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Adaptive Tolerance to Ocean Acidification in the Marine Sponge: Chondrilla nucula

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ADAPTIVE TOLERANCE TO OCEAN ACIDIFICATION IN THE MARINE SPONGE:

CHONDRILLA NUCULA

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ABSTRACT

The dramatic increase in atmospheric carbon dioxide since the Industrial Revolution has led to a 30% increase in ocean acidification over pre-industrial levels. Although most ocean acidification research thus far has focused on calcifying organisms such as corals, the potential of this increase in acidity (H^+ ions) to cause acid-base imbalances in soft-bodied animals such as sponges has been grossly overlooked. Furthermore, many studies on ocean acidification have not considered the elevated temperatures that are predicted to accompany future climate change conditions. Sponges are crucial components to coral reef systems, providing food, nutrients, structure, and support. The sponge *Chondrilla nucula* is a common member of Caribbean coral reef communities, and is occasionally found in conditions exhibiting natural environmental hypercapnia, such as caves and dark portions of mangroves. We sought to test the hypothesis that such acclimation to acidic conditions *in situ* translates to a degree of tolerance to simulated near-future conditions of ocean acidification under laboratory conditions. In the summer of 2011, we conducted two experiments in the Exuma Cays, Bahamas, assessing the ability of *Chondrilla nucula* to adapt to "acidified" conditions. The first experiment examined sponges transplanted from a shallow reef site into a cave site ("Cave Hole" of variable pH (=8.2-7.7)), the reef immediately outside the cave ("Cave Reef " (pH=8.2)), and back-transplanted to the reef of origin ("Control Site" (pH=8.2)). Non-polar lipid fraction ratios increased significantly at the Cave Hole and Control sites, but not at the

Cave Reef site. However, total lipids increased at the Cave Reef site, while remaining unchanged at the Cave Hole and Control sites. Fluorescent yield, chlorophyll *a*, soluble protein, carbohydrate, refractory material, ash, and total energetic content were unchanged across the treatment sites, suggesting some acclimation to acidified conditions in the Cave Hole sponges after 2 months. In a second experiment, we utilized a subset of the sponges from the field experiment to examine simulated near-future climate change effects of low pH and high temperature under laboratory conditions. There were no significant effects of treatment across all biochemical constituents except for ash, which showed a significant site temperature interaction. The only other significant effects observed were site effects on the Cave Reef sponges, most likely due to elevated irradiance or other conditions in the field. These findings suggest that *Chondrilla nucula* is very tolerant of acidified conditions in the field and simulated near future conditions of ocean acidification and increased temperature.

Dedication

To my parents,

Peng and Maria Lee,

for everything

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harsh life lessons, fights, laughs, smiles, and tearful moments. Needless to say I would not have made it here without you. To my girlfriend, Jennifer Lam, for all your love, patience, intelligence, nerdy sense of humor, and putting up with me. You are truly a gem.

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TABLE OF CONTENTS

LIST OF TABLES

TABLE PAGE

LIST OF FIGURES

FIGURE PAGE

CHAPTER I

INTRODUCTION

1. Climate change: Ocean Acidification and Elevated Sea Surface Temperatures

Ocean acidification is the ongoing decrease in pH and increase in acidity in the Earth's oceans due to the uptake of anthropogenic CO_2 from the atmosphere. In recent years, ocean acidification has drawn much concern as the amount of atmospheric carbon dioxide (CO_2) has increased dramatically. Since the Industrial Revolution (1850), ocean pH has decreased by 30% (a 30% increase in H^+ ions, causing a decline in pH 8.18 to 8.10), which is 100 times faster than any other period in the past 300 million years (IPCC 2007). At the current rate of change, ocean pH is predicted to reach 7.8 by 2100 (IPCC 2007).

$$CO_2 + H_2O \Rightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \Leftrightarrow CO_3^{2-} + H^+$$

