-1} and filled with 130 L of filtered (1.0 μm) seawater maintained at a salinity of ~33.4 (YSI 3100 Conductivity Meter, YSI Inc., Ohio, USA) by 20% water changes (~26 L) each evening. Temperatures were maintained independently by microsensor-based regulators (AquaController, Neptune Systems, San Jose, California, USA) connected to a 300-W heater (Taikong Corp.), and chiller (Aquatek, Aquasystems, Taiwan), and the seawater was mixed with a pump (1451 L h^{-1}, Rio 1100, TAAM, Inc., California, USA). Light was provided to each tank by two 18-W fluorescent bulbs (TL-D Blue, Phillips, Maryland) and two 150-W metal halide bulbs on a 12h light : 12h dark cycle that created mean irradiances ranging from 251–279 μmol photons m^{-2} s^{-1} as measured daily beneath the surface of the seawater using a spherical light sensor (Li-Cor LI-193, Lincoln, Nebraska, USA). The mean irradiance (± SE, n = 88) across experimental treatments was 245 ± 5 μmol photons m^{-2} s^{-1}.

pCO_2 treatments were maintained by bubbling ambient air or CO_2-enriched air into the tanks. To prepare pCO_2 treatments, pure CO_2 was mixed with ambient air by solenoid-controlled gas mixing technology (Model A352, Qubit Systems, Ontario, Canada). Pure CO_2 (99% CO_2 + ≤ 1% air) and ambient air were mixed in a chamber and

the pCO_2 measured using an infrared (IR) gas analyzer (S151, Qubit Systems) calibrated against certified reference CO_2 gas (1793 ppm CO_2, San Ying Gas Co., Taiwan). pCO_2 treatments were maintained dynamically by the IR gas analyzer regulating a solenoid valve that controlled the flow of CO_2 gas. The final concentration of CO_2 distributed to treatment tanks was logged in parts per million (ppm) on a PC using LabPro software (Vernier Software and Technology, Oregon, USA), and a second pump delivered the gas mixture to the high-pCO_2 tanks at ~15 L min^{-1}; ambient-pCO_2 tanks received air at a similar flow rate.

Treatments were monitored three times d^{-1} (9:00, 12:00, 17:00 hrs) for stability of temperature and salinity; irradiance was measured daily (12:00 hrs). pH (on the total scale, pH_T) and carbonate chemistry were determined daily on individual seawater samples (~250 mL) taken from all tanks at 9:00 hrs. Temperature was measured using a certified digital thermometer (Fisher Scientific 15-077-8, ± 0.05 °C), and seawater was assessed for total alkalinity (TA, μmol kg^{-1}) and pCO_2 (μatm) by potentiometric titrations following standard operating procedures (SOP 3, Dickson et al. 2007); pH_T was determined spectrophotometrically using *m*-cresol purple dye (SOP 6B, Dickson 2007). Seawater samples were titrated using an open cell autotitrator (Model DL50, Mettler-Toledo, Ohio, USA) equipped with a DG115-SC pH probe (Mettler-Toledo) that was three-point calibrated (4.00, 7.00, 10.00 NBS buffers, Panreac, Spain) and filled with certified acid titrant (~0.1 mol L^{-1} HCl and 0.6 mol L^{-1} NaCl, from A. Dickson, Scripps Institution of Oceanography). TA was evaluated for precision and accuracy using certified reference materials (CRM) of known TA (Batches 98, 107 and 110 from A.

Dickson Laboratory, Scripps Institution of Oceanography) with our analyses being consistently < 0.9 % above certified values (ranging 2.4 – 19.3 μmol kg^{-1}). pH_T, salinity, temperature, and TA were used in CO2SYS software in Microsoft Excel (Fangue et al. 2010) to calculate the remaining seawater carbon chemistry parameters (e.g., HCO_3^-, CO_3^{2-}, pCO_2).

Juvenile Colony Collection

Sixty juvenile *Seriatopora caliendrum* were collected on 22 July 2011 from Hobihu Reef (21°56.799'N, 120°44.968'E), Nanwan Bay, with juveniles defined as colonies ≤ 4.0 cm diameter (Bak and Engel 1979). Colonies were collected from 3 – 4 m depth and transported to a flow-through aquarium at the National Museum of Marine Biology and Aquarium (NMMBA) where they were allowed to recover from the collection for 24 h. The recovery tank (1050 L) received filtered seawater (50 μm) at 6.0 L min^{-1} and was mixed with a pump (1451 L h^{-1}). Temperature was maintained at ambient conditions (28.07 ± 0.10 °C, ± SE, n = 24) and was supplied with light at 164 ± 4 μmol photons m^{-2} s^{-1} on a 12h : 12h light : dark cycle using fluorescent bulbs and a metal halide lamp. Due to logistical constraints, light conditions in the recovery tank were below those experienced over *in situ* (~660 μmol photons m^{-2} s^{-1}), although near light levels used in the subsequent laboratory experiment (245 μmol photons m^{-2} s^{-1}). Therefore the recovery tank provided a dual function of post-collection recovery and a period of photoacclimation to the *in vitro* light regime.

One day post-collection, colonies of *Seriatopora caliendrum* were suspended in the recovery tank using monofilament nylon and left to continue their recovery and photoacclimation for an additional 5 d. On 28 July 2011, corals were placed randomly into the treatment tanks (n = 7 tank^{-1}) for incubations lasting 14 d. At the conclusion of the experiment (11 August 2011) corals were processed for the dependent variables described below, with this process taking an additional 3 d; corals were retained in experimental conditions during this time, therefore the experiment lasted from 14 – 17 d. To minimize the disparity in exposure period between temperature treatments, corals from the high-temperature treatments were processed first, followed by corals in the ambient-temperature treatments, with corals randomly selected for processing within each temperature treatment.

Photochemical Efficiency

The effects of temperature and pCO_2 on photochemical efficiency were tested by measuring the maximum photochemical efficiency of open RCIIs in the dark (F_v/F_m) and the effective photochemical efficiency of RCII in the light ($\Delta F/F_m'$) using pulse amplitude modulation (PAM) fluorometry. PAM fluorometry is an effective tool—particularly in studies of coral bleaching—to assess non-invasively the photophysiology of *Symbiodinium in hospite* (Warner et al. 1996; 2010). F_v/F_m provides a measure of photochemical quenching (qP) reflecting the rate of charge separation across PSII in the open (i.e., dark adapted) state, while $\Delta F/F_m'$ accounts for photochemical and nonphotochemical quenching (NPQ), including mechanisms for the thermal dissipation of excess absorbed light energy (Johnson et al. 1993; Hill et al. 2005). NPQ is of

biological importance as a mechanism of photoprotection and avoidance of photoinhibition under peak daily irradiance (Hoegh-Guldberg and Jones 1999; Hill and Ralph 2005) and under bleaching conditions (Warner et al. 1996; Jones et al. 1998; Hill et al. 2005).

Photochemical efficiency was assessed using a Diving-PAM (Waltz, GmbH, Effeltrich, Germany) operated at a gain of 6, intensity of 9, and a slit-width of 0.8. Prior to the start of the experiment, PAM settings were adjusted to obtain a range of F_o between 200 – 400 (arbitrary units) and a peak stabilization of F_m. Effective photochemical efficiency of RCIIs ($\Delta F/F_m' = (F_m' - F') / F_m'$) was measured to quantify changes in quantum yield relative to the dark-adapted state due to excess thermal energy dissipation and NPQ, and the maximal photochemical efficiency ($F_v/F_m = (F_m - F_o) / F_m$) was measured to quantify the maximum photochemical efficiency of open RCIIs in the dark-adapted state. In these calculations, F_m' and F' are the maximum and minimum fluorescence yield in actinic light, whereas F_m and F_o are the maximum and the minimum fluorescence yield following dark adaptation (Cosgrove and Borowitzka 2010).

Photochemical efficiency was measured using a 5-mm diameter fiberoptic probe held ~5 mm above the tissue and ~1 cm behind branch tips. $\Delta F/F_m'$ was measured under actinic irradiance (~245 μmol photons m^{-2} s^{-1}) and F_v/F_m under weak indirect red lighting ($\leq$ 2.0 μmol photons m^{-2} s^{-1}). PAM measurements were taken every second day of the incubation at 12:30 hrs ($\Delta F/F_m'$) and 1 h after sunset at 17:30 hrs (F_v/F_m). A pilot study was used to determine the duration of dark-adaptation necessary to stabilize values of

maximum photochemical efficiency, identify effects of prolonged darkness on F_v/F_m (i.e., dark-induced reduction of the PQ pool; Hill and Ross 2008), and to test whether weak indirect red light affected F_v/F_m. F_o stabilized after < 0.5 h of darkness and F_v/F_m was statistically indistinguishable when measured following dark adaptation lasting 0.5, 1.0 or 2.0 h ($F_{2, 27} = 0.137$, $P = 0.872$), or measured with and without weak red light ($F_{1, 18} = 0.352$, $P = 0.561$).

Photosynthesis-Irradiance (P/I) Curves

To test the effects of pCO_2 and temperature on photosynthesis, net photosynthesis (P^{net}) was measured under a series of irradiances using three corals selected randomly from each treatment tank ($n = 6$ treatment^{-1}). The photosynthetic-irradiance (*P/I*) response can be divided into three regions of photosynthesis, describing light-limitation, light-saturation, and photoinhibition. The light-limited region, described by its slope alpha (α), exhibits a linear, proportional relationship between photosynthesis and irradiance (e.g., two-fold increase in irradiance ≈ two-fold increase in photosynthesis) and is related to the functional absorption cross section of PSII (σ_{PSII}) and the number of photosynthetic units (n) (Falkowski and Raven 1997). In the light-saturated region of the curve, photosynthesis and irradiance are no longer proportionally related, and rates of P^{net} rise to a saturation point where photosynthesis is maximized (P^{net}_{max}). Beyond P^{net}_{max}, increasing light intensity does not result in increased P^{net}. Here, photon absorption exceeds the steady state electron transport from H_2O at the oxygen evolving complex to the terminal electron acceptor and is related to the number of photosynthetic units (n) and the maximum rate of electron turnover through PSII ($1/\tau_{PSII}$). The light-limited and light-saturated regions can be related by the saturating irradiance (I_k). At I_k, the rate of

photochemistry (e.g., photon absorption) equals the maximum rate of photosynthetic electron transport. Finally, the photoinhibited region describes a departure of P^{net} from the maximum rate of photosynthesis (P^{net}_{max}) as a product of excessive absorbed photon energy reducing $1/\tau_{PSII}$, or the number of photosynthetic units, and leading to a reduction in functional RCIIs (Falkowski and Raven 1997).

To measure the *P/I* response, two respirometers were used to measure P^{net}, and each housed a single coral during a measuring period lasting ~ 1.5 – 2.0 h. Measurements of P^{net} began on the 14^{th} day of incubations, and 3 d were required to process all corals in the experiment. Temperatures were maintained in the respirometers by placing them in a temperature regulated water bath, the effectiveness of which was evaluated using a certified digital thermometer to measure the temperature in the respirometer at various times during the incubations. Water motion within each chamber was provided by a magnetic stir-bar, and the flow rate was quantified by photographing hydrated *Artemia* spp. eggs (Sebens and Johnson 1991), revealing the mean flow rate near the center of the respirometer to be 5.43 ± 0.32 cm s^{-1} (± SE, n = 20). Prior to each trial, corals were maintained in darkness for 1 h to avoid the stimulatory effect of light on respiration (Edmunds and Davies 1988), and then were sealed in the respirometer while O_2 flux was measured at ten irradiances supplied in an ascending sequence between 0 and 747 μmol photons m^{-2} s^{-1}. Light intensities were created by adjusting the height of a 400-W metal halide lamp (Osram Sylvania, Massachusetts, USA) above the respirometer and measuring the irradiance using a cosine corrected light sensor that recorded photosynthetically active radiation (PAR). The light sensor was 1.0-mm diameter and

attached to a Diving-PAM (Waltz, GmbH, Effeltrich, Germany), and was calibrated using a Li-Cor LI-192 quantum sensor. The small light sensor was threaded into the respirometer while it was filled with seawater, thereby measuring the light received by a coral in the same chamber. O_2 fluxes were adjusted for changes in O_2 concentrations in control chambers filled with seawater alone, and controls were run at each combination of temperature and pCO_2 for each irradiance and during darkness (n = 3 treatment^{-1}).

The O_2 saturation of seawater was measured using an optrode (FOXY-R, 1.58 mm diameter, Ocean Optics, Dunedin, Florida, USA) connected to a spectrophotometer (USB2000, Ocean Optics), which logged O_2 concentrations on a laptop computer running Ocean Optics software (OOISensors, version 1.00.08, Ocean Optics). The optrode was calibrated using water-saturated air at the measurement temperature and a zero solution of sodium sulfite (Na_2SO_3) and 0.01 mol L^{-1} sodium tetraborate ($Na_2B_4O_7$). O_2 saturation during the trials was maintained between 80 – 100% by replenishing chambers with filtered (1.0 μm) seawater from the respective temperature and pCO_2 treatments. Percentages of air saturated O_2 were converted to O_2 concentrations (μmol) using tabulated gas solubility at a known temperature and salinity [N. Ramsing and J. Gundersen at Unisense, http://www.unisense.com/Default.aspx?ID=1109, based on Garcia and Gordon (Garcia and Gordon, 1992)]. Rates of change in O_2 concentrations were determined by regressing O_2 concentration against time and standardizing to the surface area of the coral tissue (cm^2) and the chlorophyll *a* content (mg). The relationship between P^{net} and irradiance was described with a hyperbolic tangent function

that included an exponent for photoinhibition induced at high irradiances:

Equation 3:

$$P^{\beta} = P_s^{\beta}(1 - e^{-a})e^{-b}$$

where $a = \alpha I/P_s^{\beta}$, $b = \beta I/P_s^{\beta}$, P^{β} is the rate of net primary productivity (P^{net}), P_s^{β} is the maximum rate of net photosynthesis (P_s) accounting for photoinhibition (β), α is the initial slope of the light-limited portion of the curve, and I is the irradiance in μmol photons m^{-2} s^{-1} (Platt et al. 1980). The hyperbolic tangent function described by Platt *et al.* (1980) was fit to the productivity data by non-linear regression using the Gauss-Newton estimation method (Platt et al. 1980). *In situ* PAR measurements (collected 6 March – 10 March 2011) at Hobihu reef revealed mean (± SE, n = 148) irradiance from 09:00 – 15:00 hrs to be 660 ± 30 μmol photons m^{-2} s^{-1} and therefore P^{net} at 660 μmol photons m^{-2} s^{-1} (hereafter P_{660}^{net}) was compared among treatments instead of P_{max}. Curve parameters (α, P_{660}^{net}) together with the saturating irradiance (I_k) were compared statistically among treatments.

Chlorophyll a Concentration and Symbiodinium Density

Chlorophyll *a* concentration and *Symbiodinium* density were quantified by removing coral tissue from the skeleton using an airbrush (Iwata-Medea, Inc., Oregon, USA) equipped with a reservoir of filtered seawater (1.0 μm). Colonies were airbrushed into a plastic bag, producing 8 – 40 mL of slurry that was homogenized (Polytron PT2100, Kinematica, New York, USA) prior to separating the *Symbiodinium* by

centrifugation (1500 x *g*). The *Symbiodinium* pellet was resuspended by vortexing in filtered seawater and used to measure chlorophyll *a* concentration and symbiont density.

Symbiodinium used for chlorophyll determinations were frozen (-4 °C for 24 hrs) and filtered onto a cellulose acetate membrane filter (3-μm pore size, Critical Process Filtration, New Hampshire, USA) to which 3.0 mL of 90% acetone was added. Samples were refrigerated (4 °C) in darkness for 36 hrs, centrifuged (1500 x *g* for 3 min), and absorbances at 630 nm and 636 nm measured and used to calculate chlorophyll *a* concentration using the equations for dinoflagellate from Jeffery and Humphrey (1975). Chlorophyll *a* concentration was standardized by algal cells (pg $cell^{-1}$) and by the surface area (μg cm^{-2}) of the coral tissue determined by wax dipping (Stimson and Kinzie 1991). *Symbiodinium* density (cells cm^{-2}) was determined by counting *Symbiodinium* in the homogenized slurry stripped from the coral colonies, with the counts completed using a hemocytometer (n = 4 counts). Preliminary data showed that the mean and standard deviation of replicate determinations of *Symbiodinium* density stabilized after four counts.

Statistical Analysis

Response variables were compared among treatments using three-way mixed model ANOVA in which pCO_2 and temperature were treated as fixed factors, and tank as a random factor nested within treatment. The physical and chemical conditions in the treatments also were analyzed with this statistical model. Tank was removed from the model when not significant at $P \geq 0.25$ (Quinn and Keough 2002). To test the statistical assumptions of ANOVA, graphical analyses of residuals were employed. Analyses were

performed using Systat 11 software (Systat, Inc., Illinois, USA). Calculations of statistical power were performed on select results according to Cohen (1988).

Results

Tank Parameters

Physical and chemical conditions in the treatment tanks were maintained precisely (Table 1). Mean pCO_2 treatments were 840 ± 5 μatm and 445 ± 2 μatm (± SE, n = 55 – 56) with mean pCO_2 across treatment tanks ranging from 430 – 464 μatm (A–CO_2) and 821 – 860 μatm (H–CO_2). pCO_2 differed among replicate tanks ($F_{4,103} = 3.673$, P = 0.008) and treatments ($F_{1,4} = 1798.18$, P = < 0.001), and pH_T differed among replicate tanks ($F_{4,103} = 5.956$, $P < 0.001$) and between pCO_2 treatments ($F_{1,4} = 1120.272$, P = < 0.001). The tank effects for pCO_2 and pH reflected differences of < 0.04 pH_T and ≤ 40 μatm pCO_2. For seawater temperatures tank effects were not detected ($F_{4,176} = 1.261$, $P = 0.287$), and mean temperature treatments (± SE, n = 23) were 27.65 ± 0.04 °C (ambient) and 30.53 ± 0.05 °C (high). In summary, corals were exposed to treatments of pCO_2 (445 versus 840 μatm) and temperature (27.65 versus 30.53 °C) for 14 – 16 d and maintained under a mean irradiance of 245 μmol photons m^{-2} s^{-1}.

Photochemical Efficiency

Prior to being introduced to treatment tanks, F_v/F_m was measured in 10 randomly selected corals and values were shown to be stable 5 d after field collection. Once placed into experimental treatments, $\Delta F/F_m'$ and F_v/F_m were measured in all corals in all treatments every 2nd day (data not shown) and were observed to decline in corals held at

30.53 °C after 7 d. After 13 d, corals at 30.53 °C experienced a 15% (HT–HCO_2, 0.61 ± 0.13) and 16% (HT–ACO_2, 0.60 ± 0.02) reduction in $\Delta F/F_m'$ compared to corals in the AT–ACO_2 treatment (0.72 ± 0.004). F_v/F_m was depressed 13% (0.64 ± 0.02) and 9% (0.66 ± 0.01) in HT–ACO_2 and HT–HCO_2 treatments versus AT–ACO_2 (0.73 ± 0.003) [all values mean ± SE, n = 12 – 14] (Figure 1). No significant effect ($P > 0.25$) of tank was detected for F_v/F_m, and tank was dropped from this analysis; while the tank effect also was not significant for $\Delta F/F_m'$ ($P = 0.193$) it was retained in the analysis. $\Delta F/F_m'$ was affected by temperature ($F_{1,4} = 96.726$, $P = 0.001$) being reduced at 30.53 °C relative to control corals at 27.65 °C. However, $\Delta F/F_m'$ was not affected by pCO_2 ($F_{1,4} = 0.775$, $P = 0.428$), and there was no temperature x pCO_2 interaction ($F_{1,4} = 0.0001$, $P = 0.993$). Simialrly, corals at 30.53 °C exhibited reduced F_v/F_m and was affected by temperature ($F_{1,48} = 63.711$, $P < 0.001$) but not pCO_2 ($F_{1,48} = 2.595$, $P = 0.114$), and there was no temperature x pCO_2 interaction ($F_{1,48} = 0.307$, $P = 0.582$).

P/I Curves

Net photosynthesis standardized to surface area (cm^2) and chlorophyll *a* (mg) increased with increasing irradiances and developed asymptotes at > 400 μmol photons m^{-2} s^{-1} in 7 cases, but showed photoinhibition in 2 cases. In 14 cases, P^{net} did not reach a clear asymptote, although rates of photosynthesis did stabilize at > 400 μmol photons m^{-2} s^{-1}. Since absolute maximum rates of P^{net} were not obtained for all corals, calculation of I_k (e.g., $I_k = P^{net}_{max}/\alpha$) was not performed. The hyperbolic tangent function (Eq. 1) fit the productivity data well (mean $r^2 = 0.95$), and curve parameters (α, P_{660}^{net}) were calculated (n = 5 – 6 $treatment^{-1}$) as described previously (Table 2). For area-normalized

parameters, tank effects were not significant for respiration or P_{660}^{net} ($P > 0.25$) and therefore tank was dropped from these analyses, although tank was retained in the statistical model for α (Tank: $P = 0.216$). For data normalized to mg chlorophyll *a* (hereafter mg Chl *a*), the tank effect was not significant ($P > 0.25$) for all parameters and was therefore dropped from the analyses. All curve parameters are summarized in Table 2 and Figure 2 and the results of statistical analyses in Table 3.

When normalized to area, mean respiration ranged from -0.96 ± 0.06 μmol O_2 cm^{-2} h^{-1} to -0.73 ± 0.07 μmol O_2 cm^{-2} h^{-1} (± SE, Table 2) and was not affected by temperature, pCO_2, or the interaction between the two (Table 3). However, when normalized to mg Chl *a,* mean respiration was affected by temperature ($P < 0.001$), with mean values (± SE) ranging from -185.93 ± 19.99 μmol O_2 (mg Chl *a*) h^{-1} to -58.22 ± 3.00 μmol O_2 (mg Chl *a*) h^{-1} in HT–ACO_2 and AT–HCO_2 treatments, respectively (Table 2). Temperature significantly affected P_{660}^{net} standardized to both surface area ($P < 0.001$) and mg Chl *a* ($P = 0.001$); no effect of pCO_2 or the temperature by pCO_2 interaction was detected (Table 3). Area-normalized P_{660}^{net} decreased 95% at 30.53 °C versus 27.65 °C at ambient pCO_2 (445 μatm), and was affected similarly at elevated pCO_2 (840 μatm) being reduced by 89% at 30.5 °C compared to 27.65 °C. P_{660}^{net} normalized to mg Chl *a* was reduced 86% (HT–ACO_2 versus AT–ACO_2) and 75% (HT–HCO_2 versus AT–HCO_2) at 30.53 °C compared to 27.65°C. Alpha based on area-nortmalized rates was affected by temperature ($P < 0.001$) but not pCO_2 or the interaction between the two. At 30.65 °C, area-normalized α showed a decrease in responsiveness to changes in PAR resulting in the slope of the light-limited portion of the curve declining

by 69% at 445 μatm (HT–ACO_2 vs. AT–ACO_2), and 64% at 840 μatm (HT–HCO_2 vs. AT–HCO_2). However, α normalized to mg Chl *a* exhibited similar slopes across temperature and pCO_2 treatments, and no treatment effects were detected (Table 3).