The equation above explains the link between the increase in oceanic CO_2 absorption with an increase in acidity. As CO_2 interacts with H_2O, H_2CO_3 (carbonic acid) is formed. This dissociates into HCO_3^- (hydrogen carbonate, a.k.a. bicarbonate) and H^+ ions, which further dissociates into CO_3^{2-} (carbonate) and H^+ ions. It is the abundance of H^+ production that is the cause for acidity increase. As the amount of CO_2 increases, the equilibrium shifts to an increased production of HCO_3^- (bicarbonate) and H^+ ions, thus lowering the availability of carbonate. Carbonate saturation is a crucial element determining the rate of $CaCO_3$ (calcium carbonate) production that can occur in coral reef systems. Calcifying organisms such as corals, coccolithophores, foraminifera, echinoderms,

crustaceans, and mollusks utilize $CaCO_3$ (calcium carbonate) to build their skeletal structures.

Much of the research on the impacts of ocean acidification has focused on such calcifying organisms. Overall, it has been found that calcification rates are altered with increases in ocean acidification and the accompanying decreases in aragonite saturation (a metastable form of calcium carbonate), decreasing in most cases (Riebesell et al. 2000, Orr et al. 2005, Kuffner et al. 2007, Anthony et al. 2008), while a few studies have found an increase in calcification, although at a metabolic cost (Fine & Tchernov 2007, Wood et al. 2008). Ocean acidification has also been implicated in causing many other stress responses in calcifying organisms, such as increasing the prevalence of bleaching events in coral (Anthony et al. 2008).

While ocean acidification can impact calcifying organisms, the increase in H^+ ions has the potential to cause many acid-base regulation imbalances in non-calcifying organisms. Studies on non-calcifying organisms such as fish have found disruption of the innate abilities of fish to detect predator olfactory cues (Dixson et al. 2010). It is plausible that such disruption is occurring on a cellular level, and is being influenced by acid-base regulation processes. Acid-base regulation occurs on systemic (extracellular), cellular, and subcellular levels (Pörtner et al. 2010). This regulation is an energy dependent process, as some acid-base equivalents are transported by H^+-ATPases or by processes exploiting the Na^+ gradient established by Na^+/K^+-ATPases, such as Na^+/H^+ exchangers (Pörtner et al. 2010). The activities of many intracellular enzymes that are a part of cell metabolism are pH sensitive such as phosphofructokinase, the rate-limiting enzyme of glycolysis (Madshus 1988). Furthermore, soluble protein, DNA, and RNA synthesis are affected by pH

oscillations. The synthesis of DNA and RNA increase with increasing intracellular pH within the physiological range (Gerson 1982). Low intracellular pH is common to both prokaryotic and eukaryotic resting cells, perhaps explaining why these cells have low metabolic activities (Pörtner et al. 2010). A rapid increase in intracellular pH may be important to bring cells from G_o/G_1 into S phase (Madshus 1988). Any disruption of these pH sensitive structures and processes has the potential to wreak havoc on acid-base regulation and the overall fitness of an organism.

According to the IPCC Fourth Assessment Report, most of the observed increase in global average temperatures since the mid-20th century is very likely due to the observed increase in anthropogenic greenhouse gas concentrations (IPCC 2007). At the time the report was assessed, the years 1995-2006 ranked among the twelve warmest years in the instrumental record of global surface temperature (since 1850) (IPCC 2007). Sea surface temperatures are rising with the increase of global temperatures, and this is causing dramatic impacts on marine organisms (Brown 1997, Hoegh-Guldberg 1999, IPCC 2007). The loss of photosymbiotic zooxanthellae in corals, a phenomenon termed "bleaching", has been well documented due to the increase in sea surface temperatures (Porter et al. 1989, Glynn 1993, Goreau & Hayes 1994, Lesser 1996, Brown 1997, Hoegh-Guldberg 1999). Mass coral-bleaching events have greatly increased in frequency and intensity over the past 30 years (Wellington et al. 2001). The loss of zooxanthellae results in decreased chlorophyll concentrations (Porter et al. 1989, Glynn 1996), which results in reduced photosynthesis (Porter et al. 1989, Glynn 1996), the arrest of skeletal growth (Tudhope et al. 1992), and loss of tissue energy reserves (Szmant & Gassman 1990, Fabricius 1999). When temperatures reach levels greater than 2°C above local long-term summer maximum levels,

many colonies of bleaching-sensitive species die (Glynn 1996, Loya et al. 2001). Although surviving colonies may eventually become repopulated by zooxanthellae, reproduction may continue to fail for up to three consecutive years until energy stores are replenished, thus decreasing the overall productivity of the community (Michalek-Wagner & Willis 2000).

Elevated sea surface temperatures also can cause bleaching in sponges, which leads to stress and, occasionally death (Vicente 1990, Fromont & Garson 1999, Cowart et al. 2006, López-Legentil et al. 2008). Many sponges possess a wide range of photosynthetic endosymbionts including zooxanthellae, eukaryotic rhodophytes, diatoms, dinoflagellates, chlorophytes, and cyanobacteria (Wilkinson 1987, Rützler 1990, Taylor et al. 2007). Bleaching in sponges is characterized by the loss of the photosynthetic endosymbionts and/or by the partial loss of endosymbiont pigments (Trench & Blank 1987, Hoegh-Guldberg & Smith 1989). There are two types of bleaching documented in sponges. The first and more common is cyclic bleaching, which is characterized by a loss of coloration and eventual recovery (Cowart et al. 2006). The second type, fatal bleaching, results in a total whitening of sponge tissue, and consequently the complete disintegration of tissue, followed by mortality (Cowart et al. 2006).

While many studies have focused solely on either ocean acidification or elevated sea surface temperature as stressors on coral reefs, the reality is that future climate change conditions are predicted to encompass both of these stressors. This study examined the effects of both ocean acidification and concomitant elevated sea surface temperature in order to provide more realistic, real-world results.

2. *Study organism*

Chondrilla nucula (chicken liver sponge) is a common Caribbean sponge that is part of the Tetractinomorpha family, Hadromerida order, and Chondrosidae subclass (Kelly-Borges & Pomponi 1992). In coral reef communities, *Chondrilla nucula* grows in yellowish-brown, smooth, flat encrusting sheets, with a firm and cartilaginous texture (figure 1). It possesses microsclere type glass spicules (pycnaster-euaster) (Ferguson & Davis 2008). It is gonochoric and oviparous (Liaci et al. 1973), but also reproduces asexually (Gaino & Pronzato 1983).

Chondrilla nucula contains endosymbiotic cyanobacteria that are photosynthetically active and provide energy to the host sponge, and in return gain shelter and varying levels of nutrients (Kayley 2008). Cyanobacteria provide a wide range of services to the sponge host, such as N_2 fixation, chemical defense, provision of a sunscreen, and ammonia conversion (Usher 2008). Cyanobacteria have a flexible photosynthetic apparatus that allows them to utilize light at very low and high levels, making them ideal symbionts for a wide range of organisms and conditions (Usher 2008). Phycobilisomes are the major accessory light-harvesting complexes of cyanobacteria (Edwards & Gantt 1971, Wildman and Bowen 1974). They exist on the thykaloid membranes, harvesting light and facilitating energy migration toward photosystem I and II reaction centers (MacColl 1998). Phycobilisomes frequently detach and re-associate with reaction centers, changing their distribution between Photosystem II and I (Sarcina et al. 2001, Joshua & Mullineaux 2004).

Such mobility may allow for flexibility in light harvesting; in very intense light, these state transitions allow cyanobacteria to maximize their use of available energy, and reduce photo-damage to Photosystem II by absorbing excess energy. *Synechococcus* spp. are the most common cyanobacterial symbionts found in sponges (Usher 2008), and 'C*andidatus Synechococcus spongiarum'* has been described by Usher et al. (2004) in *Chondrilla nucula*. The *Synechococcus* genus does not fix nitrogen, but can utilize ammonium as an inorganic nitrogen source, which is a limiting nutrient on coral reefs (Glover 1985). Vertical transmission of these cyanobacteria has been indicated in the eggs *Chondrilla nucula* (Scalera Liaci et al. 1971, 1973). *Chondrilla nucula* inhabits a wide range of habitats, existing on reefs, in mangrove swamps, and in marginal systems such as marine caves, which exhibit natural hypercapnia. Sponges found in dark regions of mangroves or caves are completely white in appearance, presumably lacking photosynthetic cyanobacteria (Gaino 1977, Swearingen 1998). Furthermore, in past studies, reef *Chondrilla nucula* transplanted to dark caves underwent metabolic collapse, presumably from the absence of light (Arillo 1993).