Chlorophyll a and Symbiodinium Density

After two weeks in the treatments, corals at 30.53 °C showed signs of bleaching and loss of pigmentation. When normalized to area, mean concentration of chlorophyll *a* ranged from 13.35 ± 0.43 μg cm^{-2} in AT–ACO_2 to 3.73 ± 0.43 μg cm^{-2} in HT–ACO_2 (± SE, n = 12 – 14) (Figure 3). The interaction of temperature and pCO_2 was significant ($F_{1,48}$ = 5.074, P = 0.029) due to a 13% reduction in chlorophyll *a* cm^{-2} between AT–ACO_2 and AT–HCO_2 (13.35 versus 11.61 μg chlorophyll *a* cm^{-2}) and a 25% increase in HT–HCO_2 compared to HT–ACO_2 (4.67 versus 3.73 μg chlorophyll *a* cm^{-2}). Chlorophyll a concentration was affected by the main effect of temperature ($F_{1,48}$ = 886.763, P < 0.001), but not pCO_2 ($F_{1,48}$ = 0.457, P = 0.502). However, a post hoc power analysis showed the power ($1 - \beta$) of the test to detect a significant effect of pCO_2 on chlorophyll *a* content was low (< 0.11) at the level of replication and observed difference in biological response (3% of means), equivalent to 0.24 μg chl *a* cm^{-2}. When normalized to *Symbiodinium* cell, chlorophyll *a* concentration was unaffected by temperature ($F_{1,28}$ = 0.143, P = 0.709), pCO_2 ($F_{1,28}$ = 0.181, P = 0.673), or the interaction between the two ($F_{1,28}$ = 0.001, P = 0.975) (Figure 3).

Symbiodinium densities standardized to area (cells cm^{-2}) were affected by temperature ($F_{1,28}$ = 104.676, P < 0.001) but not pCO_2 ($F_{1,28}$ = 0.315, P = 0.579) or the

interaction between the two ($F_{1,28} = 0.343$, $P = 0.563$). Mean *Symbiodinium* densities decreased 69% in HT–ACO_2 ($0.87 \pm 0.14 \times 10^6$ cells cm^{-2}) compared to AT–ACO_2 ($2.81 \pm 0.14 \times 10^6$ cells cm^{-2}), and 65% in HT–HCO_2 ($1.08 \pm 0.98 \times 10^6$ cells cm^{-2}) compared to AT–HCO_2 ($2.80 \pm 0.28 \times 10^6$ cells cm^{-2}) (Figure 3).

Discussion

In this study, juvenile *Seriatopora caliendrum* corals from Nanwan Bay in southern Taiwan were exposed to two temperatures (27.65 versus 30.53 °C) and two levels of pCO_2 (445 versus 840 μatm). I tested the hypotheses that: (1) OA induces coral bleaching through disruption of photochemical efficiency of RCIIs leading to photodamage of PSII, and that (2) OA additively interacts with elevated temperature to affect the thermal bleaching response by reducing photochemical efficiency of *Symbiodinium* RCIIs, photosynthetic performance [e.g., capacity (P_{max}) and efficiency (α)], and the concentrations of *Symbiodinium* and chlorophyll *a* greater than high temperature alone. My results indicate that 14 – 17 d of exposure to 840 μatm pCO_2 had no effect on algal photochemistry (e.g., $\Delta F/F_m'$ or F_v/F_m), coral respiration, photosynthesis (e.g., P_{660}^{net}, α), or on symbiont or photopigment concentrations, whereas exposure to 30.53 °C resulted in bleaching and impairment of photosynthesis. Taken together, these results suggest that 14 – 17 d exposure to OA at 840 μatm pCO_2 does not induce coral bleaching, and that coral bleaching in response to elevated temperature (30.53 °C) is not additively or synergistically affected by pCO_2 (445 or 840 μatm).

OA and Coral Bleaching

Large-scale coral bleaching represent the largest single phenomenon acting to drive reef decline worldwide (Wilkinson 2008). Conversely, OA is believed to represent an unprecedented geochemical perturbation that may impair the ability of coral reefs to remain calcifying systems in the coming century (Silverman et al. 2009; Kerr 2010). Therefore, reports that elevated pCO_2 and lowered pH induce coral bleaching at a magnitude equivalent to thermally induced bleaching (Anthony et al. 2008) has received considerable attention. However, there is limited experimental evidence in support of OA-induced bleaching, with only a single report of corals bleaching in response to OA (Anthony et al. 2008). Furthermore, quantitative data characterizing the onset and progression of OA-induced bleaching remains to be collected, leaving hypothesized mechanisms behind OA-mediated bleaching conditions untested. However, in recent years studies have attempted to connect reduced *Symbiodinium* performance, as has been reported for bleaching under excess irradiance or elevated temperatures (Warner et al. 1996; Fitt et al. 2001), with the initiation of OA-induced coral bleaching. Findings by Crawley *et al.* (2010) suggest that OA disrupts photoprotective mechanisms of *Symbiodinium* (e.g., photorespiration) and reduces the capacity for thermal dissipation of excess energy away from PSII. Exposure to pH treatments (pH 7.85 and 7.6) led to increased xanthophyll de-epoxidation compared to controls (pH 8.1) and decreased activity of a key enzyme in photorespiration, PGPase (phosphoglycolate phosphatase), at pH 7.6 (Crawley et al. 2010). These findings indicate a potential pathway of OA-mediated disruption of the photophysiology of *Symbiodinium* through blockage of carbon

fixation and may represent an effect of OA on the photochemical efficiency of *Symbiodinium* that manifests as bleaching under prolonged exposures.

The results from this study are contrary to reports that OA and elevated pCO_2 lead to coral bleaching (Anthony et al. 2008), declines in net productivity (Reynaud et al. 2003; Anthony et al. 2008; Crawley et al. 2010), and increased chlorophyll *a* algal cell^{-1} (e.g., photoacclimation) (Crawley et al. 2010). However, there are few OA experiments designed to test for the effects of pH, pCO_2, or altered carbonate chemistry on the photochemical efficiency of *Symbiodinium*, photosynthesis, or coral bleaching (Crawley et al. 2010), and in those available, the outcomes are inconsistent (Godinot et al. 2011; Edmunds 2012). Similar to this study, pH_T treatments (8.05, 7.58 and 7.41) did not lead to bleaching or change in chlorophyll *a* cell^{-1} in *Porites australiensis* (Iguchi *et al.* 2011) or reduced *Symbiodinium* density in *Acropora formosa* (pH 8.10, 7.85 and 7.60; Crawley et al. 2010) or recently settled *Porites panamensis* (pH on NBS scale, pH_{NBS} 7.83) (Anlauf et al. 2011). Alternatively, Reynaud *et al.* (2003) reported that pCO_2 (460 versus 760 μatm) led to increased density of *Symbiodinium* per animal cell (e.g., cell-specific density) and a reduction in net photosynthesis (mg protein)$^{-1}$ in *Stylophora pistillata*, although there was a trend for increased chlorophyll *a* (mg protein)$^{-1}$ at 760 μatm CO_2. In the present study, OA did not result in coral bleaching, but the potential for OA to cause bleaching in reef corals may be species-specific or dependent upon the length of the exposure to low-pH or high-pCO_2, as well as other abiotic factors (e.g., light-intensity) (Anthony et al. 2008; Godinot et al. 2011).

Effects on Photochemical Efficiency of Symbiodinium

Coral bleaching under elevated temperature is initiated by oxidative damage to RCIIs (Lesser 1997) and impairment of carbon-fixation (Jones et al. 1998). This is revealed through photoinhibition of electron transport through PSII and decreased quantum efficiency of RCIIs. Concomitant with reductions in photochemical efficiency are reductions in photosynthetic function (Iglesias-Prieto et. al. 1992; Fitt and Warner 1995; Warner et al. 1999). This study supports these findings and is consistent with past studies describing damage to PSII as a point of initiation for coral bleaching under elevated temperature or irradiance (Warner et al. 1996; Brown 1997). In this study, significant declines in $\Delta F/F_m'$ and F_v/F_m in corals exposed to 30.5 °C may be a result of damage to the D1 protein associated with PSII leading to chronic photoinhibition (Jones et al. 1998; Warner et al. 1999; Fitt et al. 2001), however a detailed quantification of the mechanism of coral bleaching is beyond the scope of the present study. The declines in $\Delta F/F_m'$ at the ambient irradiance (~245 μmol photons m^{-2} s^{-1}) suggest a reduction in photochemical quenching (qP) and increased dissipation of absorbed light energy from RCIIs through NPQ processes as a mechanism of photoprotection (Demmig-Adams 1990). While corals exposed to 30.53 °C exhibited reduced photochemical efficiency compared to 27.65 °C, pCO_2 treatment (445 versus 840 μatm) did not affect $\Delta F/F_m'$ or F_v/F_m at either 27.65 °C or 30.53 °C.

Reported effects of OA on photochemical efficiency in tropical reef corals are equivocal. The reason for disparate results across studies may depend upon the duration of exposure to OA treatments, differences in light intensities among studies, and the coral

species studied (Godinot et al. 2011; Iguchi et al. 2011; Edmunds 2012). For example, Godinot *et al.* (2011) observed no effect of pH_T (8.09, 7.78, and 7.46) on F_v/F_m in branches of *Stylophora pistillata* exposed to 10 d of CO_2-enrichment at an irradiance of 110 μmol photons m^{-2} s^{-1}, while F_v/F_m declined with pH_T (8.05, 7.58 and 7.41) in *Porites australiensis* exposed to 8-weeks of pH-treatments under 130 μmol photons m^{-2} s^{-1} (Iguchi *et al.* 2011). Conversely, 12 d exposure to elevated pCO_2 (861 μatm) at an irradiance of 545 μmol photons m^{-2} s^{-1} reduced $\Delta F/F_m'$ and F_v/F_m in juvenile massive *Porites* spp., although photochemical efficiency at 756 μatm CO_2 was not different from ambient conditions (423 μatm CO_2) (Edmunds 2012).

In this study, the charge separation between $\Delta F/F_m'$ and F_v/F_m, termed the maximum excitation pressure over PSII (i.e., $[1-(\Delta F/F_m' / F_v/F_m)]$) (Iglesias-Prieto et al. 2004), was noticeably low compared to Edmunds (2012) who reported an upward trend in the excitation pressure at 861 μatm CO_2 (compared to 423 and 756 μatm CO_2). Alternatively, Crawley *et al.* (2010), reported decreased excitation pressure in corals exposed to pH 7.85 but not 7.60 compared to controls (pH 8.1). High charge separation across PSII indicates more RCIIs were in the reduced (e.g., closed) state, and that photoinhibition may have been occurring (Iglesias-Prieto et al. 2004; Edmunds 2012). In the present study, the low excitation pressure over PSII suggests a majority of RCIIs were in the oxidized (e.g., open) state, and therefore that photosynthesis was light limited. This may potentially indicate that while light levels of ~245 µmol photons m^{-2} s^{-1} were above the area-normalized saturation irradiance determined prior to the start of the experiment (I_k = ~140 μmol photons m^{-2} s^{-1}, data not shown), these conditions may have

been undersaturating to induce significant NPQ as would be occurring under natural irradiances (> 1500 μmol photons m^{-2} s^{-1} noon-irradiance).

The effect of OA on the photochemical efficiency of *Symbiodinium* in *Porites* spp. reported by Edmunds (2012) may be attributed to the ecologically relevant light-intensities (545 μmol photons m^{-2} s^{-1}) employed and a potential threshold pCO_2 concentration of 861 μatm for *Porites* spp. This is in agreement with the hypothesis of Anthony *et al.* (2008) that prolonged exposure to OA may cause bleaching under naturally high irradiances experienced by corals *in situ*. For instance, 8-weeks of exposure to pH-treatments of pH_T 7.58 and 7.41 at a light-intensity of 130 μmol photons m^{-2} s^{-1} did not cause bleaching in *Porites asutraliensis* (Iguchi *et al.* 2011). Conversely, 8-weeks of pH-treatments (pH on seawater scale, pH_{SW} 7.90 and 7.65) under naturally high-irradiances (noon irradiance > 1200 μmol photons m^{-2} s^{-1}) led to bleaching in *Acropora intermedia* at 25.5 °C and *Porties lobata* at 28.5 °C (Anthony et al. 2008). The influence of light on coral physiology and performance is well documented (Muscatine et al. 1984; Edmunds and Davies 1986; Dennison and Barnes 1988; Gattuso et al. 1999) and appears to play a significant role in the response of reef corals and *Symbiodinium* to OA (Comeau et al. in review; Dufault et al, in review). Therefore, future studies need to incorporate ecologically relevant light-intensities in their experimental designs in order to accurately quantify the effects of OA on the coral holobiont.

Effects on the Photosynthesis-Irradiance Response

Concurrent with decreased photochemical efficiency of RCIIs at 30.53 °C were decreases in area-normalized photosynthetic capacity (e.g., P_{660}^{net}) and photosynthetic efficiency (α) and decreases in respiration and P_{660}^{net} normalized to mg Chl a^{-1}. Under prolonged temperature or light stress, photoprotective mechanisms designed to shift energy away from PSII begin to breakdown, resulting in photo-oxidative damage to PSII, reduced photosynthetic productivity and ultimately coral bleaching (Lesser 1997; Warner et al. 1999; Müller 2001; Smith et al. 2005). In the present study, reduced P_{660}^{net} normalized to cm^{-2} and mg Chl *a* can be attributed to several concurrent processes: the quantitative reductions in the number of algal cells and functional photosynthetic units (PSU), and a reduction in the turnover rate of PSII ($1/\tau_{PSII}$) (Prezelin 1987; Falkowski and Raven 1997), a response commonly reported with the earliest signs of coral bleaching (Iglesias-Prieto et al. 1992) and in photoautotrophs undergoing photoinhibition (Behrenfeld et al. 1998). Additionally, increased temperature drove decreases in area-normalized α, which suggest strong photoinhibition and a diminished integrity of RCIIs (potentially from damage to the D1-protein) leading to fewer photons absorbed by the antennae complex being transferred to the acceptor-side of PSII (Falkowski and Raven 1997); photoinhibition observed in 18% of corals at 30.5 °C support this hypothesis.

All metrics of photosynthetic performance (P_{660}^{net}, α) and coral net respiration (μmol O_2 cm^{-2} h^{-1}) in the dark were not affected by pCO_2 treatment (445 μatm or 840 μatm). Area-normalized dark respiration was not affected by pCO_2 or temperature treatments, however dark respiration normalized to mg Chl *a* (μmol O_2 (mg Chl *a*)$^{-1}$ h^{-1})

was affected by temperature. OA effects on the respiration and photosynthesis in reef corals show conflicting trends. For instance, high-pCO_2 (735 and 797 μatm) stimulated P^{net} and had no effect on dark respiration (μmol O_2 (mg protein)$^{-1}$ h^{-1}) in *Stylophora pistillata* (Reynaud et al. 2003), while high-pCO_2 (861 μatm) depressed dark respiration normalized to area in juvenile massive *Porites* spp. but had no effect on biomass-normalized dark respiration. Anthony *et al.* (2008) reported low-pH (pH_{SW} 7.90 and 7.65) had no effect on rates of dark respiration in *Acropora intermedia* and *Porites lobata*. However, low-pH (pH_{SW} 7.65) reduced rates of P^{net} (μmol O_2 cm^{-2} d^{-1}) in both species at 25.5 °C and 28.5 °C, yet rates of P^{net} were stimulated in *A. intermedia* and reduced in *P. lobata* at pH_{SW} 7.90 at 28.5 °C (compared to pH_{SW} 7.90 at 25.5 °C) (Anthony et al. 2008). Considering the disparity in responses of reef corals and their symbionts to changes in pH and pCO_2, it is difficult to say with certainty whether OA in the future will significantly alter these responses in meaningful ways. Therefore, identifying the drivers of the variability in the effects of OA on respiration and photosynthesis, and to what degree temperature mediates these responses, should be a goal of future research.

Effects on Symbiodinium and Chlorophyll a Content

Terminal effects of coral bleaching are a reduction in photopigment content per area of coral tissue or modulation of photopigment concentration per *Symbiodinium* cell and the expulsion of symbionts from the host's endodermal tissue (Brown 1997). Evidence for the mechanism of algal cell expulsion from the host is hypothesized as a response to algal cells becoming metabolically compromised (Douglas 2003) from the production of reactive oxygen species (ROS) from photodamage to PSII (Smith et al.

2005; Perez and Weis 2006). While smaller corals have been hypothesized to possess increased transfer rates of ROS from coral tissue to the environment (Nakamura and van Woesik 2001), 14 d of exposure to 30.53 °C in the present study led to substantial bleaching, presumably from photochemical disruption and photo-oxidative damage. However, the expulsion of algal cells has also been shown to occur through host-cell detachment and the loss of cell adhesion in the host (Gates et al. 1992). Additionally, *Symbiodinium* expulsion is not necessarily indicative of a loss of algal photosynthetic function (Gates et al. 1992; Ralph et al. 2001). For instance, Ralph *et al.* (2001) showed algal cells expelled at 33 °C were not photosynthetically impaired and remained photosynthetically competent until heated to 37 °C, a temperature far beyond what most corals experience during seasonal increases in temperature (Fitt et al. 2000).

Modulation of chlorophyll content per algal cell also is reported in response to changes in light-intensity and depth (Titlyanov et al. 2001). Crawley *et al.* (2010) reported an increase in chlorophyll *a* content per algal cell in response to OA (pH 7.85 and 7.60) compared to control conditions (pH 8.10) and determined that OA may elicit a photoacclimation response by *Symbiodinium.* However, the OA-induced photoacclimation response reported by Crawley *et al.* (2010) may be misleading due to: (1) a short period (24 hrs) of photoacclimation to experimental light-intensities (110 μmol photons m^{-2} s^{-1}) following collection in the field, and (2) a short experimental duration (4 d). For instance, Titlyanov *et al*. (2001) showed that photoacclimation to reductions in light-intensities (65% reduction in PAR) can lead to a doubling of chlorophyll *a* content $cell^{-1}$ within 4 d. Therefore, an unidentified portion of the photoacclimation response

observed by Cralwey et al. (2010) must be partitioned as an effect of experimental methodology and not necessarily pCO_2 treatment alone. Indeed, Crawley *et al.* (2010) observed an increase in chlorophyll *a* $cell^{-1}$ for all corals across pH treatments (8.01, 7.85, 7.60). However, the values for chlorophyll *a* concentrations were not directly reported by Crawley *et al.* (2010), and are therefore problematic for comparison to the minor variation in chlorophyll *a* content among corals held at 445 μatm and 840 μatm pCO_2 observed in the present study. In the present study, 6 days of post-collection recovery and acclimation were provided, allowing for a stabilization of Fv/Fm and a sufficient period of photoacclimation (chlorophyll *a* content $cell^{-1}$) with ~60% reduction in PAR, as demonstrated in past studies (Titlyanov 2001). Additionally, a long experimental period (14 – 16 d) allowed for further photoacclimation and stability, revealing no effect of pCO_2 on chlorophyll *a* content per area or algal cell. In this study, chlorophyll *a* (pg) algal $cell^{-1}$ did not change in response to pCO_2 treatments (445 versus 840 μatm), therefore the change in chlorophyll *a* cm^{-2} content is likely an effect of symbiont expulsion and not a product of a photoacclimation response.

Conclusion

Increasing atmospheric pCO_2 represents a salient threat to scleractinians through ocean warming and ocean acidification (Hoegh-Guldberg 1999; IPCC 2007). However, seasonal increases in SST and decadal events (i.e., ENSO events) will continue to drive the decline in coral reefs prior to OA inhibiting reef accretion. Efforts to reduce atmospheric pCO_2 and stave off further increases in SST are the best bet for reducing coral bleaching episodes. In the present study, I report that OA at 840 μatm pCO_2 does

not result in bleaching in juvenile *S. caliendrum*, and OA and elevated temperature do not interact additively or synergistically to exacerbate thermally-induced coral bleaching. I suggest that further research aimed at quantifying the effect of OA on scleractinians needs to be tested in combination with temperature treatments (Brading et al. 2011) and ecologically-relevant light conditions in order to (1) accurately quantify OA effects on reef corals and (2) to determine interactive effects of light and temperature that may be drivers of the deleterious effects reported for scleractinians under OA conditions.

Table 1. Summary of physical and chemical conditions in the 8 treatment tanks between 28 July 2011 and 11 August 2011. Seawater chemistry was assessed daily and temperature three times daily (9:00, 12:00, 17:00 hrs) in all tanks. Values displayed are mean ± SE (*n*); † = SE < 0.1. T = Temperature (°C); TA = total alkalinity; AT–ACO_2 = Ambient temperature–Ambient pCO_2; HT–ACO_2 = High temperature–Ambient pCO_2; AT–HCO_2 = Ambient temperature–High pCO_2; HT–HCO_2 = High temperature–High pCO_2.

Treatment	Tank	T (°C)	pH_{total} †	TA (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})
AT–ACO_2	2	27.6 ± 0.02 (23)	7.99 (14)	2196 ± 8 (14)	448 ± 3 (14)	1724 ± 5.1 (14)	192 ± 1.7 (14)
	4	27.7 ± 0.13 (23)	7.99 (14)	2184 ± 7 (14)	440 ± 3 (14)	1712 ± 4.5 (14)	192 ± 1.4 (14)
HT–ACO_2	3	30.4 ± 0.14 (23)	8.01 (14)	2217 ± 8 (14)	430 ± 4 (14)	1687 ± 6.3 (14)	216 ± 1.8 (14)
	8	30.6 ± 0.02 (23)	7.98 (14)	2219 ± 7 (14)	464 ± 4 (14)	1711 ± 5.1 (14)	207 ± 1.7 (14)
AT–HCO_2	1	27.7 ± 0.03 (23)	7.76 (14)	2208 ± 7 (14)	849 ± 6 (14)	1904 ± 4.6 (14)	124 ± 1.2 (14)
	7	27.7 ± 0.03 (23)	7.75 (13)	2200 ± 8 (13)	860 ± 9 (13)	1903 ± 6.3 (13)	121 ± 1.4 (13)
HT–HCO_2	5	30.6 ± 0.13 (23)	7.77 (14)	2227 ± 6 (14)	821 ± 12 (14)	1884 ± 3.4 (14)	141 ± 2.1 (14)
	6	30.5 ± 0.05 (23)	7.77 (14)	2222 ± 7 (14)	831 ± 8 (14)	1883 ± 4.7 (14)	139 ± 1.5 (14)

Table 2. Dark respiration and parameters describing the relationship between net photosynthesis (μmol O_2 cm^{-2} h^{-1}) and irradiance (μmol photons m^{-2} s^{-1}) (*P/I*) standardized by area of coral tissue (cm^2) and chlorophyll *a* (mg) for juvenile *Seriatopora caliendrum.* Corals were incubated for 14 d in combinations of temperature (°C) and pCO_2 (μatm) as described in *Table 1*. Parameters were obtained from the best-fit line of data to a hyperbolic tangent (Platt et al. 1980) (see *Materials and Methods*). Values displayed are mean ± SE (*n* = number of corals treatment^{-1}). Respiration was measured following 1 h of darkness; *R* = respiration; Chl *a* = chlorophyll *a*; P_{660}^{net} = rate of net photosynthesis as measured at 660 μmol photons m^{-2} s^{-1}; α = the initial slope of the light-limited portion of the curve.

Normalization factor	Parameter	Experimental Treatments			
		AT–ACO_2	AT–HCO_2	HT–ACO_2	HT–HCO_2
Area (cm^2)					
	R (μmol O_2 cm^{-2} h^{-1})	-0.96 ± 0.06 (6)	-0.75 ± 0.04 (5)	-0.73 ± 0.07 (5)	-0.75 ± 0.08 (6)
	P_{660}^{net} (μmol O_2 cm^{-2} h^{-1})	1.80 ± 0.44 (6)	1.96 ± 0.29 (6)	0.08 ± 0.09 (5)	0.22 ± 0.09 (6)
	α (μmol O_2 cm^{-2} h^{-1}) (μmol photons m^{-2} s^{-1})$^{-1}$	0.013 ± 0.0007 (6)	0.011 ± 0.0004 (6)	0.004 ± 0.0005 (5)	0.004 ± 0.0003 (6)
Chl *a* (mg)					
	R (μmol O_2 (mg) h^{-1})	-71.07 ± 6.43 (5)	-58.22 ± 3.00 (5)	-185.93 ± 19.99 (5)	-156.05 ± 24.29 (6)
	P_{660}^{net} (μmol O_2)$(mg)^{-1}$ h^{-1})	162.70 ± 18.26 (5)	147.75 ± 23.09 (6)	23.25 ± 21.15 (5)	36.48 ± 16.63 (6)
	α (μmol O_2 $(mg)^{-1}$ h^{-1}) (μmol photons m^{-2} s^{-1})$^{-1}$	0.97 ± 0.10 (5)	0.85 ± 0.05 (6)	1.18 ± 0.13 (5)	0.78 ± 0.11 (6)

Table 3. Photosynthesis versus irradiance (*P/I*) curve parameters standardized by surface area of the coral tissue (cm^2) and chlorophyll *a* (mg) for juvenile *S. caliendrum* among treatments. Analyses were performed using a partly nested ANOVA with two fixed factors (pCO_2 and Temp) and one nested factor (Tank). Tank was dropped from the analysis when $P > 0.25$; significant values ($P < 0.05$) are in bold. Chl *a* = chlorophyll *a,* df = degrees of freedom, MS = mean sum of squares, detailed parameter definitions in *Table 2*.