Figure 1. Image of *Chondrilla nucula*, the "chicken liver sponge."

Chondrilla nucula Schmidt, 1862.

3. *Significance of research*

While assessments of calcifying organisms have garnered the vast majority of attention in climate change and ocean acidification studies, the potential acid-base imbalances that arise from this increase in acidity (H^+ ions) have been grossly overlooked. Ocean acidification can cause many acid-base regulation imbalances in soft-bodied animals such as marine sponges. Sponges play a crucial role in coral reef systems, providing food, nutrients, shelter, and structure to these communities (Diaz & Rutzler 2001, Wulff 2001, Bell 2008). They fill a similar ecological niche to the hard corals, but are more extensive in distribution (Usher 2008), yet they have been overlooked in the race to understand the more obvious impacts of ocean acidification on calcifying organisms.

Many climate change studies have only focused on a single factor of predicted climate change conditions: increased temperatures or increased acidity (Kuffner et al. 2007, Wood 2008, Dixson et al. 2010). However, the reality is that future climate conditions are predicted to encompass both an increase in temperature and increase in acidity, as well as the interactions of these stressors. Studies that do analyze the combined effects of increased temperatures and acidity are more representative of future real world scenarios, and sometimes provide surprising, insightful results (Anthony et al. 2008, Crawley et al. 2009, Brennand et al. 2010). Brennand et al. (2010) found that an increase in acidity caused significantly reduced larval growth and skeletal length in the sea urchin, *Tripneustes gratilla*. However, increased temperatures stimulated larval growth, diminishing the negative effects of acidification and hypercapnia.