Normalization factor	Dependent variable	Effect	df	*MS*	*F*	*P*
Area (cm^2)	Respiration (μmol O_2 cm^{-2} h^{-1})	pCO_2	1	0.044	1.823	0.194
		Temp	1	0.070	2.930	0.104
		pCO_2 x Temp	1	0.070	2.912	0.105
		Error	18	0.024		
	P_{660}^{net} (μmol O_2 cm^{-2} h^{-1})	pCO_2	1	0.132	0.291	0.596
		Temp	1	17.078	32.728	**<0.001**
		pCO_2 x Temp	1	0.0001	0.001	0.974
		Error	19	0.453		
	α (μmol O_2 cm^{-2} h^{-1}) (μmol photons m^{-2} s^{-1})$^{-1}$	pCO_2	1	4.00×10^{-6}	2.162	0.215
		Temp	1	3.43×10^{-4}	171.417	**<0.001**
		pCO_2 x Temp	1	2.00×10^{-6}	1.103	0.353
		Tank(pCO_2 x Temp)	4	2.00×10^{-6}	1.639	0.216
		Error	15	1.00×10^{-6}		

Table 3 (**continued**). Photosynthesis versus irradiance (*P/I*) curve parameters standardized by surface area of the coral tissue (cm^2) and chlorophyll *a* (mg) for juvenile *S. caliendrum* among treatments. Analyses were performed using a partly nested ANOVA with two fixed factors (pCO_2 and Temp) and one nested factor (Tank). Tank was dropped from the analysis when $P > 0.25$; significant values ($P < 0.05$) are in bold. Chl *a* = chlorophyll *a,* df = degrees of freedom, MS = mean sum of squares, detailed parameter definitions in *Table 2*.

Normalization factor	Dependent variable	Effect	df	*MS*	*F*	*P*
Chlorophyll *a* (mg)	Respiration	pCO_2	1	6.633×10^{3}	2.458	0.134
	(μmol O_2 cm^{-2} h^{-1})	Temp	1	4.701×10^{4}	17.42	**0.001**
		pCO_2 x Temp	1	135.891	0.05	0.825
		Error	18	2.699×10^{3}		
	P_{660}^{net} (μmol O_2 cm^{-2} h^{-1})	pCO_2	1	415.313	0.156	0.697
		Temp	1	7.686×10^{3}	28.944	**0.001**
		pCO_2 x Temp	1	126.808	0.048	0.829
		Error	19	2.655×10^{3}		
	α	pCO_2	1	1.129	3.938	0.062
	(μmol O_2 cm^{-2} h^{-1})	Temp	1	0.124	0.378	0.546
	(μmol photons m^{-2} s^{-1})$^{-1}$	pCO_2 x Temp	1	0.033	0.101	0.754
		Error	19	0.328		

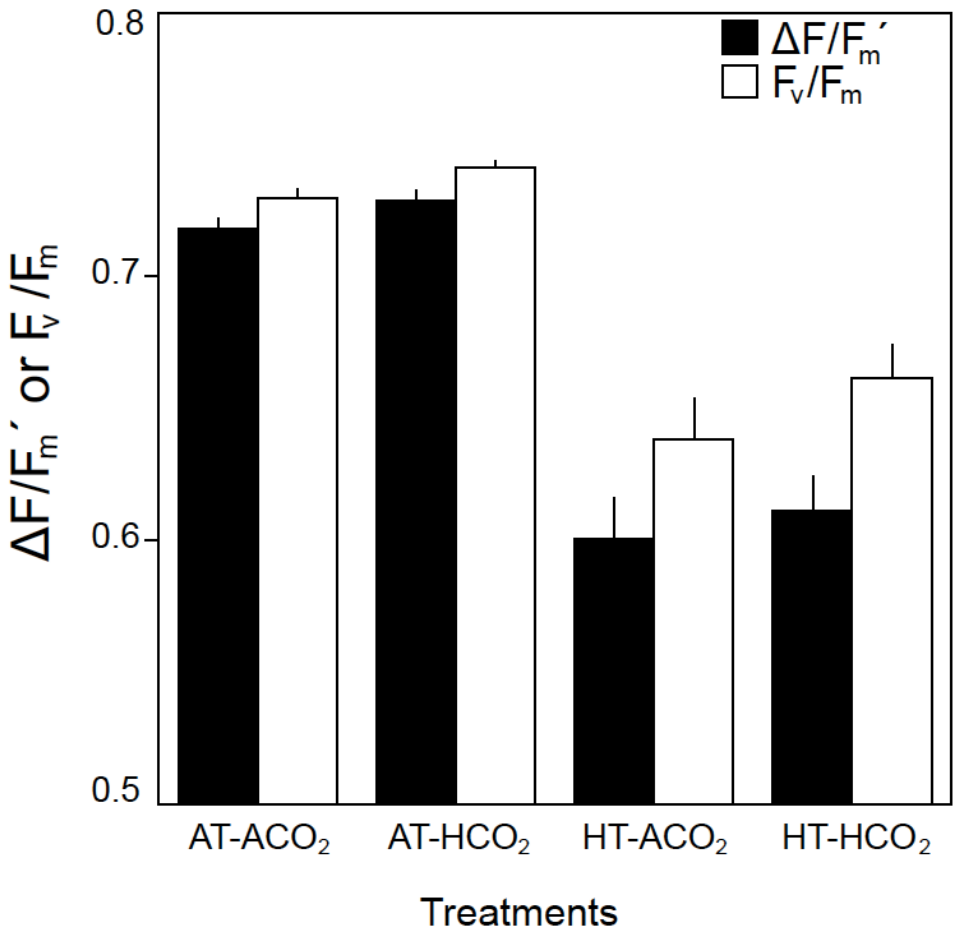

Figure 1. Effective photochemical efficiency of RCIIs in actinic light ($\Delta F/F_m'$) (~245 μmol photons m^{-2} s^{-1}) and maximum photochemical efficiency of open RCIIs (F_v/F_m) for juvenile *Seriatopora caliendrum* exposed for 14 d to combinations of temperature and pCO_2, as described in *Table 1*. $\Delta F/F_m'$ measured at 12:30 hrs and F_v/F_m measured following 1 h darkness adaptation at 19:30 hrs (n = 13–14 $treatment^{-1}$). Values displayed are means ± SE.

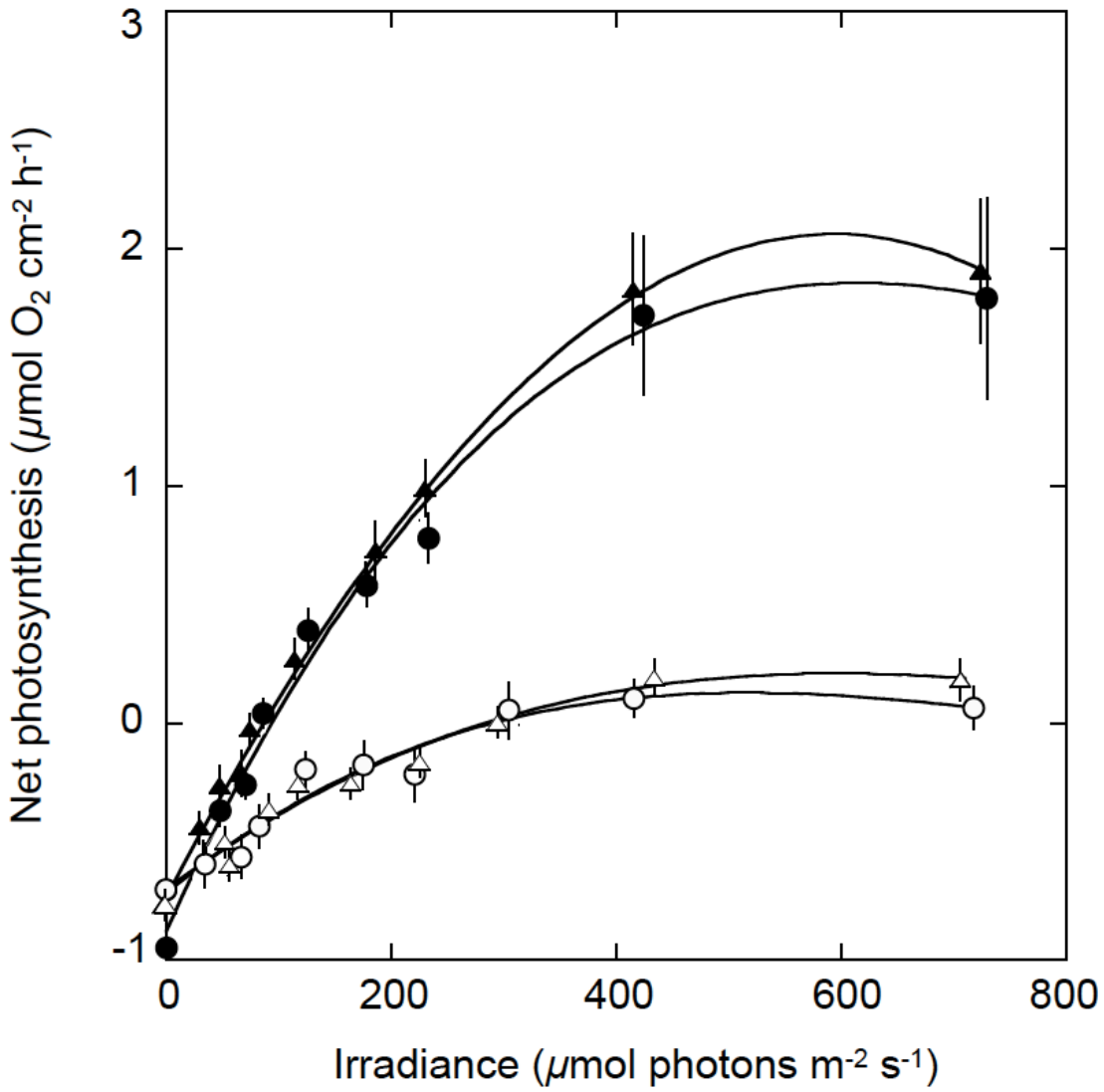

Figure 2. Net photosynthesis (P^{net}) versus irradiance (*P/I*) curves for juvenile *Seriatopora caliendrum* exposed for 14 d to combinations of temperature and pCO_2 as described in *Table 1*. Symbols correspond to treatments AT–ACO_2 (dark circles), HT–ACO_2 (open circles), AT–HCO_2 (dark triangles), and HT–HCO_2 (open triangles). At each irradiance, values are mean P^{net} ± SE (n = 3–4); best-fit lines are fit to mean P^{net} values.

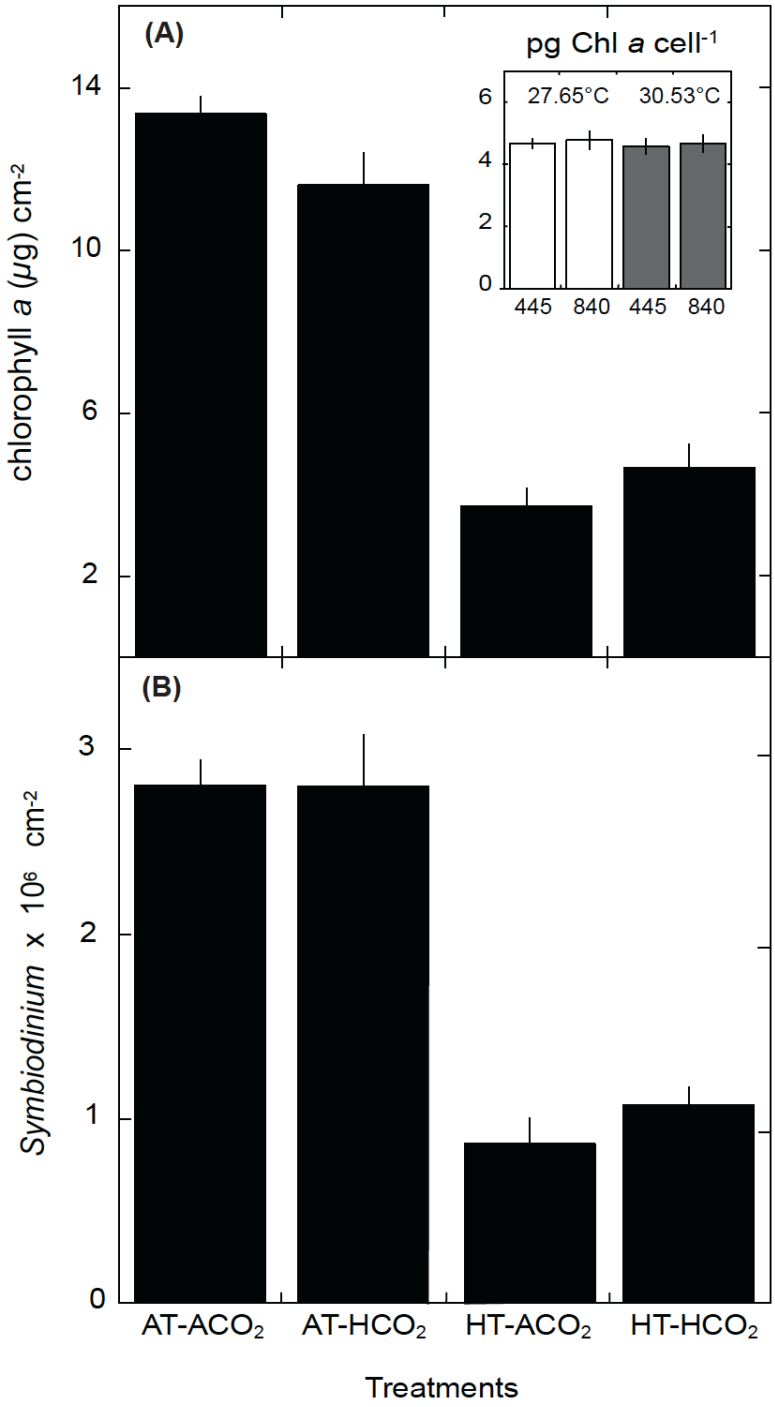

Figure 3. Chlorophyll *a* content per area and *Symbiodinium* cell, and the density of *Symbiodinium* in juvenile *Seriatopora caliendrum* exposed for 14 d to combinations of temperature and pCO_2, as described in *Table 1*. **(A)** Chlorophyll *a* (μg cm^{-2}) (n = 12–14 treatment^{-1}) with inset showing chlorophyll *a* content per *Symbiodinium* cell at 27.63 °C (white columns), 30.53 °C (grey columns), and 445 μatm and 840 μatm CO_2; **(B)** *Symbiodinium* spp. density per surface area of coral tissue (cm^{-2}) (n = 8 treatment^{-1}). Values displayed are means ± SE.

Chapter 3

In situ effects of low-pH and elevated-DIC on the calcification and respiration of juvenile massive *Porites* spp. in Moorea, French Polynesia.

Introduction

Biomineralization and the Physical Environment

Coral reefs are important components of tropical ecosystems and are built largely by biomineralization of scleractinian corals. Despite the importance of coral reefs, the process by which coral calcify remains poorly understood (Allemand et al. 2004). In part, many of the uncertainties surrounding calcification in reef corals can be attributed to the strong influence of the physical environment (e.g., pCO_2, light, temperature, water motion, nutrients, $CaCO_3$ saturation state) on biomineralization (Dennison and Barnes 1988; Kleypas et al. 1999) and the tightly coupled nature of calcification and photosynthesis in symbiotic scleractinians (Gattuso et al. 1999).

Increased global concentration of atmospheric carbon dioxide (pCO_2) is predicted to reduce the health and calcification of reef corals by increasing global atmospheric and sea surface temperatures (SST) and by causing ocean acidification (OA) (Hoegh-Guldberg et al. 2007; IPCC 2007). OA refers to the reduction in the pH of seawater and the perturbation of ocean carbonate chemistry from the equilibration of atmospheric pCO_2 with seawater (Kleypas et al. 1999) and impairs the ability for corals—and other marine calcifiers—to biomineralize effectively (Hofmann et al. 2010). Concerns surrounding the effects of anthropogenic climate change on coral reefs have motivated research to identify drivers of decreased calcification under conditions of OA (Gattuso et

al. 1998; Leclercq et al. 2000; Kroeker et al. 2010) and to identify the mechanism of biomineralization in scleractinians (Marubini et al. 2008; Tambutté et al. 2011; Venn et al. 2011).

Carbon Chemistry and Climate Change

Dissolved inorganic carbon (DIC) in seawater is present in three forms: 90% bicarbonate (HCO_3^-), 10% carbonate (CO_3^{2-}), and < 1% of dissolved carbon dioxide (CO_2 (aq) and carbonic acid (H_2CO_3) (Gattuso et al. 1999). As atmospheric pCO_2 increases, CO_2 in the atmosphere equilibrates with seawater, altering the pH of seawater, the DIC concentration and the proportion of the various dissolved carbon species. The dissolution of CO_2 with seawater leads to the formation of H_2CO_3, which quickly dissociates into a proton (H^+) and HCO_3^-. Increasing $[H^+]$ leads to acidification of seawater and a reduction in $[CO_3^{2-}]$ as more CO_3^{2-} binds with H^+ to form HCO_3^-. Meta-analysis across diverse groups of marine organisms has revealed organism performance to be affected negatively by OA (Kroeker et al. 2010). The negative effects of OA on marine calcifiers, including reef corals, may be attributed to the shift in the concentrations of dissolved carbon species and/or altered energetic costs of performance (e.g., metabolism, pH regulation) under environmental stress (Sibly and Calow 1989; Pörtner 2008).

The supply of DIC to the coral holobiont for physiological processes (e.g., calcification and photosynthesis) can come directly from the surrounding seawater or can be supplied from respiration by the coral (Goreau 1977; Furla et al. 2000). While CO_2 (aq) can be transported transcellularly (e.g., across cells) or paracellularly (e.g., between

cells) across cellular membranes driven by its chemical gradient, HCO_3^- and CO_3^{2-} require active transport across or between cell membranes with this accomplished by specific carrier proteins (Gattuso et al. 1999; Allemand et al. 2004). To increase the DIC pool at the site of calcification below the calicoblastic epithelium, carbon concentrating mechanisms (CCMs) (e.g., carbonic anhydrase) function by increasing equilibration of CO_2 with seawater to HCO_3^-, which can be utilized for calcification and intracellular pH buffering (Allemand et al. 2004; Venn et al. 2011). OA potentially may reduce the efficiency of CCMs or increase the cost of intracellular buffering and/or ion transport, thereby negatively affecting the biomineralization of reef corals.

Effects of Ocean Acidification on Coral Calcification

In laboratory experiments, the rate of precipitation of inorganic calcium carbonate ($CaCO_3$) declines with declining $[CO_3^{2-}]$ and aragonite saturation state (Ω_{arag}). Ω_{arag} is equal to the product of $[Ca^{2+}] \times [CO_3^{2-}]$ divided by (K'_{sp}), the apparent stoichiometric solubility constant for aragonite (Gattuso et al. 1998). A trend for decreasing rates of calcification with decreasing (Ω_{arag}) has been reported for major groups of marine calcifiers (Doney et al. 2009). In scleractinian corals, this trend has been noted for experimental reef assemblages (Langdon et al. 2000; Leclercq et al. 2000), adult reef corals (Gattuso et al. 1998; Ohde and Hossain 2004; Langdon and Atkinson 2005), and newly settled coral recruits (Albright et al. 2008; Anlauf et al. 2011; de Putron et al. 2011). However, the response of reef corals to changes in pH, $[HCO_3^-]$, $[CO_3^{2-}]$ and Ω_{arag} is dissimilar across coral genera (Edmunds et al. 2012). For example, the massive corals within the genus *Porites* are able to maintain high rates of calcification under decreased

Ω_{arag} and elevated pCO_2 predicted for the next century (Fabricius et al. 2011; Pandolfi et al. 2011; Edmunds et al. 2012). Furthermore, the negative effects of OA on calcification can be ameliorated by increased concentrations of HCO_3^- (Marubini and Thake 1999; Herfort et al. 2008), dissolved inorganic nutrients (e.g., nitrogen, phosphorous, iron) (Holcomb et al. 2010), or increased heterotrophic feeding (Edmunds 2011), and it may demonstrate a potential mechanism by which corals can maintain high rates of calcification under future OA conditions of low Ω_{arag} (Holcomb et al. 2010).

In situ Approaches to OA Research

Field or *in situ* physiological experiments are advantageous because they provide natural exposures of temperature, carbonate chemistry, and the light environment (e.g., intensity, spectral quality, and diurnal flux), which are inherently difficult to mimic *in vitro* (Patterson et al. 1991). Understanding the interactive effects of OA with other abiotic physical parameters (e.g., temperature, irradiance) will be important in predicting the response of reef corals to environmental change *in situ* (Reynaud et al. 2003; Langdon and Atkinson 2005; Anthony et al. 2008). In recent years OA research has begun to venture out from the laboratory and into the field with *in situ* manipulation of pH and seawater carbon chemistry (Kline et al. 2012). However, *in situ* OA experimentation currently is in a nascent state, with few examples of studies examining the physiological responses of corals to *in situ* CO_2-enrichment. In designing *in situ* experiments, water motion, irradiance, and temperature are important physical parameters to consider due to their effects on primary productivity, calcification, and metabolism (Dennison and Barnes 1988; Edmunds and Davies 1988; Patterson et al. 1991). *In situ*

experiments have been essential tools to quantify the physiological responses of corals in their natural environment for over 30 years (Porter 1980). Past microcosm or aquarium-based experiments have been performed on marine algae and reef corals within sealed chambers (e.g., respirometers) circulated with seawater and maintained *in situ*. These experiments utilized automated respirometers (Porter 1980) surge-driven paddles (Carpenter 1985), oscillating sample platforms (Wheeler 1980; Carpenter et al. 1991) and centrifugal pumps (Schneider et al. 2009) to provide water motion and maintain treatment conditions. For instance, Patterson *et al.* (1991) on the Hydrolab submarine research station measured the effects of water motion on coral metabolism *in situ*. Other studies have used *in situ* experiments to quantify the relationship between water motion and photosynthetic electron transport (Finelli et al. 2007) and to measure the diel cycles of photosynthesis and calcification of corals in the field (Schneider et al. 2009). One recent example of an *in situ* analysis of OA effects on calcification and photosynthesis was performed by Chauvin et al. (2011) using coral nubbins of *Acropora murcata* from the western Indian Ocean. In this study, coral nubbins were sealed in vials containing seawater manipulated to low-pH and high-DIC conditions and placed on the reef with water agitation provided by gentle rocking.

Alternatives to microcosm or aquarium-based approaches to studying *in situ* effects of OA on benthic communities are field experiments using natural marine CO_2 vents (e.g., CO_2 seeps) (Hall-Spencer et al. 2008; Cigliano et al. 2010; Fabricius et al. 2011) and *in situ* manipulation of pH and seawater carbonate chemistry by Coral Proto-Free Ocean Carbon Enrichment (CP-FOCE; Klein et al. 2012) or FOCE designs

(Kirkwood et al. 2007). However, CO_2 seeps are subject to high variability in pH and pCO_2, recruitment from adjacent non-seep communities, and are a finite regional resource (Hall-Spencer et al. 2008), and CP-FOCE designs are costly and labor-intensive. Thus, the broad application for CO_2 seeps and CP-FOCE experiments is limited. Albeit imperfect, CO_2 seeps and CP-FOCE designs are at the forefront of research on the ecological and physiological effects of OA on marine ecosystems *in situ*, and represent an important step in the interconnection of *in vitro* laboratory-based manipulative experiments and ecosystem modeling approaches to climate change research (Fabricius et al. 2011).