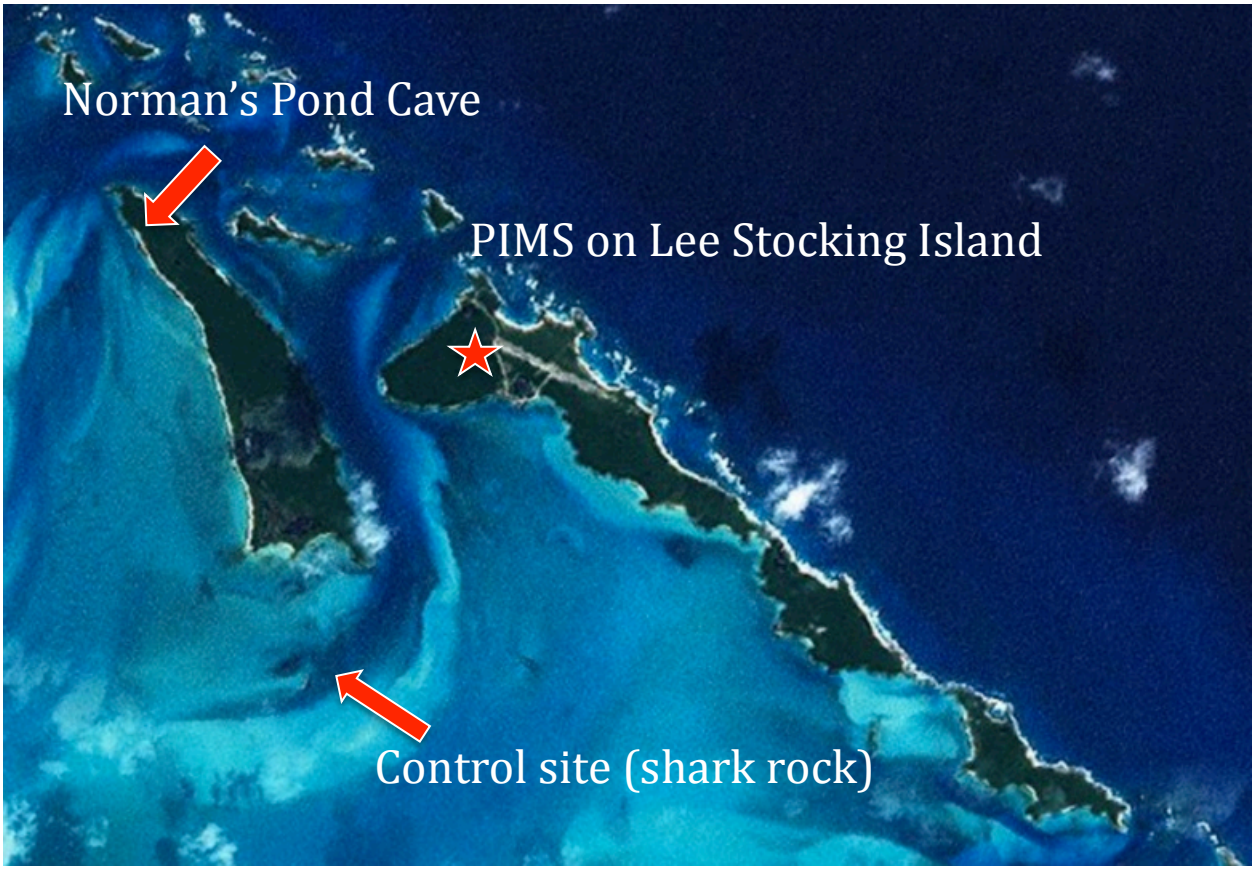
To date, no study has been published analyzing the impacts of ocean acidification and elevated temperature on marine sponges. My research focused on the potential for adaptive tolerance to acidified conditions in the field, and to simulated climate change conditions of ocean acidification and elevated temperature in the laboratory in the marine sponge *Chondrilla nucula. Chondrilla nucula* is a shallow water sponge occasionally found in marginal systems such as marine caves that exhibit environmental hypercapnia. High metabolic activities in small, confined spaces, and dissolution of limestone contribute to the acidification