Experimental Overview and Objectives

The objective of this study was to examine the effects of low-pH and elevated-DIC on the calcification and dark respiration of juvenile colonies of massive *Porites* spp. under *in situ* conditions. To accomplish this objective, I placed individual juvenile corals in submersible acrylic chambers filled with DIC-enriched seawater and a stirring motor and placed them on a fringing reef at 1.5-m depth and assessed the effects on coral calcification and respiration after 28 h. I used massive *Porites* spp. because this functional group of reef corals is tolerant to environmental disturbance (Loya et al. 2001) and in laboratory studies is resistant to OA-effects on calcification as a consequence of changes in the carbonate chemistry of seawater (Edmunds et al. 2012). Further, massive *Porites* are a functionally important group on coral reefs in the Indo-Pacific (Done and Potts 1992). Juvenile corals were used because of the importance of this life history stage

in the coral life cycle (Bak and Engel 1979), and because they are tractable to manipulations in small chambers.

My goal was to test three hypotheses regarding the calcification, respiration, and the metabolic expenditure concurrent with calcification of juvenile corals to OA. First, as rates of coral respiration may be stimulated, depressed, or unchanged in response to OA, I tested the null hypothesis that coral respiration in *Porites* spp. are not affected by reduced pH and DIC-enrichment due to the resistance of this functional group to environmental stress. Second, I tested the null hypothesis that manipulation of pH and DIC concentration will not affect calcification in *Porites* spp. and that changes in respiration are not associated with reduced calcification rates. Impaired calcification has been reported for stony corals under OA conditions, potentially as a result of increased costs of calcification (Pandolfi et al. 2011) and/or calcification being thermodynamically unfavorable under decreased Ω_{arag} (Atkinson and Cuet 2008). Additionally, altered rates of aerobic respiration may affect the rate at which corals calcify by stimulating or depressing metabolism (Sibly and Calow 1989; Pörtner 2008). Therefore, using the rates of coral respiration and calcification, I evaluated the energetic expenditure concurrent with calcification in corals under low-pH and enriched DIC as an indirect measure of the cost of calcification (after Edmunds 2012). Finally, high nutrient and/or DIC concentrations can increase rates of coral calcification and can ameliorate the negative effects of OA on calcification (Herfort et al. 2008; Holcomb et al. 2010), therefore I tested the null hypothesis that low-pH and decreases in Ω_{arag} would not impair

calcification under very-high DIC concentration (1000 μmol kg^{-1} SW above ambient seawater) and that very-high DIC would not stimulate rates of coral calcification.

Materials and Methods

Experimental Design

Nine acrylic chambers (2.47 L) were made from custom-cast ultraviolet transmitting (UV-T) acrylic tubing and sheeting (Industrial Plastics, California, USA). Experimental chambers allowed transmission of ultraviolet radiation (UV-R) and photosynthetically active radiation (PAR) to provide corals with irradiances similar to that experienced *in situ.* UV-T acrylic allows > 90% transmission of UV-R (280 – 400 nm), including UV-A and UV-B radiation, as well as PAR (400 – 700 nm) (Gleason et al. 2006). In this study, initial tests were performed using a spectrophotometer (USB-2000, Ocean Optics, Dunedin, Florida, USA) and logging software (Spectra Suite, Ocean Optics) to contrast ambient light (UV-R and PAR) and light transmitted through the acrylic polymer, measured as the intensity of light across the light spectrum (nm) corrected against a known color temperature (°Kelvin) (e.g., the relative spectral irradiance). Graphical representation of relative irradiance intensity across the light spectrum showed no discernable difference in the relative irradiance of ambient light or light transmitted through the UV-T polymer.

To construct experimental chambers, sections of UV-T acrylic tubes (0.3 cm thickness, 20.3 cm inner diameter, 7.6 cm tall) were cemented to a 23 x 23 cm base of UV-T acrylic sheeting with an identical piece serving as the lid. Chambers were sealed

with four stainless steel threaded rods and nuts and a polyvinyl gasket was fixed to the top of the chamber to ensure a seal. To provide flow within the chambers, a battery powered (1.25 V) stirring motor (Playmobil, Zirndorf, Germany) was attached to the inside of each chamber with Velcro™, corals were held in place by threading the plastic base attached to each colony through a small plastic screw. Flow inside the chambers where the corals were attached was determined by photographing hydrated *Artemia* spp. eggs (Sebens and Johnson 1991), which revealed a mean (± SE, n = 12) flow speed of 15.8 ± 1.3 cm s^{-1}.

Experiments were performed over three days (4, 6, and 8 February 2012) using six 2.47 L UV-T acrylic chambers representing three treatments of manipulated pH and DIC with one juvenile coral per chamber (n = 2 per treatment). Three additional chambers were filled with ambient or manipulated seawater alone and used as controls (n = 1 control CO_2-treatment^{-1}). Each morning of the experiment, 6 corals were removed from the flow-through acclimation tank and assigned randomly to an UV-T acrylic chamber filled with filtered seawater (50 μm) from one of three pH- and DIC-treatments. The nine UV-T acrylic chambers were sealed and placed at 1.5-m depth on a fringing reef adjacent the Richard B. Gump South Pacific Research Station. Chambers were attached to cinderblocks and remained on the reef for 28 hrs. Throughout the incubations, the chambers were removed from the reef at 4-h intervals to flush the chambers with fresh treatment seawater and replace batteries in the stirring motors. Light intensity and seawater temperature adjacent to the chambers were recorded at 10-min intervals using Hobo Water Temp Pro v2 loggers (± 0.2 °C, Onset Computer, Massachusetts, USA) to

record temperature, and a MkV-L logging light sensors (JFE Advantech Co., Kobe, Japan) that measured PAR with a 4-π spherical quantum PAR sensor.

Juvenile Coral Collection

Eighteen juvenile colonies (≤ 4.0-cm diameter) of massive *Porites* spp. were collected from 2 – 3 m depth in the back reef of Moorea, French Polynesia, between 28 January 2012 and 1 February 2012. Identification of massive *Porites* spp. to species requires examination of the corallite structure by microscopy once tissue has been removed from the colonies. However, the coral skeletal of *Porites* spp. is remarkably plastic, with recent genetic analyses finding three evolutionarily divergent groups within corals taxonomically identified as *Porites lutea* (Forsman et al. 2009). For this reason, a functional group approach was taken using *Porites* spp. as a species complex that previously has been shown to consist of 85% *P. lutea* and 15% *P. lobata* in this location (Edmunds 2009). After collection, colonies were transported to the Richard B. Gump South Pacific Research Station and attached to plastic bases with epoxy (Z-Spar A788). Corals were placed in a flow-through seawater table maintained at ambient seawater temperature (~28 °C) receiving indirect natural irradiance (4 μmol photons m^{-2} s^{-1}) and rigorous water motion. Corals remained in the seawater table while the epoxy cured (~36 hrs), before being transferred to a flow-through acclimation aquarium.

Thirty-six hours following collection and mounting in epoxy, corals were transferred to a separate flow-through acclimation tank (1000 L) receiving filtered seawater (50 μm) at ~27.5 °C and a mean irradiance (PAR) of 515 ± 30

μmol photons m^{-2} s^{-1} (± SE, n = 75) provided by two 75-W light emitting diode (LED) lamps (Model: Sol White, Aquaillumination, Iowa, USA). These conditions were similar to those experienced by the corals at the site of their collection. Corals were retained in the flow-through acclimation tank (~3 d) until used for *in situ* experiments beginning on 4 February 2012.

Experimental Treatments and Carbon Chemistry Manipulation

Three seawater treatments were designed to test the effects of elevated DIC on the calcification and dark respiration of juvenile massive *Porites* spp. Experimental seawater treatments contrasted: (1) ambient pH and DIC (A–DIC); (2) low pH and high DIC (H–DIC); and (3) low pH and very high-DIC (VH–DIC). The A–DIC treatment reflects the present chemical composition of seawater at current atmospheric conditions (~pH_T 8.0, ~2000 μmol kg^{-1} DIC, ~390 μatm pCO_2), and the H–DIC treatment reflects the seawater conditions projected under representative concentration pathway (RCP) 8.5 (~pH_T 7.7, ~2150 μmol kg^{-1} HCO_3^-, ~950 μatm pCO_2) for the year 2100 (van Vuuren et al. 2011). The VH–DIC treatment was designed to reflect low pH, very high DIC and pCO_2 (~3000 μmol kg^{-1} DIC, ~1400 μatm pCO_2) specifically to test the hypothesis that elevated DIC can increase rates of calcification under OA conditions of decreased Ω_{arag} and low pH (Marubini et al. 2008).

CO_2 enriched seawater was created by bubbling ambient air or CO_2-enriched air into two reservoirs supplied with filtered seawater (50 μm) from Cook's Bay, as detailed in Edmunds *et al.* (2012). DIC treatments were maintained using a solenoid-controlled

gas mixing system (Model A352, Qubit Systems, Ontario, Canada) mixing 99% pure CO_2 (at 17 kPa) with ambient air from an air compressor (103 kPa) in a mixing chamber that was analyzed by an infra-red (IR) gas analyzer (Model SI51 Qubit Systems). The final gas mixture was provided to the experimental microcosms at 10 – 15 L min^{-1} using a pump (Gast Model DOA-P7O4-AA). A–DIC treatments were bubbled with ambient air at a similar flow rate. To manipulate DIC in the VH–DIC treatment, 100% $Na(HCO_3)_2$ (Church and Dwight Co., California, USA) at a concentration of 0.5 mmol L^{-1} was added to seawater drawn from the H–DIC treatments to increase [DIC] to ~3000 μmol kg^{-1}.

Seawater Chemistry and Dependent Variables

Salinity, pH on the total scale (pH_T) and total alkalinity (TA, μmol kg^{-1}) were monitored daily using a single seawater sample (~400 mL) collected from each CO_2 treatment. Titrations were performed using an autotitrator (Model T50, Mettler-Toledo, Ohio, USA) filled with certified acid titrant (~0.1 mol L^{-1} HCl and 0.6 mol L^{-1} NaCl, from A. Dickson Laboratory, Scripps Institute of Oceanography) and equipped with a DG115-SC pH probe (Mettler-Toledo) following standard operating procedure (SOP) 3b (Dickson et al. 2007). To ensure both precision and accuracy of titrations, certified reference material (CRM) of known TA (Batch 105 from A. Dickson Lab, Scripps Institute of Oceanography) were titrated daily with a mean accuracy over the course of the experiment were 0.1% (ranging 0.3 – 4.3 μmol kg^{-1}) below certified TA values. pH was determined by using the DG115-SC pH probe calibrated with a certified Tris-buffer (Batch 8, A. Dickson Lab). Final values for pH_T and carbon chemistry were calculated

for seawater samples using the seacarb script in *R* (The R Foundation for Statistical Computing, http://www.R-project.org).

After 24 h of *in situ* incubations, the response of the coral to treatments was assessed as rates of calcification and dark respiration. Calcification was measured *in situ* as the change in total alkalinity of seawater within the 2.47 L acrylic chambers over a 4 h period using the alkalinity anomaly technique (Smith and Key 1975). TA anomaly measurements began at 09:10 – 11:00 hrs and culminated between 14:40 – 15:40 hrs with corals exposed to a mean (± SE, $n = 86$) irradiance of 1,769 ± 60 μmol photons m^{-2} s^{-1} that ranged from 515 – 2,927 μmol photons m^{-2} s^{-1} as measured 6 times h^{-1} with a 4-π spherical quantum PAR sensor (Advantech Co). Calcification over the 4 h incubation was calculated as:

Equation 4:

$$(\mu\text{mol } CaCO_3 \text{ cm}^{-2} \text{ h}^{-1} = [(\Delta TA*0.5)(SW_{volume}/(time* SA_{coral}))*SW_{density}])$$

using a seawater density calulated from tables of known temperature and salinity [N. Ramsing and J. Gundersen at Unisense, http://www.unisense.com/Default.aspx?ID=1109, based on Garcia and Gordon (Garcia and Gordon, 1992)]. Calcification rates (μmol $CaCO_3$ cm^{-2} h^{-1}) were converted to mg of $CaCO_3$ and scaled to the rates occurring over a 24 h day assuming a ratio of 3.0 for light : dark calcification rates (Gattuso et al. 1999). Calcification rates then were normalized to surface area of the coral tissue (cm^2), which was estimated by using the aluminum foil technique at the conclusion of the experiment (Marsh 1970).

Following measurements of calcification, the chambers were removed from the reef and dark respiration of the corals was measured in a laboratory respirometer. Once in the laboratory, corals were kept in darkness for 30 min to avoid stimulatory effects of light on respiration (Edmunds and Davies 1988). Dark respiration was determined in two respirometers (240 mL) filled with filtered seawater (50 μm) representing the same conditions as the *in situ* treatments, and maintained by a water bath at ambient temperature (28 °C). Flow within respirometers was provided by a magnetic stir bar with a mean (± SE, n = 16) flow speed of 3.0 ± 0.2 cm s^{-1} at the margins of the circular chambers. Changes in oxygen partial pressure (pO_2) in seawater were determined using optrodes (Foxy-R, 1.58 mm diameter, Ocean Optics) connected to spectrophotometers (USB2000 and NeoFox, Ocean Optics) that logged O_2 saturation onto personal computers running Ocean Optics software (OOISensors, version 1.00.08 and NeoFox Viewer version 2.3, Ocean Optics). Optrodes were calibrated with water-saturated air (100%) and a zero solution of sodium sulfite (Na_2SO_3) and 0.01 mol L^{-1} sodium tetraborate ($Na_2B_4O_7$). O_2 saturation was maintained between 80 – 100% to avoid effects of hypoxia on respiration. O_2 saturation was converted to μmol O_2 mL^{-1} using tabulated values for O_2 solubility [N. Ramsing and J. Gundersen at Unisense, http://www.unisense.com/Default.aspx?ID=1109, based on Garcia and Gordon (Garcia and Gordon, 1992)]. The rate of O_2 consumption was determined by regressing change in O_2 concentration against time, correcting for the rate of change in [O_2] of respirometers filled with filtered seawater alone, and standardized to the surface area of the coral tissue (cm^2).

Estimation of the energetic expenditure of corals exposed to pH- and DIC-treatments were made using rates of O_2 consumption that were converted to units of energy (Joules) through the oxy-joulimetric conversion of 440 J mmol O_2^{-1} (Elliot and Davison 1975) and normalized to grams of calcium carbonate deposited over a similar period (i.e., J mg^{-1} $CaCO_3$) (after Edmunds 2012). Oxygen consumption can be used to estimate the aerobic energetic equivalent of the chemical and metabolic work performed by an organism and is therefore not a direct measure of the energetic costs of calcification (Edmunds 2012). However, calcification is expensive energetically and may account for 13 – 30% of energetic costs in scleractinians (Allemand et al. 2011). Therefore, insight into changes in the cost of calcification may be gleamed through changes in aerobic respiration.

Statistical Analysis

Calcification, dark respiration and carbon chemistry (pH_T, pCO_2, $[HCO_3^-]$ and $[CO_3^{2-}]$) were compared among treatments using a one-way ANOVA with treatment as a fixed factor. As the experiment was performed *in situ* with potential differences in physical conditions among days, a one-way ANOVA was used to test for differences in light and temperature among days. Post hoc analyses were performed using Tukey's Honestly Significant Difference (HSD) test, and statistical assumptions of normality and homogeneity of variance required for ANOVA were tested by graphically analysis of residuals. Analysis of data was performed using the statistical software Systat 11 (Systat,

Inc., Illinois, USA) using a Windows operating system. Power analysis was performed on select results according to Zar (2010).

Results

Treatment Conditions

The conditions of pH- and DIC-treatments were achieved with a high degree of precision (Table 1) with a mean irradiance of 1,278 ± 52 μmol photons m^{-2} s^{-1} measured from 5:40 – 16:40 hrs at 1.5 m depth and salinity of 35.94 ± 0.02 (pooled across days and treatments, ± SE, n = 308 and 16, respectively). However, during measurements of calcification (~9:00 – 15:00 hrs) corals experienced a mean irradiance of 1,769 ± 60 μmol photons m^{-2} s^{-1} (see *Materials and Methods*). The mean temperature during the 28 h *in situ* exposure period across the three days the experiment was performed was 28.8 ± 0.2 °C (n = 514). Treatments differed significantly in terms of pH_T ($F_{2,15}$ = 44.84, $P < 0.001$), pCO_2 ($F_{2,15}$ = 41.29, $P < 0.001$), HCO_3^- ($F_{2,15}$ = 720.79, $P < 0.001$), and CO_3^{2-} ($F_{2,15}$ = 54.79, $P < 0.001$). Light intensities did not differ over the 3 d experiment ($F_{2,87}$ = 1.99, P = 0.142) but seawater temperature was significantly different among days ($F_{2,86}$ = 35.75, $P < 0.001$). Mean seawater temperatures varied from 28.2 °C on 4 February to 29.4 °C on 8 February (± 0.2 °C, n = 171 and 170, respectively). The pH_T and DIC (μmol kg^{-1} SW) concentrations for treatments were pH_T 8.04 and 2008 μmol kg^{-1} DIC (A–DIC), pH_T 7.73 and 2177 μmol kg^{-1} DIC (H–DIC), and pH_T 7.69 and 2938 μmol kg^{-1} DIC (VH–DIC) (Table 1).

Calcification and Respiration

Mean calcification ranged from 0.84 ± 0.14 mg $CaCO_3$ cm^{-2} d^{-1} in H–DIC to 1.97 ± 0.29 mg $CaCO_3$ cm^{-2} d^{-1} in the VH–DIC treatment (± SE, n = 6 and 4, respectively) and was affected significantly by seawater chemistry ($F_{2,13}$ = 6.56, P = 0.011). Calcification in H–DIC corals was reduced 23% compared to A–DIC corals (0.84 versus 1.087 mg $CaCO_3$ cm^{-2} d^{-1}) and by 58% compared to VH–DIC corals (Figure 2). Increased [DIC], primarily in the form of [HCO_3^-], strongly stimulated calcification in the VH–DIC treatment despite a low pH_T of 7.69 (Table 2). VH-DIC corals exhibited an 81% increase in calcification compared to A–DIC (2938 versus 2008 μmol kg^{-1} DIC) (Figure 2). Respiration (μmol O_2 cm^{-2} hr^{-1}) was not affected by seawater chemistry ($F_{2,15}$ = 0.37, P = 0.697). Mean respiration ranged from 0.46 ± 0.06 to 0.39 ± 0.05 μmol O_2 cm^{-2} h^{-1} in VH–DIC and H–DIC treatments, respectively (Table 2). However, a post hoc power analysis showed the power of the test (1- β) to detect a significant effect of DIC-treatments on respiration rates at the observed effect size of 0.04 μmol O_2 cm^{-2} hr^{-1} (10% of grand mean) and sampling size (n = 6) was low (< 0.30).

Converting O_2 consumption to energy revealed a daily energetic expenditure of 57.00 to 159.78 J $coral^{-1}$ across treatments for corals depositing between 7.29 mg to 54.99 mg $CaCO_3$ d^{-1}. These values suggest the daily costs for a juvenile massive *Porites* spp. to deposit a gram of $CaCO_3$ were between 1,606 and 9,068 J (Table 2), with mean values ranging from 2,706 J to 5,192 J d^{-1} in H–DIC and VH–DIC treatments, respectively (Figure 3). Manipulation of seawater chemistry across the three treatments did not affect the cost of calcification ($F_{2,13}$ = 2.35, P = 0.134). However, a post hoc power analysis

showed the power of the test (1- β) to detect a significant effect as a result of the DIC-treatments with a 50% change in kJ g^{-1} $CaCO_3$ at this sampling size (n = 6) was low (< 0.55).

Discussion

In the present study, I exposed juvenile massive *Porites* spp. to three seawater treatments of manipulated pH and [DIC] within sealed chambers that were maintained *in situ* under ecologically relevant exposures of temperature and irradiance. The effects of reduced pH and increased [DIC] on rates of dark respiration, calcification, and the cost of calcification were determined to test the hypothesis that seawater carbonate chemistry under OA conditions (e.g., low pH, low Ω_{arag}, high pCO_2) reduces rates of coral respiration and calcification, and increases the cost of calcification. Alternatively, I hypothesized that increases in [DIC] (~1000 μmol kg^{-1} above ambient [DIC]) can stimulate coral calcification under OA conditions by increasing the availability of carbon for calcification, thereby reducing the total energetic expenditure concurrent with calcification. Results from my study indicate the OA conditions do not affect calcification or respiration and does not result in changes in the total metabolic expenditure concurrent with calcification (e.g., indirect cost of calcification) in juvenile massive *Porites* spp. Additionally, my results suggest that increased [DIC] can increase rates of coral calcification under conditions of low pH and Ω_{arag}. These findings suggest that coral calcification in massive *Porites* spp. is DIC-limited at ambient DIC concentrations (~2000 μmol kg^{-1}) and that large increases in [DIC] (~1000 μmol kg^{-1} above ambient [DIC]) stimulate calcification. Additionally, my findings suggest that

reduced calcification rates observed under OA conditions of low pH and low Ω_{arag} can be ameliorated by increasing seawater [DIC], predominately by increasing [HCO_3^-]. However, moderate increases in DIC (~200 μmol kg^{-1}) predicted to occur under OA over the present century (RCP 8.5; van Vuuren et al. 2011) are not sufficient to enhance calcification through altered seawater carbonate chemistry (e.g., low CO_3^{2-}, low Ω_{arag}), low pH and high pCO_2. These findings also support previous studies that have identified HCO_3^- as the DIC species taken up by corals from the external environment to support biomineralization (Furla et al. 2000; Al-Horani et al. 2003).

OA Effects on Calcification

In the present study, calcification rates (mg $CaCO_3$ cm^{-2} d^{-1}) across the three treatments (A–DIC, H–DIC, VH–DIC) were within the range previously reported for massive *Porites* spp. (Lenihan and Edmunds 2010; Edmunds et al. 2011; 2012). Calcification rates of massive *Porites* spp. were not different between corals exposed to ambient seawater carbonate chemistry (A–DIC) at 405 μatm pCO_2 and the carbonate chemistry predicted to occur by the year 2100 under an atmospheric pCO_2 of 976 μatm pCO_2 (pH_T 8.04 versus 7.73). My results suggest that calcification in massive *Porites* spp. is resistant to reductions in pH and Ω_{arag}, and moderate increases in DIC, commensurate with 976 μatm pCO_2. These results are in agreement with Edmunds (2011), who showed area-normalized calcification rates in massive *Porites* spp. to be unresponsive to changes in seawater carbonate chemistry at 805 μatm pCO_2. However in a subsequent study, Edmunds (2012) reported rates of area-normalized calcification in juvenile massive *Porites* spp. were reduced at 861 μatm pCO_2 but unchanged at 756 μatm pCO_2, relative to

control corals at 423 μatm pCO_2. The pCO_2 effect on calcification at 861 μatm pCO_2 suggests a potential pCO_2 threshold concentration $\geq$ 861 μatm in massive *Porites* spp. A non-linear relationship between calcification and Ω_{arag} was also found in *Favia fragum* and *Porites astreoides* (de Putron et al. 2011) suggesting reef corals may be able to maintain high rates of calcification under decreased Ω_{arag} to the point of a threshold saturation state, beyond which coral calcification declines.

It has long been accepted that the geochemical characteristics (e.g., $\Omega_{arag.}$, pH, CO_3^{2-}) of seawater exert a strong influence on the calcification of marine organisms (Gattuso et al. 1998; Leclerq et al. 2000). However, the biology of the coral holobiont affects calcification directly in ways that only recently are being understood (Hoffman et al. 2010; Tambutté et al. 2011). Calcification in corals is stimulated in the light, believed to be a response to photosynthesis providing energy for calcification (Anthony et al. 2002), cellular alkalinization from the removal of waste (e.g., CO_2, H^+) produced by the coral (Al-Horani et al. 2003), or photosynthetic-induced OH^- secretion into the coelenteron (Allemand et al. 2004). The relationship between calcification and Ω_{arag} has been reported for a variety of corals (Gattuso et al. 1998; Kleypas et al. 1999; Langdon and Atkinson 2005), experimental reef mesocosms (Leclerq et al. 2000; 2002), coralline algae (Borowitzka 1981) and coccolithophores (Riebesell et al. 2000; Beaufort et al. 2011). However, the relationship between Ω_{arag} and calcification rates may be more variable than once thought (Leclerq et al. 2000; Pandolfi et al. 2011), and the role of DIC species, pH, and pCO_2 may play significant roles in affecting the calcification rates of a variety of corals (Jury et al. 2010; Jokiel 2011; Edmunds et al. 2012). For instance, meta-

analyses of the relationship between seawater carbonate chemistry and calcification across six coral genera revealed pH, [HCO_3^-], and [CO_3^{2-}] were capable of accounting for variation in the calcification response within the genus *Acropora*, *Favia*, and *Madracis*, while *Porites* showed no relationship to pH, [HCO_3^-], or [CO_3^{2-}] (Edmunds et al. 2012).

Complicating the geochemical relationship with coral calcification are abiotic factors (i.e., temperature and irradiance) that strongly influence calcification rates in scleractinians (Dennison and Barnes 1988; Edmunds 2005; 2006; 2008). For instance, 760 μatm pCO_2 had no effect on calcification in *Stylophora pistillata* at 25 °C, but reduced calcification by 50% at 28 °C (Reynaud et al. 2003). In the present study, an *in situ* approach was used to ensure that corals received ecologically relevant irradiances under field conditions. Mean irradiance in this study (1,278 μmol photons m^{-2} s^{-1}) were 2 – 12 times higher than the irradiances reported in other OA studies (Crawley et al. 2010; Edmunds 2012). The direct effect of light-intensity on coral calcification (Barnes and Chalker 1990) suggests accurate prediction of the effects of OA on coral calcification requires the use of irradiances relevant to the environment from which the experimental corals are collected. For instance, Marubini *et al.* (2001) reported calcification in *Porites compressa* at 5.05 Ω_{arag} and 2.48 Ω_{arag} were indistinguishable at 80 μmol and 150 μmol photons m^{-2} s^{-1}, however rates of calcification substantially increased at 5.05 Ω_{arag} compared to 2.48 Ω_{arag} when exposed to 700 μmol photons m^{-2} s^{-1}. Reef corals are quintessentially multitrophic systems (Ferrier 1991; reviewed in Houlbrèque and Ferrier-Pagès 2009), and enhancing the nutritional status of a coral through increased autotrophic or heterotrophic (e.g., micromolecules to zooplankton) contributions to metabolism can

stimulate coral calcification under ambient and OA conditions (Holcomb et al. 2010; Edmunds 2011). Therefore, in the present study, an increase in calcification under low Ω_{arag} may be due to increased [DIC] providing more carbon substrate to be used in the process of biomineralization. Alternatively, the lack of an effect of pCO_2 between A–CO_2 and H–CO_2 corals may be an effect of high PAR maximizing calcification rates, or to resistance of massive *Porites* spp. to OA effects on calcification.

Together, the present findings support the hypothesis that corals possess beneficial physiological strategies that may be used to respond to changes in carbonate chemistry predicted under anthropogenic climate change. Although the extent to which these strategies can be sustained in order to ameliorate deleterious OA effects remain to be determined. Furthermore, studies assessing the effects of manipulated seawater carbonate chemistry on reef corals may be obtaining biased estimates of the calcification responses if quantified in the laboratory under irradiances sub-saturating with regard to photosynthesis and calcification. Alternatively, corals from low-light environments routinely experience sub-saturating photosynthetic photon flux densities (PPFD), may be more vulnerable to OA than conspecifics from high-PPD, shallow reef environments. Therefore, unraveling the responses of corals to OA under a range of physical conditions (e.g., temperature, PAR irradiance, water motion) will assist in accurately determining the effects of OA on reef corals across the reef environment.

Effects of DIC-Enrichment on Coral Calcification

High levels of DIC in seawater promote growth and photosynthesis in the coral holobiont (Marubini and Thake 1998), although the extent to which calcification and photosynthesis are saturated at the ambient concentration of DIC in seawater is contentious (Furla et al. 2000; Marubini et al. 2008). My results indicate that calcification rates in juvenile massive *Porites* spp. are not saturated under ambient [DIC] of 2008 μmol kg^{-1}. In the present study, addition of $Na(HCO_3)_2$ (0.5 mmol kg^{-1}) to pH_T 7.73 seawater—reducing the mean pH_T to 7.69 and increasing mean pCO_2 to 1445 μatm—increased mean [DIC] by 761 μmol and 935 μmol kg^{-1} relative to A–DIC and H–DIC treatments, respectively. The increase in [DIC] led to stimulation of calcification rates in VH–DIC corals relative to A–DIC and H–DIC corals.

Past studies show coral calcification to be stimulated by increased [DIC] (Marubini and Thake 1999; Herfort et al. 2008), suggesting that coral calcification is under-saturated at ambient [DIC]. However, photosynthesis, which is also dependent upon DIC for carbon fixation, responds to increased [DIC] more equivocally, with studies showing photosynthesis being under-saturated (Herfort et al. 2008; Marubini et al. 2008) and saturated at ambient [DIC] (Burris et al. 1983; Furla et al. 2000; Schneider and Erez 2006). Photosynthesis and calcification appear tightly coupled in reef corals (Barnes and Chalker 1990), with calcification being stimulated during light exposure and potentially as a direct effect of photosynthesis. However, the coral and the algal symbiont may compete for a shared DIC pool (Langdon and Atkinson 2005), and therefore high demand for DIC for *Symbiodinium* photosynthesis may limit DIC for use by the coral for

calcification under ambient [DIC] (Stambler 1991; Hoegh-Guldberg and Smith 1989; Marubini and Thake 1999; Chauvin et al. 2010). However, the hypothesis that the corals and *Symbiodinium* compete for an internal DIC pool is contentious. Furla *et al.* (2000) disputed the hypothesis that photosynthesis and calcification compete for a shared internal DIC pool, reporting that metabolically liberated CO_2 accounted for > 70% of DIC used in calcification with < 30% originating from the external seawater; conversely, algal photosynthesis was shown to be dependent upon an internal DIC supply maintained by carbon concentrating mechanisms. However, in the present study the increase in calcification in VH–CO_2 corals was not concurrent with an increase in respiration rates, therefore it is hypothesized that increased [DIC] in the external seawater stimulated calcification by increasing DIC used for calcification.

Calcification under moderate DIC-enrichment (~200 μmol kg^{-1}) occurring in pCO_2-enrichment and OA experiments does not stimulate calcification (Andersson and Gledhill 2013) and reveals mixed effects on photosynthetic rates (Reynaud et al. 2003; Langdon and Atkinson 2005; Marubini et al. 2008). The lack of a DIC stimulatory effect on calcification under OA conditions is hypothesized to be due to the far more significant decrease in pH and Ω_{arag} overshadowing the relatively minor increase in [DIC]. Interestingly, supplementing [DIC] (0.5 – 4.0 times ambient [DIC]) increases rates of calcification in several studies using *Stylophora pistillata* (Marubini et al. 2008), *Porites porites* (Marubini and Thake 1998), *Acropora* sp., (Herfort et al. 2008), and *Madracis auretenra* (Jury et al. 2010), and in some cases independent of pH and Ω_{arag} (Marubini et al. 2008; Jury et al. 2010). These findings suggest that: (1) ambient [DIC] does not

saturate calcification in all reef corals, and that (2) elevated [DIC] enrichment can decouple competition for DIC between photosynthesis and calcification. Increased [DIC], predominately in the form of HCO_3^-, may increase calcification by increasing the pH buffering capacity of seawater and cellular compartments, by directly increasing the availability of DIC used in $CaCO_3$ biomineralization (Herfort et al. 2008), or by increasing the influx of calcification into the coral ectoderm as a product of DIC transport for calcification (Marshall and Clode 2003). Alternatively, elevated [DIC] may increase the deposition of the $CaCO_3$ crystalline framework within the organic matrix at night, and subsequently the nucleation of new crystals in the day, leading to increased skeletal extension and density (Marubini and Thake 1998). However, in the context of OA, reduced pH and Ω_{arag} outweigh the positive effects of elevated [DIC] and lead to declines in calcification rates.

Effects on Dark Respiration

In the present study, I show that coral dark respiration was unaffected by pH- and DIC-treatments. This is in agreement with past studies showing no effect of pCO_2 on dark respiration of reef corals. CO_2-enrichment (298, 613, 1184 μatm pCO_2) did not affect dark respiration in *Porites lobata* and *Acropora intermedia* (Anthony et al. 2008) and had no effect on dark respiration in *Acropora formosa* (360, 695, 1155 μatm pCO_2) (Crawley et al. 2010) or *Acropora eurystoma* (370 versus 560 μatm CO_2) (Schneider and Erez 2006). In *Stylophora pistillata*, Reynaud *et al.* (2003) and Godinot *et al.* (2011) showed dark respiration to be unaffected by pCO_2 as high as 2039 μatm. However, my findings are in contrast to reports that pCO_2-enrichment (861 μatm pCO_2) reduce dark

respiration in corals (Edmunds 2012). Conversely, other studies reported elevated pCO_2 led to a trend for increased light-enhanced dark respiration (e.g., respiration rates quantified immediately following illumination) in *A. formosa* at 695 μatm and 1155 μatm pCO_2 compared to 360 μatm pCO_2 (Crawley et al. 2010).

Using the juvenile massive *Porites* spp. in Moorea, Edmunds (2012) reported rates of respiration were reduced 36% at 861 μatm pCO_2 compared to 423 μatm and 756 μatm pCO_2. Conversely, in the present study, I report that pH and DIC treatments had no effect on respiration. Interestingly, the values I report for respiration rates are considerably lower than those reported by Edmunds (2012) for the massive *Porites* spp. I suggest the differences in mean respiration rates and responses to pCO_2-enrichment in juvenile *Porites* spp. reported herein and in Edmunds (2012) are attributed to disparities in treatment exposure periods and seasons in which the corals were collected. The pCO_2-manipulation procedure and the temperature experienced by corals were identical (28.0 °C) between the present study and Edmunds (2012). However, corals in the present study experienced a short exposure period to treatments (28 hrs) while a 12 d exposure was applied in Edmunds (2012). Perhaps more importantly, colonies in the present study were collected during the austral summer (January) and experienced a mean irradiance of 1,356 μmol photons m^{-2} s^{-1}, while corals in Edmunds (2012) were collected near the start of the austral winter (May) and exposed to a mean irradiance of 545 μmol photons m^{-2} s^{-1}. Seasonal changes affect the densities of *Symbiodinium* and chlorophyll, as well as the tissue thickness of corals (Fitt et al. 2001). Changes in tissue thickness were hypothesized by Edmunds (2012) to contribute to disparities in the calcification response

of massive *Porites* spp. to OA among years (Edmunds 2011; 2012). Together, the differences in season and coral characteristics may have affected the rates of dark respiration in massive *Porites* spp. between the present study and Edmunds (2012) and the observed responses to elevated pCO_2. This example illustrates that coral respiration can be considerably variable across studies using a single species collected from identical locations (Edmunds et al. 2011).

Effects on the Cost of Calcification

My results indicate a trend for reduced energetic expenditure concurrent with calcification (J g^{-1} $CaCO_3$) in VH–DIC corals compared to A–DIC and H–DIC corals, although this trend was not significant due to a small sample size and low statistical power (< 0.55). The indirect measure of the cost of calcification employed in the present study must be interpreted with caution, as measurements of calcification and respiration were not measured concurrently due to logistical limitations. Measurements of calcification and respiration were staggered by ~1 – 2 h, and therefore are useful only as a first approximation of the cost of chemical and mechanical work performed by the coral (Edmunds 2012). Inferred mean costs of calcification between A–DIC and H–DIC corals do not support the hypothesis that OA increases the cost of calcification (Atkinson and Cuet 2008). Interestingly, preliminary data shown here suggests increased [DIC] (1000 μmol kg^{-1} above ambient [DIC]) may reduce the inferred costs of calcification without a change in respiratory cost (e.g., O_2 consumed), however due to low statistical power this hypothesis requires further testing. Mean values of cost of calcification in this study are lower than those reported by Edmunds (2012), reflective of the higher rates of respiration

of *Porites* spp. measured in that study. Edmunds (2012) reported a non-significant trend for decreased cost of calcification in corals exposed to 861 μatm pCO_2, but no difference among corals at 423 μatm and 756 μatm pCO_2. The decrease in calcification and concurrent reductions in respiration reported by Edmunds (2012) suggest that *Porites* spp. may be experiencing metabolic depression as a consequence of environmental hypercapnia (Pörtner 2008). However, due to the confounded nature of seawater chemistry manipulation, it is difficult to identify whether pH or pCO_2 is the causative agent underlying metabolic depression under OA conditions. My results do not suggest metabolic depression in *Porites* experiencing conditions of OA under 976 μatm pCO_2. Together, the findings of Edmunds (2012) and the present study do not support the hypothesis that coral calcification is reduced under OA conditions in response to increased energetic costs (Pandolfi et al. 2011). Rather, my results indicate that calcification, respiration, and the inferred costs of calcification in *Porites* spp. are unaffected by changes in seawater chemistry between 405 μatm and 976 μatm pCO_2 .

Conclusions

Corals in the genus *Porites* have been shown to be resistant to environmental stress, including changes in seawater chemistry that reduce rates of calcification in other corals (Loya et al. 2001; Anthony et al. 2008; Edmunds et al. 2012). In part, this may be explained by the increased tissue thickness in massive *Porites* compared to more environmentally sensitive reef corals (e.g., acroporids) and by *Porites* harboring *Symbiodinium* from clades tolerant to environmental perturbations (e.g., clade C15) (Fitt et al. 2000; LaJeunesse 2002; Fitt et al. 2009; Putnam et al. 2012). Albeit resistant to

environmental stress, calcification in the massive *Porites* spp. is reduced under declining Ω_{arag} (Ohde and Hossain 2004), however, the effects of OA and high-pCO_2 on calcification in massive *Porites* remain equivocal. (Edmunds 2012). Edmunds (2011) determined 804 μatm pCO_2 had no effect on area-normalized calcification but reduced calcification normalized to mg biomass. The following year, however, this trend was reversed, and 862 μatm pCO_2 reduced area-normalized calcification and had no effect on calcification mg biomass^{-1} (Edmunds 2012). Calcification in *Porites australiensis* (Igushi et al. 2011), *Porites lobata* (Anthony et al. 2008), *Porites lutea* (Ohde and Hossain 2004) and in newly settled *Porites astreoides* recruits (Albright et al. 2008) decreased with decreasing pH and Ω_{arag}. Furthermore, the skeletal density and linear extension of massive *Porites* spp. on nearshore regions of the northern Great Barrier Reef have been decreasing over the last two decades, hypothesized as a response to ocean warming and acidification (Cooper et al. 2008; De´ath et al. 2009). However, the perception of massive *Porites* spp. as an "ecological winners" (sensu Loya et al. 2001) is reinforced through the ability of this genus to outcompete other coral genera under persistent sub-optimum conditions (Green et al. 2008; Fabricius et al. 2011) and under stochastic stress events (Loya et al. 2001).

Table 1. Summary of physical and chemical conditions for three pH- and DIC-treatments maintained within 2.47 L acrylic chambers *in situ* between 4 February 2012 and 9 February 2012. Seawater chemistry was assessed daily at 9:00 hrs with pH and carbonate chemistry calculated using the program *R*. *In situ* temperature (T °C) was recorded 6 time h^{-1} using loggers (± 0.2 °C resolution). Values displayed are mean ± SE (n = 6 for carbonate chemistry variables; n = 514 for temperature); DIC = dissolved inorganic carbon; TA = total alkalinity; Ω_{arag} = aragonite saturation state; A–DIC and H–DIC n = 6; VH–DIC n = 4. A–DIC = ambient–DIC, ambient–pH; H–DIC = high–DIC, low–pH; VH–DIC = very high–DIC, low–pH

Treatment	T (°C)	pH_{total}	TA (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	DIC (μmol kg^{-1})	Ω_{arag}
A–DIC	28.8	8.04 ± 0.01	2359 ± 3	405 ± 8	1751 ± 6	246 ± 2	2008 ± 4	3.95 ± 0.03
H–DIC	28.8	7.73 ± 0.04	2358 ± 2	976 ± 94	2011 ± 25	141 ± 10	2177 ± 17	2.27 ± 0.15
VH–DIC	28.8	7.69 ± 0.03	3131 ± 8	1445 ± 104	2730 ± 21	171 ± 8	2938 ± 16	2.75 ± 0.12

Table 2. Comparison of calcification, respiration, colony size, and energetic cost of calcification for juvenile massive *Porites* spp. exposed to three pH- and DIC-treatments maintained within 2.47 L acrylic chambers *in situ* between 4 February 2012 and 9 February 2012. Treatments are described in *Table 1*. Values displayed are mean ± SE; $n = 6$ for all variables, except † $n = 4$.

Treatment	calcification mg $CaCO_3$ cm^{-2} d^{-1}	respiration μmol O_2 cm^{-2} h^{-1}	surface area cm^{-2}	cost of calcification Joules g^{-1} $CaCO_3$
A–DIC	1.09 ± 0.23	0.43 ± 0.65	25.08 ± 4.06	5013 ± 1032
H–DIC	0.84 ± 0.14	0.39 ± 0.05	19.85 ± 1.97	5192 ± 866
VH–DIC	1.97 ± 0.29 †	0.49 ± 0.85	18.27 ± 2.16	2706 ± 390 †

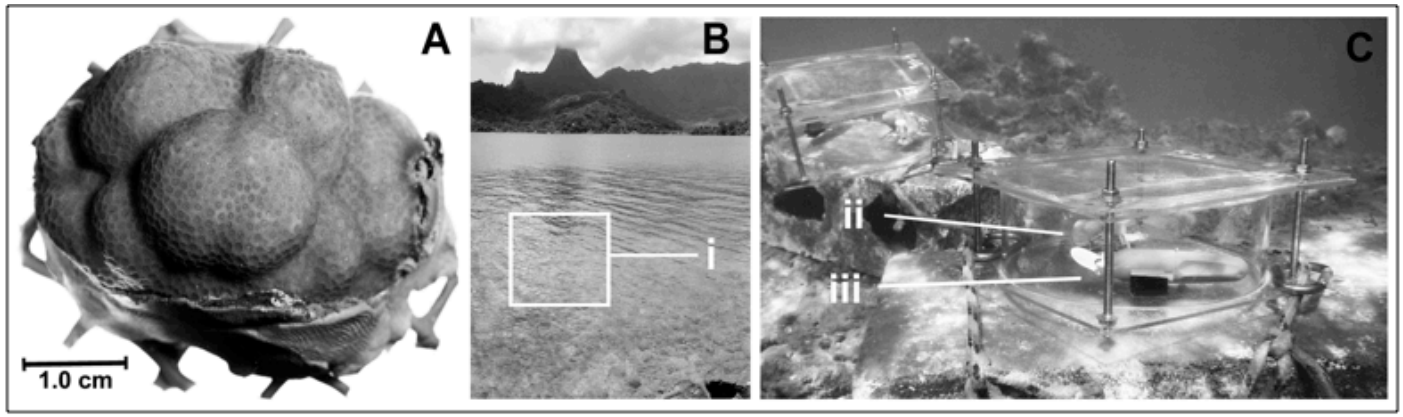

Figure 1. Photo panel of **(A)** juvenile massive *Porites* spp., **(B)** fringing reef off the Richard B. Gump South Pacific Research Station in west Cook's Bay, Moorea French Polynesia, and **(C)** the 2.47 L experimental chambers (23 x 23 x 8 cm) housing coral colonies *in situ*; (i) location of chambers on the fringing reef at 1.5 m depth, (ii) juvenile colony attached to mesh with epoxy, (iii) submersible stirring motor. *Photo credits* **(A)** D. Liittschwager, **(B-C)** C. Wall

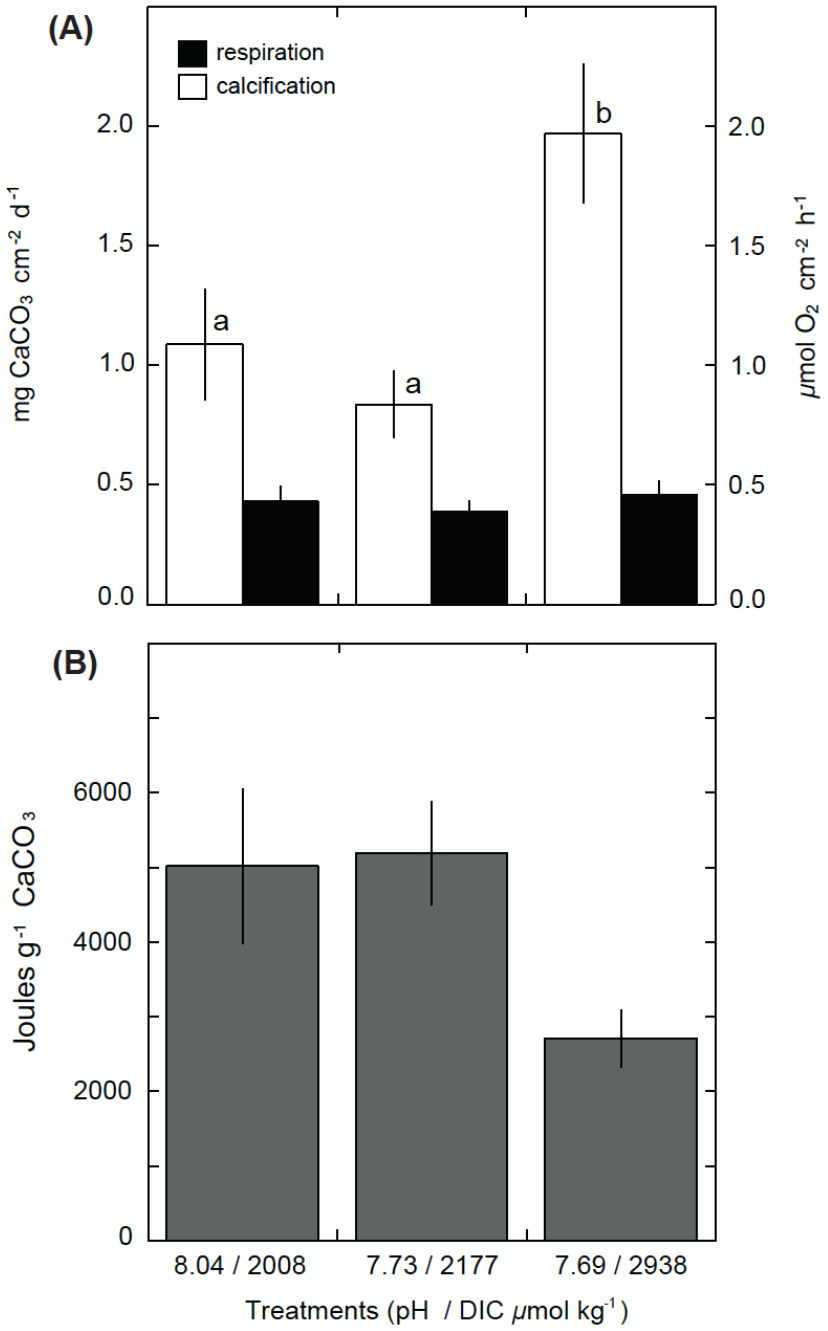

Figure 2. Mean rates of calcification, respiration, and the metabolic expenditure concurrent with depositing 1 g of calcium carbonate in juvenile massive *Porites* spp. exposed for 28 h to pH- and DIC-treatments in closed chambers and maintained *in situ*. **(A)** Calcification (*left ordinate*; pH 8.04 $n = 6$; pH 7.73 $n = 6$; pH 7.69 $n = 4$) and respiration (*right ordinate;* $n = 6$ treatment^{-1}) are normalized to surface area (cm^2). **(B)** Metabolic cost concurrent with calcification (J g^{-1}) (n = 4–6 treatment^{-1}). Treatments significantly affected calcification ($P = 0.011$) with *letters* indicating treatments that differed as determined from *post hoc* analysis; there was no effect of treatment on respiration ($P = 0.697$) or cost of calcification ($P = 0.134$); values displayed are mean ± SE.