in such caves (Barton & Northup 2007). Caves are also characterized by a myriad of additional variable factors such as low light levels, low oxygen levels, low temperatures, and high levels of nutrients (Sket 1996, Lapointe et al. 2003). Additionally, suspended particulate matter from caves serves as a source in heterotrophic food webs in the surrounding area (Fichez 1991).

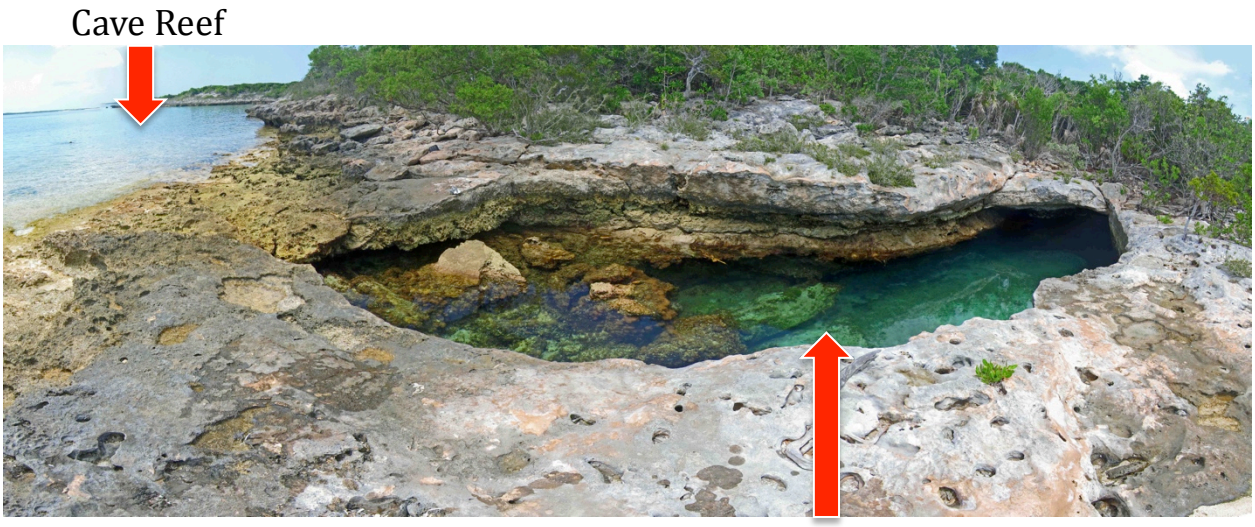
Because *Chondrilla nucula is* occasionally found in such marginal systems, I sought to assess its capacity to tolerate simulated near-future conditions of ocean acidification and elevated temperature. The objective of my study was two fold.

1.) In order to test the hypothesis that *Chondrilla nucula* could acclimate to acidified conditions under field conditions, I carried out a field transplant experiment to three sites: Norman's Pond Cave ("Cave Hole", a site of tidally variable pH (7.7-8.2)), the reef directly outside the Cave Hole ("Cave reef (pH 8.2)), and the reef of origin ("Control Reef" (pH 8.2)) (figure 2). The pH of Norman's Pond Cave fluctuated on a semi-diurnal basis, reaching 7.7 at low tide and rebounding back up to 8.2 at high tide (figure 3). The Cave Hole and Control Reef were both at the same approximate depth (~2m). The Cave Reef site was situated in

very shallow water depth (~0.5m) directly outside of the Cave Hole, which likely equated to almost twice the irradiance levels as the other two sites (figure 4) (Lesser et al. 2010).

Figure 2. Field transplant experiment study sites





Norman's Pond Cave

Figure 3. pH fluctuation at all 3 sites

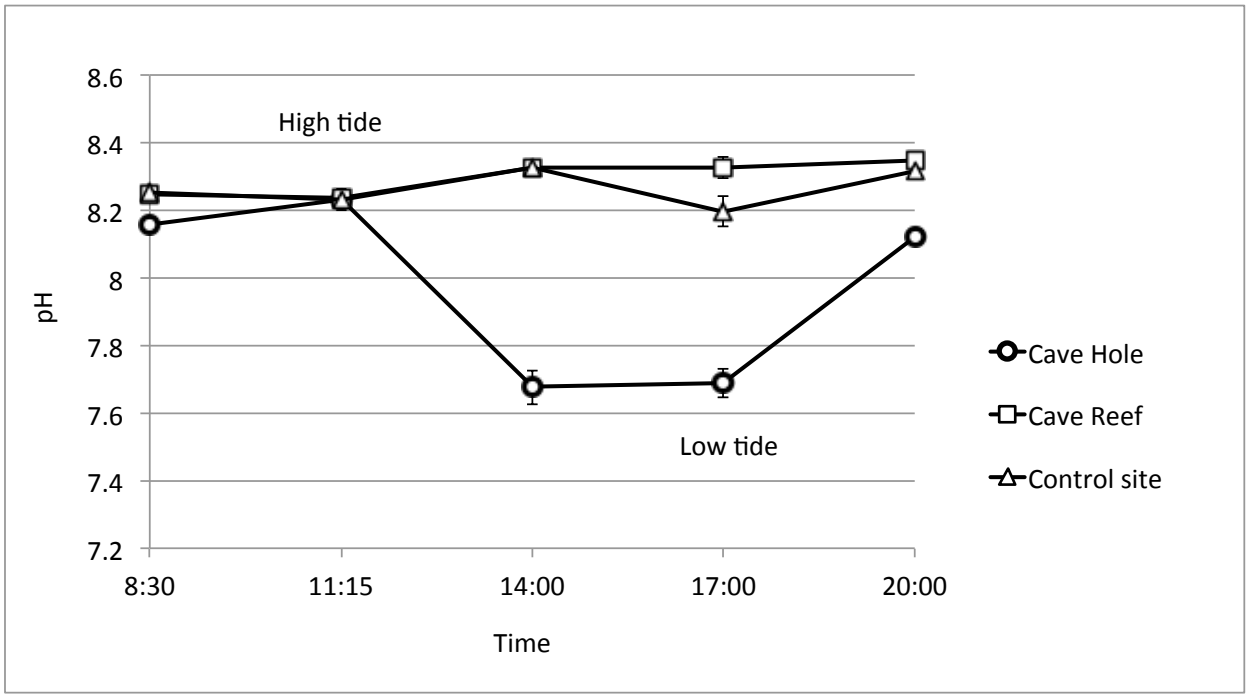
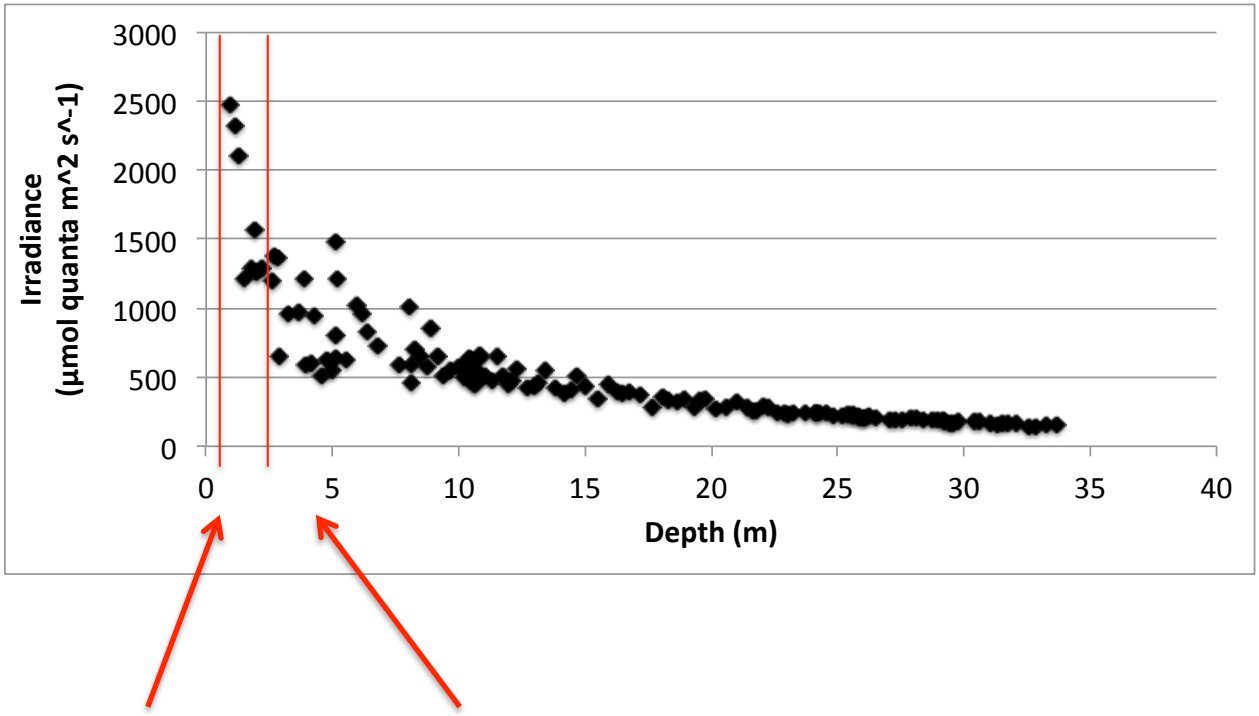