Chapter 4

Ocean acidification effects on oxygen consumption and nitrogen excretion in the juvenile reef coral *Seriatopora caliendrum*

Introduction

Metabolism and Environmental Change

Metabolism, as a complex series of biochemical pathways describing the processing of materials and transfer of energy, is a unifying process of life (Brown et al. 2004). The fate of metabolic products, following the laws of thermodynamics and energy conservation (Wieser 1994), may be partitioned to fuel somatic growth, reproduction, daily activity, or allocated to costs of maintenance (e.g., pH regulation, ion pumping, protein turnover) (Koehn and Bayne 1989). Metabolic maintenance and costs associated with maintenance of the basal metabolic rate (BMR) are hypothesized to be costly, equaling 20 – 40% of the metabolic rate in free-living individuals (Hulbert and Else 2000).

To a degree, the cost of maintenance in living organisms is determined genetically, however rates of metabolism are a function of the life stage of an organism (e.g., adult versus juvenile), environmental condition, and efficiency of energy absorption (Koehn and Bayne 1989; Koehn 1991; Hulbert and Else 2000). Stress, as an environmental conditions that reduces Darwinian fitness or performance (Sibly and Calow 1989), may alter the metabolic rate of organisms through two pathways: (1) increasing rates of metabolism to cope with increased costs of functioning, or alternatively, (2) reduce

metabolic rates through metabolic energy conservation as an adaptive strategy for survival during prolonged stress (Hand and Hardewig 1996). In the context of global climate change, increased ocean warming and ocean acidification (OA) may affect the metabolism of marine organisms as environmental conditions are perturbed. As the cost of functioning (e.g., BMR) increases in sub-optimum conditions, costs of maintenance increase and the energy available for growth (i.e., scope for growth) decreases (Koehn and Bayne 1989). Therefore, environmental stress may reduce energy available for tissue and skeletal growth, as well as signifying a strain on energetic reserves.

The positive relationship of elevated temperature and metabolism is well described (Brockington and Clarke 2001). However, the effects of OA on metabolism remain inconclusive (Langenbuch and Pörtner 2002; Thomsen and Melzner 2010; Nakamura et al. 2011). In reef corals, the complex nature of the coral-algal symbiosis and the recycling of nutrients, including excretory products in the form of ammonium, have made determination of excretion rates problematic, with only a few studies directly quantifying rates of excretion (Kawaguti 1953; Muscatine and D'Elia 1978; Szmant-Froelich and Pilson 1984; Rahav et al. 1989; Szmant et al. 1990). Albeit difficult, excretion is a valuable component of the energy budget of organisms as it represents loss of energy from protein metabolism or amino acid deamination. When excretion is concurrently determined with rates of aerobic respiration, the ratio of oxygen consumed to nitrogen loss (O:N) can be useful in quantifying the costs of metabolism and changes in energetic substrates (e.g., protein vs. lipids or carbohydrates) used to fuel respiration, growth or maintenance (Szmant et al. 1990).

The Coral-Algal Symbiosis and Nutrient Recycling

The prevalence of scleractinian corals in oligotrophic tropical seas for > 200 million years is, in part, attributed to the association with the dinoflagellate alga *Symbiodinium* spp. (Yonge 1968; Musactine and Porter 1977). *Symbiodinium* contribute to the nutritional status of the coral host through the uptake and recycling of essential nutrients, such as nitrogen and phosphorous, and through the translocation of carbon derived from photosynthesis to the host (Muscatine and D'Elia 1978; Wilkerson and Muscatine 1984). The algal endosymbionts transfer > 95% of photosynthetically-fixed carbon to the coral to fuel growth and metabolism (Muscatine et al. 1984) while the metabolic waste products from host metabolism (e.g., ammonium) are assimilated by the algae (Muscatine and Porter 1977). While the coral animal has adapted to utilize the photoautrophic capacity of its symbiont, corals also are capable of heterotrophic feeding to meet metabolic requirements (Wang and Douglas 1999; Houlbrèque and Ferrier-Pagès 2009). Additionally, corals are able to take up dissolved materials (e.g., urea, free amino acids, phosphorous, ammonium) from the environment (Ferrier 1991; Grover and Maguer 2002; Grover et al. 2006; Holcomb et al. 2010).

Cnidarians, including the scleractinian corals, are ammonotelic and excrete metabolic waste as ammonium into seawater (Muscatine and D'Elia 1978; Rahav et al. 1989). The source of ammonium produced is largely a product of protein catabolism and deamination of amino acids, used as substrates in coral respiration and other metabolic processes (Rees and Ellard 1989; Szmant et al. 1990). However, in symbiotic corals the conservation and recycling of nutrients by *Symbiodinium* result in the retention of

nutrients and metabolic waste within the coral-algal system (Wilkerson and Trench 1986), whereas in aposymbiotic corals and symbiotic corals in low light, ammonium is excreted in the surrounding seawater (Muscatine and D'Elia 1978).

Ammonium Assimilation in Symbiotic Reef Corals

Ammonium recycling in reef corals is primarily a function of the *Symbiodinium* (Grover et al. 2002), which assimilates exogenous ammonium in seawater or from the host catabolism. However, tissues of symbiotic anemones and reef corals possess a minor capability for ammonium assimilation independent of the presence of symbiotic algae (Lipschultz and Cook 2002; Wang and Douglas 1998; Pernice et al. 2012). Ammonium uptake by the coral holobiont involves three enzymes: glutamine synthetase (GS), NADPH-dependent 2-oxoglutarate amidotransferase (GOGAT) also referred to as glutamate synthase, and NADPH-dependent glutamate dehydrogenase (GDH). Of these, the GS/GOGAT pathway is the prominent pathway of inorganic nitrogen assimilation in *Symbiodinium* (Summons and Osmond 1981).

Ammonium assimilation by the GS/GOGAT pathway begins with GS and ATP hydrolysis catalyzing the amination of glutamate with ammonium to form glutamine. The amido nitrogen of glutamine is then transferred to α-ketoglutarate (α-kg) by GOGAT to form two molecules of glutamate, one of which can be recycled further through amination by GS while the other can be used to produce other amino acids (Wilkerson and Muscatine 1984; Falkowski et al. 1993). The dual action of GS and GOGAT is key in ammonia assimilation in plants and algae, however insects are the only animal group

where GOGAT has been reported (Martinez-Bilbao et al. 1988; Scaraffia et al. 2005). Alternatively, in reef corals GS and GDH are found in both animal and algal tissues, of which GDH has been suggested as a secondary pathway for ammonium assimilation by the coral holobiont (i.e., alga + cnidarian) through the reversible NADPH-dependent amination of α-ketoglutarate (Martinez-Bilbao et al. 1988; Catmull et al. 1987; Rees 1987). However the role of the host in ammonium uptake and assimilation, and the degree to which GDH functions in the assimilation of DIN from the environment remains uncertain. The half-saturation constant, K_m, for GDH in regards to ammonium is high (10mM in GDH compared to 20 μM for GS; Rahav et al. 1989), and catalyzes the deamination of glutamate leading to ammonium production. Therefore, GDH has been suggested to serve a relatively minor role in ammonium uptake in reef corals (Falkowski et al. 1993) and often is related to ammonium excretion and not assimilation in marine invertebrates (Moyes et al. 1985; Falkowski et al. 1993; Lipschultz and Cook 2002).

Inhibition of Ammonium Assimilation

Nitrogen recycling within the coral holobiont can be inhibited during prolonged exposure to darkness (16 – 72 hrs) (Kawaguti 1953; Rahav et al. 1989) or by the use of pharmacological inhibitors of enzymes involved directly in nitrogen recycling, or by disruption of algal photosynthetic electron flow (Muscatine and D'Elia 1978; Rees 1987; Rahav et al. 1989). The three pharmacological inhibitors successfully used to inhibit ammonium assimilation are methionine sulphoximine (MSX), an inhibitor of GS (Ronzio et al. 1969); azaserine, which irreversible blocks GOGAT (Wallsgrove et al. 1977); and the herbicide Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) commonly referred to as

DCMU. DCMU inhibits acyclic or linear electron flow through photosystem II of *Symbiodinium* by blocking the transport of electrons from the primary (Q_A) to the secondary plastoquinone electron acceptor (Q_B) (Vasil`ev et al. 1988). The role of photosynthesis in ammonium uptake is unclear. However, ammonium excretion under prolonged darkness, or DCMU treatment, likely is a result of reduced energy supplies that might otherwise fuel ammonium uptake by the algae. Alternatively, ammonium excretion may take place as the availability of carbon skeletons to accept ammonium for the construction of amino acids is reduced (Muscatine and D'Elia 1978). Rahav et al. (1989) compared methods of inhibition of ammonium uptake in the reef coral *Stylophora pistillata* by using MSX, 1.0 mM azaserine, 10 μM DCMU, and prolonged darkness (24, 48, 72 hrs) and found that treatment with azaserine, DCMU and darkness (48 – 72 hrs) resulted in similar rates of ammonium excretion, but MSX resulted in coral mortality. However treatment with azaserine and MSX inhibited ammonium assimilation in the green hydra-*Chlorella* symbiosis (Rees 1987; McAuley 1995).

Objectives and Experimental Overview

Environmental stress can induce depression (Guppy and Withers 1999) or stimulation of metabolism (Sibly and Calow 1989). Determining how an organism is responding to a perturbation can be revealed indirectly through rates of aerobic metabolism (e.g., respiration) and rates of energy loss through excretion (Bayne and Widdows 1978; Hawkins et al. 1989). OA is hypothesized to lead to metabolic depression in marine organisms through extracellular acidosis and disruption of acid-base regulation (Pörtner 2008), however few studies have tested the hypothesis that OA leads

to metabolic depression in corals (Edmunds 2012). Elevated pCO_2 and reduced extracellular pH have been reported to affect aerobic metabolism and alter rates of ammonium excretion in marine invertebrates (Langenbuch and Pörtner 2002; Michaelidis et al. 2005; Thomsen and Melzner 2010). OA reduced aerobic respiration in massive *Porites* spp. (Edmunds 2012), however general trends for OA-mediated reductions in aerobic metabolism are uncertain. Reduction in metabolic rates, or increased costs of maintenance (e.g., pH regulation) required to compensate for extracellular acidosis, may lead to reductions in physiological function (e.g., calcification) as the energy budget of an organism is disrupted (Pörtner 2008). Unraveling the metabolic response of corals to OA may identify overarching physiological mechanisms that, in part, may provide a context for observed deleterious affects of OA in reef corals and other marine taxa.

In this study, I exposed juvenile *Seriatopora caliendrum* to 14 – 16 d of ambient–pCO_2 (465 μatm pCO_2) and high–pCO_2 (885 μatm pCO_2) to test the hypothesis that corals exposed to elevated pCO_2 have altered metabolic rates compared to corals in ambient–pCO_2 treatments, as determined by rates of ammonium excretion and oxygen consumption. To quantify rates of ammonium excretion, I exposed corals from two pCO_2 treatments to inhibitors of ammonium uptake (1 mM azaserine, 10 μM DCMU), and contrasted rates of excretion in inhibited corals against uninhibited control corals. Two important components of the uptake and excretion of ammonium in reef corals are the protein content of coral tissue (mg cm^{-2}), and the *Symbiodinium* content (cells cm^{-2}). Protein represents a source of ammonium from catabolic processes (e.g., protein catabolism and deamination) and *Symbiodinium* represent the capacity for ammonium

uptake. Additionally, exposure to azaserine and DCMU can result in a loss of *Symbiodinium* from intact corals (Rahav et al. 1989). Therefore, *Symbiodinium* density and total protein content were compared among corals and among inhibitor (e.g., DCMU, azaserine, controls) and CO_2 treatments (465 μatm and 885 μatm pCO_2) to test the hypothesis that *Symbiodinium* density and protein content are affected by pCO_2 or inhibitor treatment. Secondly, I hypothesized that corals exhibiting high excretion rates have reduced protein content as a result of increased protein catabolism.

Materials and Methods

Experimental Design and Coral Collection

Two experimental CO_2 treatments were designed to test the hypothesis that ocean acidification (OA) affects the metabolism of juvenile scleractinian corals by altering rates of aerobic respiration and ammonium excretion. Experimental CO_2-treatments were defined as ambient–pCO_2 (A–CO_2) and high–pCO_2 (H–CO_2), which reflected ambient seawater pCO_2 at the National Museum of Marine Biology and Aquarium (NMMBA) (465 μatm pCO_2) and elevated pCO_2 (885 μatm pCO_2) predicted for the year 2100 under the representative concentration pathway 8.5 (RCP 8.5) (van Vuuren et al. 2011); ambient conditions correspond to ambient air at NMMBA. Temperatures in treatments were maintained at ambient conditions along shallow reefs in Nanwan Bay at 3.0-m depth (~27.5 °C; T. Fan personal communication) for the time the experiment was conducted (June 2012).

Twelve juvenile corals (≤ 4-cm diameter) were collected on 4 June 2012 at a depth of 5 – 7 m from Hobihu Reef in Nanwan Bay, Taiwan, and transported back to NMMBA. Once at NMMBA, epiphytes and commensal crabs were removed from the corals, and corals placed into a 1050-L flow-through aquarium (flow rate of 7.5 L min^{-1}) receiving filtered seawater (50 μm). Temperature in the flow through aquarium was regulated by a chiller (Aquatek, Aquasystems, Taiwan) and maintained at a mean temperature (± SE, n =15) of 27.89 ± 0.10 °C; water motion was provided by a submersible pump (1451 L h^{-1}, TAAM, Inc., California, USA). Photosynthetically active radiation (PAR) in the acclimation tank was supplied by four 150-W metal halide bulbs and four 18-w fluorescent bulbs (TL-D Blue, Phillips, Maryland, USA) programmed to a 12h : 12h light : dark cycle that created a mean irradiance (± SE, n =12) of 259 ± 10 μmol photons m^{-2} s^{-1} as measured daily beneath the surface of the seawater using a 4-π spherical quantum PAR sensor (Li-Cor LI-193, Lincoln, Nebraska, USA). Corals remained in the flow-through aquarium for 5 d before being placed into pCO_2 treatment tanks on 9 June 2012.

Experimental Treatments and pCO_2 Manipulation

Ambient- and high-pCO_2 treatments were created in two 130-L tanks (77 x 77 x 30 cm) filled with filtered (1.0 μm) seawater and maintained at an ambient salinity of ~32.5 with partial water changes (~26 L) performed each evening. Seawater temperature was controlled independently for each treatment using micro-sensor based regulators (AquaController, Neptune Systems, San Jose, California, USA) connected to a 300-W heater (Taikong Corp.) and chiller (Aquatek, Aquasystems, Taiwan) with water motion

provided by a submersible pump (1451 L h^{-1}). Light was provided by two 150-W metal halide bulbs and two 18-W fluorescent bulbs programmed on a 12h : 12h light : dark cycle creating a mean (± SE, n = 36) irradiance of 263 ± 5 μmol photons m^{-2} s^{-1}. Six juvenile corals were placed into each treatment tank and exposed to CO_2-treatments for 14 – 16 d until processed between 22 and 24 June 2012.

CO_2 treatments were created by bubbling ambient air and CO_2-enriched air into treatment tanks using solenoid-controlled gas mixing technology (Model A352, Qubit Systems, Ontario, Canada), as detailed in Dufault *et al.* (2012). Ambient air from an air compressor was mixed with 99% pure CO_2 (≤ 1% air) in a mixing chamber and analyzed by an infrared gas analyzer (S151, Qubit Systems) calibrated against certified CO_2 reference gas (1793 ppm CO_2, San Ying Gas Co., Taiwan) and logged in ppm on a PC (LabPro software, Vernier Software and Technology, Oregon, USA). Flow of CO_2 into the mixing chamber was dynamically regulated through the variable duty cycle of the solenoid controller to reach a desired pCO_2. CO_2 enriched air from the Qubit system and ambient air from the air compressor were delivered to H–CO_2 and A–CO_2 treatment tanks, respectively, at a flow rate of ~15 L min^{-1}.

pCO_2 treatments were monitored regularly for stability of physical conditions and chemistry of seawater. Temperature and salinity were monitored three times d^{-1} (9:00, 12:00, 17:00 hrs) using a certified digital thermometer (Fisher Scientific, 15-077-8, ± 0.05 °C) and a conductivity meter (YSI 3100 Conductivity Meter, YSI Inc., Ohio, USA). Irradiance was measured daily (12:00 hrs) near the surface of the corals using a 4-π

spherical light sensor (LI-Cor LI-193). Samples of seawater (~400 mL) from each treatment were assessed for total alkalinity (TA, μmol kg^{-1}) and pCO_2 (μatm) by potentiometric titration following Dickson standard operating procedure (SOP) 3 (Dickson et al. 2007). pH on the total scale (pH_T) was determined spectrophotometrically by the *m*-cresol purple dye method (SOP 6B, Dickson 2007) using two replicate seawater samples from each treatment tank. The chemistry of seawater samples was determined for 13 of the 16 days of experimental exposure. Seawater samples were titrated using an open cell autotitrator (Model DL50, Mettler-Toledo, Ohio, USA) equipped with a DG115-SC pH probe (Mettler-Toledo) filled with certified acid titrant (~0.1 mol L^{-1} HCl and 0.6 mol L^{-1} NaCl, from A. Dickson, Scripps Institution of Oceanography) and calibrated against known standards (4.00, 7.00, 10.00 NBS buffers, Panreac, Spain). TA determinations of seawater samples were tested for accuracy and precision against certified reference material (CRM) of known TA (Batch 110; A. Dickson Laboratory, Scripps Institute of Oceanography) with our analyses being < 0.6% above reference materials (ranging 1.1 – 12.3 μmol kg^{-1}). Calculations of carbonate chemistry parameters (e.g., pCO_2, HCO_3^-, CO_3^{2-}, Ω_{arag}) were determined in CO2SYS software in Microsoft Excel (Fangue et al. 2010) using TA, salinity, pH_T and temperature as input parameters.

Inhibitor Incubation Treatments

Corals were exposed to two inhibitors (1 mM azaserine, 10 μM DCMU) or remained as uninhibited controls (e.g., controls). The incubation periods required for the inhibitors were 24 h for DCMU and 1 h for azaserine (Rahav et al. 1989), with comparable controls concurrently incubated in filtered seawater. After the incubations,

excretion was measured as the rate of change in ammonium concentration in seawater over 3 h, dark respiration was measured as the change in O_2 concentration of seawater over time (~30 min), and biomass was assessed as the density of *Symbiodinium* and the total protein content.

DCMU treatment

After 14 d of exposure to ambient- and high-CO_2 treatments, rates of respiration, excretion, and biomass characteristics were determined for each coral (n = 6 CO_2-treatment^{-1}). Measurements of dependent variables took 3 d, thus the final treatment exposure period across treatments was 14 – 16 d. Each day, two A–CO_2 corals and two H–CO_2 corals were incubated for 24 h prior to measuring rates of respiration and excretion. Corals were suspended in four 1.0 L beakers filled with 0.9 L filtered seawater (0.45 μm Millipore filter, EMD Millipore Corp., Massachusetts, USA) from their respective CO_2-treatments in the presence or absence of 10 μM DCMU (i.e., DCMU+ and control), following Rahav *et al*. (1989). As DCMU has limited solubility in water, DCMU was solubilized in ethanol (90% EtOH) and diluted to make a stock solution of 10 mM DCMU in 45% EtOH. The stock solution was diluted to a final concentration of 10 μM DCMU in < 0.05% EtOH (Ramanujam et al. 1981). Therefore, to test for effects of the EtOH on corals, an additional two corals (one from each CO_2 treatment) were incubated in filtered seawater with < 0.05% EtOH and used as carrier controls (hereafter 'EtOH control'). Water motion inside the beakers was provided by a submersible pump (260 L h^{-1}) and gentle aeration, which supplied ambient- or CO_2-enriched air to each beaker to maintain treatment conditions. Flow near the corals was determined by

photographing hydrated *Artemia* spp. eggs (Sebens and Johnson 1991), which revealed a mean flow rate (± SE, n = 14) of 5.34 ± 0.26 cm s^{-1}. The beakers were placed into a water bath to maintain a stable temperature over the course of the incubations. Temperature within the water bath was maintained at a mean temperature (± SE, n = 48) of 27.30 ± 0.78 °C using a micro-sensor based regulator (Neptune Systems). Corals were maintained on a 12h : 12h light : dark cycle receiving a mean (± SE, n = 12) photosynthetically active irradiance of 274 ± 4 μmol photons m^{-2} s^{-1} measured by a cosine quantum PAR sensor on a Diving-PAM (Waltz, GmbH, Effeltrich, Germany).

Azaserine Treatment

Corals to be treated with azaserine (n = 6 corals CO_2-treatment^{-1}) were removed from CO_2-treatments and placed in 1 mM azaserine dissolved in filtered seawater (0.45 μm) or filtered seawater as a control. Two corals from each CO_2 treatment were incubated for 1 h in a 1 mM azaserine solution or in filtered seawater (0.45 μm) within 70 ml glass beakers and covered with polyvinylchloride lids during the inhibitor incubation period (1 h) with water motion provided by an aerator. Beakers were placed in a water bath and maintained at mean temperature (± SE, n = 5) of 27.45 ± 0.04 °C.

Excretion Experiment

Following incubations, corals were removed from the inhibitor and control treatments and submersed in filtered seawater (0.45 μm) to rinse residual inhibitor from the corals. After rinsing, corals were individually placed into 250 mL glass beakers containing 200 mL of filtered seawater (0.45 μm). All glassware for the excretion

experiment was washed with 5% HCl and rinsed with distilled water before use. Each CO_2 treatment was represented by three beakers: one inhibited coral treatment, one uninhibited coral treatment, and one beaker containing seawater alone. The six 250 mL beakers (n = 3 CO_2-treatment^{-1}) were placed into a water bath, maintained at a mean temperature of (± SE, n = 32) of 27.55 ± 0.18 °C by a heater and chiller, with a mean (± SE, n = 11) flow rate of 4.65 ± 0.31 cm^2 s^{-1} provided by an aerator. Beakers were covered in saran wrap and remained in darkness for 3 h. Ammonium excretion for azaserine treated corals was estimated over 2 hrs in the light, at a mean (± SE, n = 3) photosynthetically active irradiance of 267 ± 6 μmol photons m^{-2} s^{-1}.

Ammonium Determination

Concentration of ammonium (NH_4^+) in seawater was measured following the method of Solorzano (1969) and Parsons *et al.* (1984). In this method, seawater samples are exposed to an alkaline citrate medium, sodium hypochlorite, phenol and the reagent catalyzer sodium nitroprusside, which produces an indophenol blue color upon reacting with ammonia. Reagents were added to freshly collected seawater samples and incubated in darkness for 30 min. Ammonium concentration (μg-at N L^{-1}) was determined spectrophotometrically by measuring the absorbance of samples at 640 nm using a 10-cm pathlength glass cuvette and regressing absorbance against NH_4^+ concentration of eleven standard solutions (range: 0.5 – 20 μM NH_4^+) of NH_4Cl in distilled water. Rates of excretion (μmol NH_4^+ h^{-1}) were converted to nmol and standardized to the surface area of the coral tissue (cm^2) determined by wax dipping (Stimson and Kinzie 1991), and finally expressed as nmol NH_4^+ cm^{-2} h^{-1}.

Respiration Rates

Respiration was measured in the dark on individual colonies with sealed respirometers (~330 mL). Respirometers were filled with filtered seawater (1.0 μm) from either A–CO_2 or H–CO_2 treatments and maintained at mean (±SE, n = 32) temperature of 27.7 ± 0.2 °C using a water bath and a heater and chiller. Water motion within the respirometers was provided by a magnetic stir bar, with a mean (± SE, n = 17) flow rate near the center of the respirometer of 5.19 ± 0.31 cm s^{-1} as determined by photographing hydrated *Artemia* eggs (Sebens and Johnson 1991). Prior to quantifying respiration, corals were placed in darkness for 30 min to reduce stimulatory effects of light and photosynthesis on animal respiration (Edmunds and Davies 1988). Following dark acclimation, colonies were maintained in darkness and respiration rates measured on two individual colonies concurrently using duplicate respirometers. Final rates of oxygen (O_2) flux were corrected for changes in O_2 in respirometer controls (n = 4 $treatment^{-1}$) filled with filtered seawater alone and maintained under identical conditions.

Respiration rates were determined by measuring the change in O_2 saturation of seawater using an optrode (FOXY-R, 1.58 mm diameter, Ocean Optics, Dunedin, Florida, USA) calibrated against water-saturated air (e.g., 100% saturation) at ~27.5 °C and a zero-percent solution of sodium sulfite (Na_2SO_3) and 0.01 mol L^{-1} sodium tetraborate ($Na_2B_4O_7$). The optrode was connected to a spectrophotometer (USB2000, Ocean Optics) logging O_2 saturation percent on a personal computer running Ocean Optics logging software (OOISensors, version 1.00.08, Ocean Optics). O_2 saturation percent

was converted to O_2 concentration (μmol L^{-1}) using tabulated values of gas solubility at known temperature and salinity [N. Ramsing and J. Gundersen at Unisense, http://www.unisense.com/Default.aspx?ID=1109, based on Garcia and Gordon (Garcia and Gordon, 1992)]. Respiration rates were determined by regressing nmol O_2 against time (nmol O_2 mL^{-1} h^{-1}) and standardizing to the area of the coral tissue (cm^2), and finally expressed as nmol O_2 cm^{-2} h^{-1}.

Symbiodinium Density and Protein Content

Following determination of excretion and respiration rates, corals were frozen at -80 °C for 24 – 48 hrs until processed for *Symbiodinium* and total protein. Corals were processed by removing tissue from the skeleton using an airbrush (Iwata-Medea, Inc., Oregon, USA) filled with filtered seawater (1.0 μm). The total volume of the coral slurry ranged from 10.0 – 37.5 mL. The slurry was homogenized for 15 s using a homogenizer (Polytron PT2100, Kinematica, New York, USA), vortexed, and two aliquot (0.8 mL and 1.0 mL) removed for analysis of *Symbiodinium* density and total protein content, respectively. *Symbiodinium* samples were preserved in 10% formalin and total protein samples were frozen at -80 °C until further processing.

Symbiodinium density of homogenized coral tissue samples was determined by adding 10 μL of the formalin-preserved coral slurry to 90 μL of phosphate buffered saline (PBS) buffer (1x strength, Invirogen, CA, USA) with cell density (cells mL^{-1}) using a hand-held automated cell counter (Sceptor 2.0, Millepore Corporation, MA, USA). The cell counter provided a histogram of cell density by cell diameter and allows for density

to be determined by programming a cell diameter range to be counted. Based on the range of *Symbiodinium* sizes found in scleractinians (Wilkerson et al. 1988), cells between 5.99 – 10.18 μm diameter were counted as *Symbiodinium*. Cell densities determined by the cell counter were checked against counts by microscopy using a hemocytometer, revealing a < 4.0% difference between the two methods.

Total protein content of the coral slurry was performed using a bicinchoninic acid (BCA) assay using a Pierce® BCA Protein Assay Kit (Pierce Biotechnology, Illinois, USA). The BCA assay involves the reduction of Cu^{+2} to Cu^{+1} by protein oxidation. In an alkaline medium, a chelate complex of copper (Cu^{+1}) and protein (peptides with > 3 amino acid residues) is formed, which reacts with BCA to form a BCA/Cu^{+1} complex, developing a color, which can be analyzed spectrophotometrically (Smith et al. 1989). Tissue samples were removed from -80 °C and kept on ice until samples returned to a liquid state and vortexed extensively. To ensure lysing of cells in the homogenized extract, an aliquot from each tissue sample (150 μL) was added to a buffer/protease inhibitor solution [3.0 ml RIPA buffer (Radio-Immunoprecipitation Assay) consisting of 50 mM TRIS buffer, 1% NonidetP40, 0.25% Na-deoxycholate, 150 mM NaCl, and 0.12 mL protease inhibitor (4% of total volume)] at a 2:3 sample : buffer ratio while on ice. Samples were vortexed and sonicated on ice for 5 min and left on ice for an additional 20 min; samples were subsequently centrifuged at 1500 x *g* for 5 min at 4 °C to remove any remaining debris. An aliquot of the protein extract (25 μL) was added to 200 μL of reagent dye (alkaline BCA solution and cupric sulfate, Pierce Biotechnology) and incubated for 30 min at 37 °C. After 30 min, protein samples were analyzed

spectrophotometrically at 562 nm using a 96-well plate reader (H4 Hybrid Reader, Biotek, Vermont, USA) and total protein concentration (mg protein mL^{-1}) determined using a standard curve prepared from BSA. Final total protein concentrations were expressed as mg total protein per surface area of coral tissue (cm^2).

Statistical analysis

Ammonium excretion and respiration, O:N ratio, *Symbiodinium* density, and protein content were compared among treatments using a two-way ANOVA with pCO_2 (465 versus 891 μatm) and inhibitor treatment (inhibitor versus controls) as fixed factors. To test for effects of the EtOH carrier used to solubilize DCMU, control corals were compared to EtOH-controls in a separate two-way ANOVA with CO_2 and inhibitor treatment (control versus EtOH-control) as fixed factors. Physical and chemical conditions of treatments were analyzed by one-way ANOVA with pH_T, pCO_2, temperature and DIC parameters as fixed factors. The homoscedacity and normality assumptions of ANOVA were tested by graphical analyses of residuals.

Results

Treatment conditions

Conditions within the pCO_2 treatment were regulated precisely. Tanks received a mean intensity of photosynthetically active radiation of 264 ± 5 μmol photons m^{-2} s^{-1} and were maintained at a salinity of 32.03 ± 0.31 (pooled across time and tanks; ± SE n = 98 and 36, respectively). Temperature did not differ between treatments ($F_{1,95}$ = 0.535, P = 0.466) and ranged from 27.50 to 27.62 °C in the two tanks (Table 1). Seawater chemistry

of the two CO_2-treatments differed in pH_T and all DIC parameters ($F_{1,24} = \geq 92.015$, $P < 0.001$), but TA was not different between treatments ($F_{1,24} = 0.176$, $P = 0.679$). The final values for pCO_2 treatments contrasted ambient conditions in laboratory seawater (465 μatm; A–CO_2) with CO_2-enriched seawater (885 μatm; H–CO_2) (Table 1).

Effect of EtOH carrier

Corals treated with the ethanol carrier (< 0.05% EtOH) and used as EtOH-controls were statistically indistinguishable from control corals incubated in seawater alone ($P > 0.100$). There was no effect of EtOH on the rate of ammonium excretion ($F_{1,7} = 3.151$, $P = 0.119$) or respiration rates ($F_{1,6} = 0.0013$, $P = 0.972$), total protein content ($F_{1,8} = 0.289$, $P = 0.606$), or *Symbiodinium* density ($F_{1,8} = 3.334$, $P = 0.105$), and no interaction of pCO_2 by EtOH-carrier treatment was observed ($P > 0.095$). Therefore, control corals ($n = 3$ CO_2-treatment^{-1}) were directly compared to corals incubated in 10 μM DCMU ($n = 3$ CO_2-treatment^{-1}) in the subsequent analyses of dependent variables.

NH_4^+ Excretion

Corals treated with 1 mM azaserine developed tissue necrosis within 5 hrs of treatment, and 100% mortality was observed within 12 h. Serial dilutions of the inhibitor (0.5, 0.25, 0.10, 0.050 mM) did not amend these symptoms. Following the first day of azaserine treatment, this treatment was dropped from the experiment and only a DCMU inhibitor treatment applied thereafter.

Juvenile corals across pCO_2- and DCMU-treatments (e.g., control vs. 10 μM DCMU) showed no visible signs of stress over the 24 h pre-incubation. NH_4^+ concentration in filtered seawater (0.45 μm) ranged from 0.823 – 4.975 μmol NH_4^+ L^{-1}. Over the 3 h excretion period, the [NH_4^+] in control beakers with seawater alone was reduced by 0.012 – 0.942 μmol NH_4^+ L^{-1}, equivalent to a mean reduction of [NH_4^+] (± SE, n = 6) of 15.00 ± 0.09 %. In total, dependent variables were measured for six corals from the A–CO_2 and H–CO_2 treatments were over the 3 d processing period, with three corals from each CO_2-treatment being allocated to DCMU+ and uninhibited control incubations.

Analysis of pCO_2 and inhibitor treatments revealed an effect of pCO_2 ($F_{1,8}$ = 11.220, P = 0.010) on NH_4^+ excretion, but no effect of DCMU ($F_{1,8}$ = 0.848, P = 0.384) or a pCO_2 by DCMU interaction ($F_{1,8}$ = 0.315, P = 0.590) (Figure 1). Regardless of DCMU treatment, corals from the A–CO_2 treatment showed a net uptake of NH_4^+ over the 3 h experiment, with net uptake ranging from 2.89 nmol – 13.29 nmol NH_4^+ cm^{-2} h^{-1}. Conversely, corals from the H–CO_2 treatment showed a net release of NH_4^+ ranging from 5.01 – 60.64 nmol NH_4^+ cm^{-2} h^{-1}. However one H–CO_2 coral in the control treatment showed a net uptake of 2.83 nmol NH_4^+ cm^{-2} h^{-1}. Mean rates of NH_4^+ uptake in A–CO_2 corals ranged from 10.72 ± 0.75 nmol to 6.84 nmol NH_4^+ cm^{-2} h^{-1} (control vs. DCMU+), while mean rates of net excretion in H–CO_2 corals ranged from 19.41 ± 13.83 nmol to 35.43 ± 16.27 nmol NH_4^+ cm^{-2} h^{-1} (control vs. DCMU+) (Table 2). Although treatment with 10 μM DCMU decreased mean values for net NH_4^+ uptake in A–CO_2 corals and increased net NH_4^+ excretion in H–CO_2 corals these differences were not significant (Table 2).

Mean respiration rates across pCO_2 and DCMU treatments ranged from 310 ± 49 nmol O_2 cm^{-2} h^{-1} (A-CO_2, control) to 475 ± 28 nmol O_2 cm^{-2} h^{-1} (HCO_2, control) with a trend for higher respiration in H–CO_2 versus A–CO_2 corals (Figure 1). However, respiration was not affected by pCO_2 ($F_{1,8}$ = 4.452, P = 0.068) or DCMU ($F_{1,8}$ = 3.220, P = 0.110) or the interaction of the two ($F_{1,8}$ = 0.112, P = 0.746). Mean protein content in corals across pCO_2 and DCMU treatments ranged from 0.215 ± 0.009 mg cm^{-2} (A–CO_2, DCMU+) to 0.273 ± 0.039 mg cm^{-2} (A–CO_2, control), and these values were not affected by pCO_2 ($F_{1,8}$ = 0.432, P = 0.530), DCMU ($F_{1,8}$ = 0.533, P = 0.486) or the interaction of the two ($F_{1,8}$ = 3.195, P = 0.112) (Table 2). Mean *Symbiodinium* densities ranged from 1.83 ± 0.24 × 10^6 cells cm^{-2} (A–CO_2, DCMU+) to 2.60 ± 0.51 × 10^6 cells cm^{-2} (H–CO_2, DCMU+) across treatments (Table 2). However there was no effect of pCO_2 ($F_{1,8}$ = 0.979, P = 0.352), DCMU ($F_{1,8}$ = 0259, P = 0.625) or the interaction between pCO_2 and DCMU ($F_{1,8}$ = 1.785, P = 0.218).

Discussion

In the present study, I exposed juvenile *Seriatopora caliendrum* for 14 – 16 d to 465 and 885 μatm pCO_2 at a 27.56 °C to test the hypothesis that ocean acidification leads to metabolic depression and changes in ammonium excretion. In accomplishing this goal, I utilized two pharmacological inhibitors (e.g., 1 mM azaserine, 10 μM DCMU) to prevent ammonium recycling by *Symbiodinium* and induce ammonium excretion in intact coral colonies, after which rates of dark respiration were determined as an estimate of aerobic metabolism. Total protein content and *Symbiodinium* density were compared

among corals to test the hypothesis that ammonium excretion is related to *Symbiodinium* density and that high ammonium excretion rates result in reduced total protein content. among inhibitor and pCO_2 treatments. Azaserine resulted in coral mortality and was not an effective inhibitor in the present system. DCMU-treated corals remained healthy, although 10 μM DCMU did not fully inhibit ammonium uptake by *Symbiodinium*. Therefore, uninhibited corals (e.g., controls) were not statistically different from inhibited corals (e.g., DCMU+) across all dependent variables.

Results from my study revealed ammonium excretion in *S. caliendrum* to be increased under 885 μatm pCO_2 compared to control corals at 465 μatm pCO_2. However, pCO_2 did not affect *Symbiodinium* density (cells cm^{-2}) or protein content (mg cm^{-2}). There was a trend for high-pCO_2 to increase respiration compared to control corals, although this trend was not significant. These results suggest that low-pH and high-pCO_2 may increase the rates of ammonium excretion in reef corals by affecting: (1) protein catabolism or amino acid deamination; (2) the ability for *Symbiodinium* to recycle ammonium, or (3) increasing the use of ammonium as an intracellular pH-buffer that functions to remove acid (e.g., H^+) from the coral tissue (Lindinger et al. 1984; Pörtner 1987). Results in the following sections will be discussed in the context of the aforementioned hypotheses.

Effectiveness of Ammonium Uptake Inhibitors

The earliest studies of ammonium excretion in symbiotic corals used prolonged darkness ranging from 8 – 96 hrs to induce excretion (Kawaguti 1953; Muscatine and

D'Elia 1978; Rahav et al. 1989; Szmant et al. 1990). The use of pharmacological agents has been effective to induce ammonium excretion in symbiotic anthozoans (Anderson and Burris 1987) and hydrozoans (Rees 1987; McAuley 1995), however pharmacological agents have not been extensively researched in scleractinians (Rahav et al. 1989). The most extensive study of ammonium metabolism and the application of pharmacological agents to *Symbiodinium* ammonium recycling in intact scleractinians was performed by Rahav *et al.* (1989). The effectiveness of ammonium uptake inhibition was tested using azaserine (1 mM), DCMU (10 μM), methionine sulphoxamine (100 μM; an inhibitor of glutamine synthetase), and darkness (24, 48, 72 hrs) using intact *Stylophora pistillata* colonies (10 – 15 cm diameter). Azaserine, DCMU, and darkness (72 hrs) were all equally effective, and ammonium excretion was consistent across the three methods. While methionine sulphoxamine was an effective inhibitor of ammonium uptake in *Pocillopora damicornis* (500 μM; Anderson and Burris 1987) and the green hydra-*Chlorella* symbiosis (200 μM; Rees 1987), 100 μM methionine sulphoxamine resulted in tissue disintegration in *S. pistillata* (Rahav et al. 1989). Similarly, corals in the present study exposed to 0.05 – 1.0 mM azaserine developed tissue necrosis and disintegration. Conversely, while 10 μM DCMU did not negatively affect the corals or lead to a reduced concentration of *Symbiodinium* as in Rahav *et al.* (1989), DCMU did not inhibit ammonium uptake in intact corals, a fact clearly observed through ammonium uptake in corals at 465 μatm pCO_2. The disparity in responses of intact corals exposed to pharmacological agents used for the inhibition of ammonium uptake suggests further research may be required to develop a more reliable methodology and effective

concentrations of inhibitors for inducing complete inhibition of ammonium recycling by *Symbiodinium in hospite*.

Effects of pCO_2 on Aerobic Metabolism

Aerobic metabolism was not affected by pCO_2 (465 versus 885 μatm pCO_2). Past studies have reported mixed effects of pCO_2-erichment on reef corals. pCO_2-enrichment to 1184 μatm had no affect on area-normalized dark respiration in *Porites lobata* or *Acropora intermedia* (Anthony et al. 2008). Likewise, dark respiration in *Stylophora pistillata* was not affected by 760 μatm pCO_2 when normalized to protein (Reynaud et al. 2003), or by 2039 μatm pCO_2 when normalized to area (cm^{-2}) or chlorophyll *a* (μg) (Godinot et al. 2011). Crawley *et al.* (2010) reported dark respiration in nubbins of *Acropora formosa* to be unaffected by pCO_2 (360, 695, 1155 μatm pCO_2), but post-illumination respiration (e.g., light-enhanced dark respiration) was stimulated by high-pCO_2 (695 versus 1155 μatm) although were not significantly different from controls (360 μatm pCO_2). However, studies using massive *Porites* spp. have shown area-normalized dark respiration rates to be unaffected by pCO_2 at 756 μatm pCO_2, yet 861 μatm pCO_2 led to 36% reduction compared to controls (423 μatm pCO_2) (Edmunds 2012).

It is difficult to make broad conclusions regarding the affects of OA on coral respiration due to the covariance of seawater chemistry parameters under OA conditions (e.g., pH, pCO_2, DIC) and the small number of studies that have tested for such effects. Moreover, physiological data for other marine invertebrates exposed to elevated pCO_2

also reveals disparate trends. Aerobic respiration increased with increasing pCO_2 (280 – 1,020 μatm) in the Arctic pteropod *Limacina helicina* under elevated temperature (0 ° versus 4 °C) (Comeau et al. 2010), while low extracellular pH (pHe) (7.2 and 7.6 versus 8.0) reduced respiration in the sediment-dwelling sipunculid *Sipunculus nudus* (Langenbuch and Pörtner 2002). Alternatively, respiration was reduced in *Mytilus galloprovincialis* exposed to pH 7.3 and ~5,206 μatm pCO_2 (Michaelidis et al. 2005), while respiration exhibited a parabolic response to hypercapnia in *M. edulis*, increasing from 385 – 2,398 μatm pCO_2 and decreasing at 3,997 μatm pCO_2 (Thomsen and Melzner 2010). Resolving changes in respiratory rates in corals (and other marine organisms) exposed to OA are further complicated by different exposure durations, characteristics of the corals, and the conditions in which respiration is measured. The aerobic demand in small corals (e.g., nubbins, juveniles, or recently settled recruits) can be affected by biomass, temperature, and water motion (Edmunds 2005), as well as, light history (Edmunds and Davies 1988). While the trend from laboratory-based studies suggests OA does not affect respiration in scleractinians, it is premature to suggest this trend is consistent across all taxa of reef corals considering the small fraction that have been used in OA experiments (~25 of 794 species; Veron 2000; Erez et al. 2011). Further research is needed to resolve the variance in this trend and to determine whether the interaction between OA and temperature may alter aerobic respiration (and other physiological processes) as occurs in other marine invertebrates.

Effects on pCO_2 on Ammonium Excretion

pCO_2 had a strong affect on the ammonium excretion of juvenile *S. caliendrum*. After 14 – 16 d at 885 μatm pCO_2, *S. caliendrum* excreted ammonium, while corals exposed to 423 μatm pCO_2 were observed to uptake ammonium in seawater. This trend that was consistent across the three days of excretion measurements and was observed in control corals, as well as corals treated with the photosynthetic electron transport inhibitor DCMU. However, total protein content and *Symbiodinium* densities were not different among corals at 423 or 885 μatm pCO_2. The null effect of pCO_2 on protein content or *Symbiodinium* density is agreement with Reynaud *et al.* (2003) and Godinot *et al.* (2011) using *Stylophora pistillata* (combined 378 – 2039 μatm pCO_2). Similarly, pCO_2 (804 μatm pCO_2) had no effect on *Symbiodinium* density in juvenile massive *Porites* spp. (Edmunds 2012) or protein content in the temperature coral *Cladocora caespitosa* (700 μatm pCO_2; Rodolfo-Metalpa et al. 2010).

In the present study, ammonium was excreted from control corals at 885 μatm CO_2. A parsimonious explanation of ammonium excretion under these conditions, and the apparent cessation of ammonium uptake by *Symbiodinium* may be that host ammonium excretion outpaced symbiont ammonium uptake. However, this hypothesis seems unlikely considering *Symbiodinium* are efficient ammonium assimilators and utilize excess ammonium to synthesize new biomass leading to increased algal densities *in hospite* (Hoegh-Guldberg and Smith 1989). Using *Acropora* sp. and *Porites lutea*, Muscatine and D'Elia (1978) exposed intact corals to 7.0 μM NH_4^+ and determined the coral holobionts were capable of removing ~3 – 5 μM NH_4^+ within 1 h. Additionally,

Pocillopora capitata sustained ammonium uptake rates of ~0.1 – 0.5 μM NH_4^+ h^{-1} in a series of diel incubations over 24 hrs (Muscatine and D'Elia 1978). In the present study, the uptake of ammonium from seawater calculated from ammonium uptake in control corals from A–CO_2 treatments (Table 2) is 11 nmol NH_4^+ cm^{-2} h^{-1}, approximately half the NH_4^+ uptake rate reported for intact *Stylophora pistillata* colonies (Rahav et al. 1989). However, when calculated as ammonium flux per hour, rates of ammonium uptake in the present study are 0.13 – 0.17 μM NH_4^+ h^{-1}, which are within the lower range of values found for *Pocillopora capitata* (Muscatine and D'Elia 1978). Interestingly, excretion rates of corals at H–CO_2 in the current study were 2 – 3 times those observed by Rahav *et al.* (1989), who reported excretion rates of 8.7 – 12.4 nmol nmol NH_4^+ cm^{-2} h^{-1} for corals exposed to various inhibitors of NH_4^+ uptake (e.g., azaserine, DCMU, darkness) under A–CO_2. However, colonies used by Rahav *et al.* (1989) were 10 – 15 cm in diameter and had a respiration rate of 400 – 600 nmol O_2 cm^{-2} h^{-1}, whereas corals in the present study were ≤ 4-cm in diameter and had respiration rates of 310 – 475 nmol O_2 cm^{-2} h^{-1}. Unfortunately, there are few published reports of ammonium excretion rates in scleractinians, and these studies often present data with disparate data normalization methods (e.g., ammonium flux per chlorophyll *a,* tissue biomass, area, time, or no normalization) making comparison of their data to data in the present study problematic (Kawaguti 1953; Muscatine and D'Elia 1978; Szmant et al. 1990). In the present study, corals exposed to 423 μatm CO_2 displayed similar area-normalized respiration rates for corals found in the literature (Edmunds and Davies 1989; Schneider et al. 2009), whereas area-normalized ammonium excretion rates were in the upper range of values reported for scleractinians (Rahav et al. 1989). While evaluating the efficacy of ammonium uptake

and the saturation kinetics of ammonium uptake enzymes in *Symbiodinium* was beyond the scope of this study, determining host and symbiont ammonium uptake and excretion rates under OA conditions should be a topic for future research.

The lack of ammonium uptake by *Symbiodinium* at 885 μatm pCO_2 conflicts with findings of Godinot *et al.* (2011) who reported no affect of pCO_2 (378 vs. 908 vs. 2039 μatm) on the uptake of ammonium, nitrate, or phosphate in *Stylophora pistillata.* Additional information on the three enzymes involved in the uptake of ammonium (e.g., GDH, GS, GOGAT) reveals that the low-pH in the present study (pH_T 7.78) or as used by Godinot et al. (2011) (pH_T 7.46 versus 7.78 versus 8.09) would not negatively affect the function of these enzymes, as their activity is typically stimulated by low-pH (Godinot et al. 2011). The optimum pH for GDH, GS and GOGAT activity in the actinobacteria *Corynebacterium callunae* and cyanobacteria *Phormidium laminosum* is 7.2 – 7.5 (GDH), 7.0 (GS) and 7.6 (GOGAT) (Martinez-Bilbao et al. 1988; Ertan 1992). GDH isolated from corals shows a pH optimum at 7.3 for the amination of α-ketoglutarate and 8.1 for the deaminating reverse reaction forming glutamate and NH_4^+ (Rahav et al. 1989). However, Venn *et al.* (2009) using *Stylophora pistillata* reported the internal pH was much more acidic than the external pH of seawater (8.1). In the light, animal cells containing symbiont algae maintained an intracellular pH of 7.41, whereas in the dark this dropped to 7.13 (Venn et al. 2009). Furthermore, regions of low-pH (< 6.0) were found directly adjacent to the algal symbionts (Venn et al. 2009). Therefore, it is difficult to infer that GDH and GS/GOGAT were in the present study were maintained under optimal or suboptimal pH ranges based solely on measurements of the pH of seawater

from the treatment tanks. However, Godinot *et al.* (2011) reported an experimental treatment of pH_T 7.46 had no affect on ammonium assimilation. Contrary to Godinot *et al.* (2011), my results indicate that the ability of *Symbiodinium* to effectively recycle ammonium may have been affected by high-pCO_2 (885 μatm) due to the clear loss of ammonium in uninhibited control corals in H–CO_2 treatments. However, more research is needed to test the hypothesis that OA affects ammonium assimilation or nutrient uptake in reef corals.

Protein and amino acids are not considered to be an energy source preferentially catabolized to fuel aerobic metabolism in metazoans, including scleractinians (Rahav et al. 1989; Szmant et al. 1990). High O:N ratios (e.g., moles of oxygen respired to moles nitrogen excreted) indicate corals have less reliance on protein or amino acid metabolism, whereas O:N ratios of ~8 indicate high reliance on amino acid catabolism (Szmant et al. 1990; Langenbuch and Pörtner 2002). Szmant *et al.* (1990) reported mean O:N values of 135 – 3,221 for *Montastrea annularis* and 80 – 368 for *Acropora cervicornis*, indicative of metabolism of carbohydrate and lipids a have low reliance on amino acid and protein metabolism (Szmant et al. 1990). However, excretion in the aforementioned study was induced by 20 – 36 hrs of darkness, and the author notes high variance in excretion rates due to excretion values near zero, suggesting ammonium recycling remained functional. Additionally, Szmant *et al.* (1990) noted large variance among individuals in tissue-specific respiration and excretion rates, potentially as an affect of temporal differences in colony characteristics (e.g., tissue biomass) including conducting experiments in summer 1986 and spring 1987 (Szmant et al. 1990). Rates of protein catabolism or ammonium

excretion show significant inter- and intra-specific variance among organisms. These disparities can arise from differences in tissue biomass, energetic status, heterotrophic feeding, and the density of *Symbiodinium* (Kawaguti 1953; Szmant-Froelich and Pilson 1984; Szmant et al. 1990). Furthermore, increased stress, prolonged starvation, or changes in tissue biomass can also affect the degree of protein catabolism (Szmant et al. 1990; Quetin et al. 1980). In the present study, it was not possible to calculate the O:N ratio for A–CO_2 corals due to a net uptake of NH_4^+, however the O:N ratio for H–CO_2 corals was ~7 – 21 (Table 2). While it is not possible to compare these values to corals at 423 μatm pCO_2, O:N values for *S. caliendrum* at 885 μatm pCO_2 suggest utilization of amino acid catabolism under OA conditions.

Another potential explanation for the increased ammonium excretion despite no change in total protein content may be found in the conditions under which the present experiment was performed. A previous study of juvenile *S. caliendrum* (Thesis: *Chapter II*) conducted July 2011 using corals collected from the identical location as the present study showed the photon capture and electron turnover through PSII in algal photosynthesis to be saturated (e.g., saturation irradiance I_k) at ~129 – 172 μmol photons m^{-2} s^{-1} (data not shown). Therefore corals in the present study held under a photosynthetically active irradiance of 263 μmol photons m^{-2} s^{-1} were likely receiving sufficient PAR exposure to maintain high levels of carbon fixation and the coral host was likely not deficient in autotrophic carbon. Additionally, during a portion of the experiment, [NH_4^+] in the seawater was > 5-times higher than [NH_4^+] normally detected in seawater from NMMBA (~5.0 μM vs. 1.0 μM NH_4^+) due to a typhoon providing high

influx of terrigenous runoff. Therefore, corals and *Symbiodinium* may have been replete in both light for photosynthesis and nitrogen for tissue production or catabolism. *Symbiodinium in hospite* are able to facultatively utilize increased ammonium content to increase chlorophyll content, *Symbiodinium* density, and increase photosynthetic rates (cm^{-2}) (Hoegh-Guldberg and Smith 1989). Potentially, an increased photosynthetic performance could have resulted in more metabolites (e.g., amino acids, glycerol) translocated to the coral to provide energy for cellular maintenance and metabolism, or allowed for increased storage of metabolites within the coral tissues in the form of carbohydrates, protein, lipids, or amino acids.

Together, a combination of higher-than-normal [NH_4^+] and potential stimulation of photosynthetic performance in all corals from A–CO_2 and H–CO_2 treatments may have contributed to a surplus of energy for the coral holobiont. The nutritional status of the organism (e.g., starved, nutrient replete) can affect how an organism responds to stress (Quetin et al. 1980). Additionally, high rates of protein turnover (e.g., continual breakdown and synthesis of intracellular proteins) and selective use of amino acids for catabolism (Langenbuch and Pörtner 2002) may have masked effects of pCO_2 on protein content resulting in apparent no net change in protein content among A–CO_2 and H–CO_2 corals. Reduced extracellular pH (7.2 and 7.6 versus 8.0) led to the preferential catabolism of monoamino dicarboxylic acids and their amines (e.g., asparagine, aspartate, glutamic acid, glutamate) in the sipunculid *Sipunculus nudus*, interpreted as the preferential catabolism of dicarboxylic amino acids to produce more bicarbonate to increase the capacity for intracellular pH-buffering (Langenbuch and Pörtner 2002). In

corals, high-pCO_2 or a reduced extracellular pH may lead to changes in rates of protein metabolism or the catabolism of amino acids as a result of additional cellular requirements (e.g., pH-regulation, ion pumping) required to compensate for OA (Pörtner 2008). However, protein metabolism and turnover rates in corals have yet to be fully determined (Gates and Edmunds 1999), and examining pCO_2 effects on amino acid catabolism and protein turnover were beyond the scope of the present study.

Finally, the increased rates of excretion in corals exposed to 885 μatm pCO_2 is difficult to resolve considering ammonium recycling by the *Symbiodinium* classically have resulted in ammonium uptake and no measurable excretion rates in corals under ambient conditions (Muscatine and D'Elia 1978; Szmant et al. 1990; Rahav et al. 1989). Additionally, respiration rates were not affected by pCO_2 treatment, therefore it is not possible to reconcile net ammonium excretion as an effect of increased oxygen consumption (e.g., inferred increases in BMR). However, resolving changes in metabolic rates that would give rise to increase ammonium excretion may have been precluded due to low replication and low statistical power. In the present study, the GS/GOGAT enzymes primarily used by *Symbiodinium* to take up ammonium from the coral and the external seawater may have been impaired at 885 μatm pCO_2, resulting in more NH_4^+ being retained in the external seawater. Alternatively, corals exposed to OA at 885 μatm pCO_2 had higher rates of NH_4^+ excretion and may indicate increased deamination of amino acids or protein catabolism that may indicate changes in the costs of metabolism, costs of regulating intracellular pH relative to extracellular pH, or the use of ammonium to buffer against changes in intracellular pH (Pörtner 1987). Under high-pCO_2 increased

amino acid deamination may have increased the capacity for acid-base regulation through the production on ammonia, which under low-pH conditions acts as a base covalently bonding to protons which can then be transported to the extracellular environment as the conjugate acid ammonium (Pörtner 1987). Ammonia (NH_3) can move freely across cellular membranes as a neutrally charged molecule, however NH_4^+ requires transporters to move across cell membranes (Davy et al. 2012). In this study, a lower extracellular pH in the external seawater (pH_T 7.78 versus 8.02) may have resulted in increased ammonia production to buffer the intercellular pH (Pörtner 1987), removing protons from intracellular tissues and being transported to the outside medium (Lindinger et al. 1984). However, further research is required to test the hypothesis that ammonia/ammonium excretion is an effective pathway of acid removal or pH-buffering in reef corals.

Conclusions

The affects of high-pCO_2 and OA on the metabolism of reef corals are poorly understood. The few studies that have investigated OA-effects on aerobic metabolism have focused upon respiration rates, although there may be other factors of metabolism may be affected by pCO_2. In this study, I found that elevated pCO_2 (885 μatm pCO_2) led to increased ammonium excretion rates and a trend for higher respiration rates in juveniles *S. caliendrum* corals. Additionally, the observed excretion in coral at 885 μatm pCO_2 in the absence of an ammonium uptake inhibitor suggests the ability for *Symbiodinium* to recycle ammonium may have been impaired under OA conditions. To data, few studies have quantified the excretion rates of corals and furthermore, no study has investigated OA-effects on ammonium metabolism in reef corals. The tropical seas

are nutrient poor environments (Muscatine and Porter 1977), therefore should OA reduce nitrogen conservation in scleractinians and *Symbiodinium,* corals may be deleteriously affected. Alternatively, should ammonium excretion prove an effective means for pH-buffering in scleractinians, increased ammonium excretion may represent a strategy for acclimating to OA. In this regard, to elucidate the affects of OA on the metabolism of reef corals future OA research should consider the affects of OA on ammonium uptake and excretion, amino acid and protein catabolism, and mechanisms of pH-regulation in scleractinians.

Table 1. Summary of physical and chemical conditions for two CO_2-treatments maintained between 9 June and 24 June 2012. Seawater chemistry was assessed daily at 9:00 hrs with pH and carbonate chemistry calculated using CO2SYS (n = 13 for all variables). Values displayed are mean ± SE, except where † = SE < 0.1; TA = total alkalinity; Ω_{arag} = aragonite saturation state; A–CO_2 = Ambient pCO_2; H–CO_2 = High pCO_2.

Treatment	T (°C)	pH_{total} †	TA (μmol kg^{-1})	pCO_2 (μatm)	HCO_3^- (μmol kg^{-1})	CO_3^{2-} (μmol kg^{-1})	Ω_{arag}
A–CO_2	27.50	8.02	2205 ± 14	465 ± 5	1751 ± 12	186 ± 2	3.04 ± 0.03
H–CO_2	27.62	7.78	2213 ± 15	885 ± 14	1922 ± 13	119 ± 1	1.94 ± 0.02

Table 2. Comparison of excretion, respiration, protein content, *Symbiodinium* spp. density and oxygen:nitrogen ratio for the juvenile reef coral *Seriatopora caliendrum* exposed for 14–16 d to ambient- or CO_2-enriched conditions and 24 hrs exposure to inhibitor treatments: control (DCMU-) or 10 μM DCMU (DCMU+). Negative values for excretion represent net NH_4^+ uptake, whereas positive values for represent net NH_4^+ excretion. Values are mean ± SE, n = 3; except * mean ± SD, n = 2. Treatments are described in *Table 1*; A–CO_2 = 465 μatm CO_2; H–CO_2 = 885 μatm CO_2; DCMU = (3-(3,4-Dichlorophenyl)-1,1-dimethyl urea).

Treatment	excretion nmol NH_4^+ cm^{-2} h^{-1}	respiration nmol O_2 cm^{-2} h^{-1}	protein mg cm^{-2}	*Symbiodinium* cells cm^{-2}	*O:N ratio mol O: mol N
A–CO_2 control	-10.72 ± 0.75	372 ± 61	0.273 ± 0.039	2.44 ± 0.34 x 10^6	NA
A–CO_2 DCMU+	-6.84 ± 3.26	310 ± 49	0.215 ± 0.009	1.83 ± 0.24 x 10^6	NA
H–CO_2 control	19.41 ± 13.83	475 ± 28	0.247 ± 0.019	2.32 ± 0.07 x 10^6	21.19 ± 14.38
H–CO_2 DCMU+	35.43 ± 16.27	385 ± 15	0.271 ± 0.012	2.60 ± 0.51 x 10^6	7.19 ± 1.82

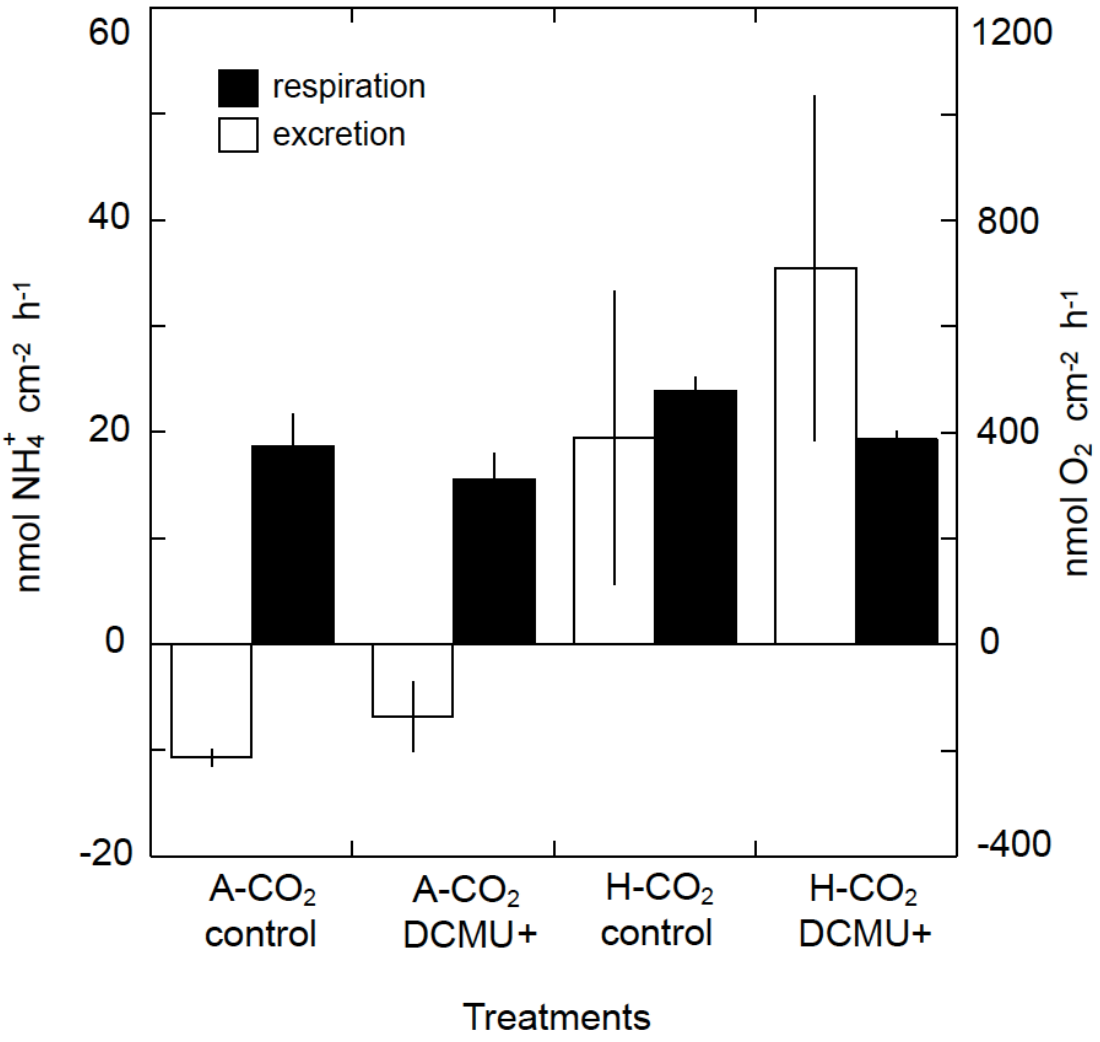

Figure 1. Comparison of excretion and respiration rates in juvenile *Seriatopora caliendrum* exposed to 14-16 d of ambient-CO_2 (A–CO_2; 465 μatm) or enriched-CO_2 (H–CO_2; 885 μatm) treatments and 24 h exposure to inhibitor treatments: control or 10 μM DCMU (DCMU+). Excretion (*left ordinate*) and respiration (*right ordinate*) are standardized to surface area (cm^2). Excretion was affected by pCO_2 (P = 0.010) but not DCMU (P = 0.384); respiration was not affected by pCO_2 (P = 0.068) or DCMU treatment (P = 0.110). Values are mean ± SE, n = 6 for all parameters; DCMU = (3-(3,4-Dichlorophenyl)-1,1-dimethyl urea).

Chapter 5

Conclusion

The progressive acidification and perturbation of carbonate chemistry occurring across the world's oceans is unprecedented in our planet's history (Kerr 2010; Zeebe and Ridgwell 2011) and is predicted to negatively affect marine organisms, particularly tropical reef corals (Kroeker et al. 2010). However, in the case of reef corals, as the number of coral species studied increases to include corals of different morphologies and life-stages (e.g., larvae, newly settled recruits, juveniles, adults) it now appears that OA effects on calcification may be more variable than previously thought (Pandolfi et al. 2011). However, the affects of OA on coral metabolism remain inconclusive. The response of reef corals to environmental stress can be affected by exposures to physical conditions (e.g., irradiance, temperature, water motion) and can show species-specific responses, as well as disparate spatial- and temporal-responses (Dennison and Barnes 1988; Gattuso et al. 1999; Fitt et al. 2000; Edmunds et al. 2011). In the past, OA studies have utilized several methods for manipulation of carbon chemistry and pH (e.g., acid addition, CO_2-bubbling), differences in exposure duration to OA-treatments (e.g., days versus months), and varying physical conditions (especially irradiance), which have likely contributed to conflicting trends among studies. While the techniques to manipulate seawater chemistry and the analytical methods to evaluate the results have become more standardized in recent years (Dickson et al. 2007), there is a need for experiments of longer duration that are modeled after ecologically relevant exposures of physical conditions (Dufault et al. in press; Dufault et al. 2012; Kline et al 2012).

The goal of my research was to examine the affects of OA on the bleaching, calcification, and metabolism of juvenile corals from Moorea, French Polynesia, and Checheng, Taiwan. Few studies have used intact juvenile colonies (≤ 4.0 cm diameter) in OA experiments (Edmunds 2012), however juvenile colonies represent an important component of the coral life cycle (Bak and Engel 1979) that ultimately affect the structure and establishment of adult coral population (Edmunds 2008). Using juvenile *Seriatopora caliendrum* from southern Taiwan, I examined the effects of OA alone and in combination with elevated temperature to test the hypothesis that OA results in coral bleaching (Chapter II). While OA has been reported to cause coral bleaching, to date, no study has corroborated this report or quantified OA bleaching or high-temperature bleaching under OA conditions with the parameters characteristic of bleaching studies (e.g., *Symbiodinium* and chlorophyll *a* density, photochemical efficiency). Subsequent (Chapter III), I used juvenile colonies of the coral massive *Porites* spp. in Moorea, French Polynesia, to experimentally evaluate the effects of OA on calcification, respiration, and the cost of calcification. The effects of OA on calcification have been a central focus to OA research, however few studies have exposed corals to pCO_2 treatments under ecologically relevant conditions as experiences on the reef. Finally in Chapter IV, using juvenile *S. caliendrum* in Taiwan, I examined the effects of OA on coral metabolism using aerobic energy respiration, nitrogen excretion, and total protein content as indicators of metabolism and stress induced by OA.

In Chapter II, elevated pCO_2 (840 μatm pCO_2) had no affect on coral bleaching under elevated temperatures (30.53 °C) and did not induce coral bleaching under ambient temperatures (27.65 °C). Moreover, high-pCO_2 had no affect on photochemical efficiency, photosynthesis, or *Symbiodinium* or photopigment concentration. While these findings contradict Anthony *et al.* (2008) who reported coral bleaching in adult corals after 8-week exposure to OA under natural irradiances, my findings agree with studies showing no affect of pCO_2 on photochemical efficiency (Godinot et al. 2011; Iguchi et al. 2011), which is a commonly used indicator of bleaching and pre-bleaching stress (Warner et al. 1999). Therefore my results suggest that OA does not cause bleaching or interact with temperature to exacerbate thermal bleaching in juvenile *S. caliendrum*, however more research is needed to determine whether these results are consistent among coral taxa and life-cycle stages (Godinot et al. 2011).

In Chapter III, juvenile *Porites* spp. were exposed to low-pH and elevated-DIC seawater within sealed respirometers and incubated *in situ.* Results revealed low-pH and high-DIC treatments affected calcification by increasing calcification rates under low-pH and elevated DIC (~1000 μmol kg^{-1} above ambient [DIC]). However treatments had no affect on respiration rates or the inferred energetic expenditure concurrent with calcification. These results suggest that massive *Porites* spp. may be resistant to changes in seawater chemistry associated with high-pCO_2 predicted for the end of the century (976 μatm pCO_2; Edmunds 2011, Fabricius et al. 2011), although the genus *Porites* exhibits conflicting calcification responses to OA (Edmunds 2011; 2012). Furthermore, while OA is hypothesized to make calcification thermodynamically unfavorable

(Allemand et al. 2011), elevated [DIC] may stimulate calcification by providing carbon for mineralizaton or by reducing competition for DIC between the photosynthesis and calcification (Herfort et al. 2008). Additionally, my study illustrates the feasibility of using enclosed chambers *in situ* for OA experiments.

In Chapter IV, high-pCO_2 (885 μatm pCO_2) increased ammonium excretion in *Seriatopora caliendrum*, but did not affect respiration, total protein content, or *Symbiodinum* density. Few studies have measured ammonium excretion in reef corals (Muscatine and D'Elia 1978; Rahav et al. 1989) and never in the context of OA. My results indicate that ammonium recycling in the coral holobiont may be affected by OA, either by affecting the capacity of *Symbiodinium* to take up nitrogen, or by increasing the rates of nitrogen excretion by the coral. Increased ammonium excretion under OA conditions may indicate changes in protein or amino acid metabolism associated with pH-buffering with bicarbonate ions, or alternatively, through the use of ammonium as a pH-buffer. However, protein and amino acid metabolism and pH-buffering in reef corals is poorly understood; elucidating these processes should be a priority for future research.

My experimental examination of the physiological responses of the juvenile corals *S. caliendrum* and massive *Porites* spp. indicate short-term exposures to OA can affect coral ammonium metabolism (*S. caliendrum*), however OA does not induce coral bleaching, interact with temperature to exacerbate bleaching, or reduce photosynthetic productivity (*S. caliendrum*). Additionally, my results suggest that OA does not increase the energetic expenditure concurrent with calcification or reduce rates of calcification

(*Porites* spp.) or respiration rates (*S. caliendrum* and *Porites* spp.). Together these findings suggest that juvenile and adult reef corals reported to be resistant to OA (e.g., *Porites*) may be well adapted to function under OA conditions, or alternatively, may possess mechanisms for acclimatizing to OA stress (Edmunds 2011; Fabricius et al. 2011). While these finding reinforces the perception of the genus *Porites* to be an ecological winner under environmental stress (sensu Loya et al. 2001), more research is needed to determine the long-term effect of OA coral community structure *in situ.* In this way, OA experiments need to incorporate ecologically relevant exposure of physical conditions to accurately determine OA effects on coral physiology and essential processes (e.g., settlement, growth, reproduction) *in situ.* Finally, my findings that ammonium metabolism is affected by OA may be attributed to direct affects of OA on metabolism or have resulted from preferential amino acid metabolism to increase pH-regulation (Lindinger et al. 1984; Langenbuch and Pörtner 2002). pH-regulation is hypothesized to contribute to OA resilience in marine calcifiers and may represent the key for adapting to OA and global warming (McCullough et al. 2012). Therefore, unraveling the effects of OA on coral metabolism and the capacity for corals to pH-regulate may provide insight into reported deleterious affects of OA on calcification and should be a priority for future research.

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