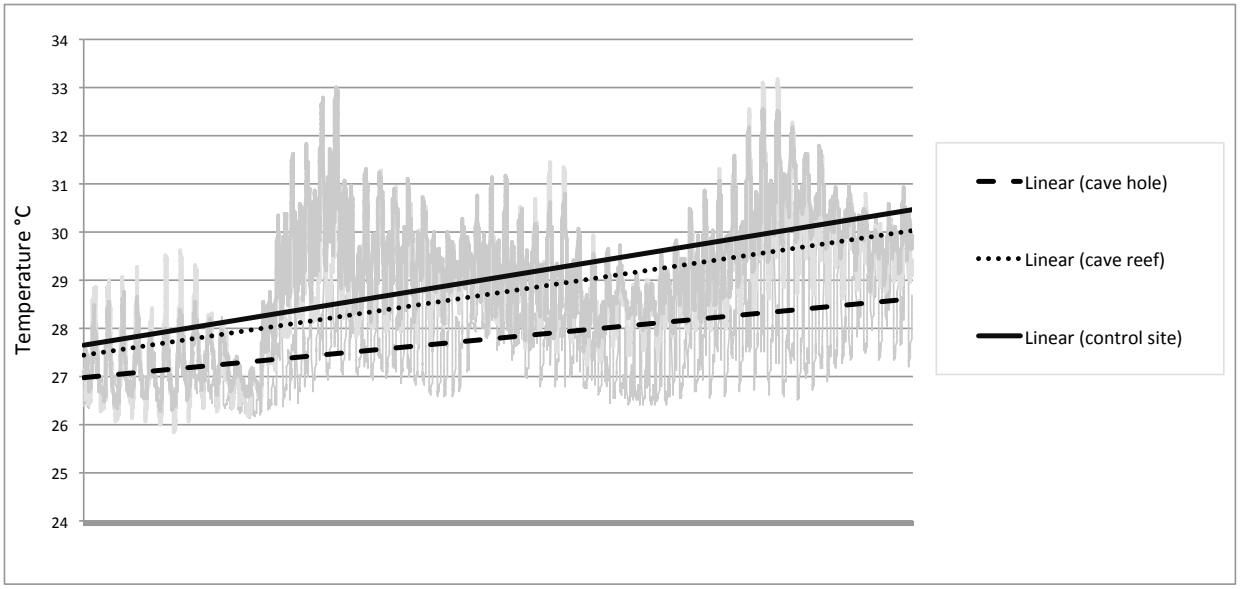
Figure 4. Representative irradiance data from reefs at Lee Stocking Island. Data from Lesser et al. 2010



Cave Reef irradiance

Cave Hole and Control sites irradiance

Figure 5. Linear Regression of temperature from May-July in the field experiment. All sites increased significantly over the course of field experiment (linear regression, P<0.0001)



2.) In order to test the hypothesis that *Chondrilla nucula* could adapt to simulated near-future conditions of climate change (low pH and high temperature), I carried out a laboratory experiment, which was performed at the Perry Institute of Marine Science on Lee Stocking Island in the Exuma Cays, Bahamas. The rational for this experiment stemmed from the conditions *Chondrilla nucula* endures in caves. The constant flux of pH in Norman's Pond Cave is arguably more stressful than a state of stable, low pH (i.e. ocean acidification) because of the constant disruptions of acid-base regulation and cellular homeostasis. Therefore, I hypothesized that sponges that could thrive in such conditions should be better able to tolerate simulated future conditions of stable low pH (7.9) and high temperatures (31^{o}C), the IPCC predicted conditions for 2050, based on the "business as usual" IS92a scenario (IPCC 2007).

In laboratory acidification experiments, there are two widely used methods to acidify water: CO_2 bubbling and acid titration. The acid titration method changes the total alkalinity of water, and fails to change total carbon (Hurd et al. 2009). The CO_2 bubbling method is effective in providing the required pH/ CO_2 concentration (Hurd et al. 2009), and since I wanted to simulate future conditions of increased acidity, and increased CO_2, naturally this was the method I chose to implement in the laboratory experiment.

Since *Chondrilla nucula* is not a calcifying sponge, the impacts of these experiments were quantified through biochemical and physiological endpoints. The health of the endosymbiotic cyanobacteria and sponge were both analyzed. Fluorescent yield of the cyanobacteria was measured via Pulse Amplified Modulating Fluorometry (PAM), which measures the fluorescent yield of the photosystem II of the phycobilisomes in the cyanobacteria. Chlorophyll *a*, a major subunit of the photosystem II complex, is responsible

for harvesting light for photosystem II usage, and also was quantified. Recent studies have found changes in chlorophyll *a* content in corals due to increases in CO_2 (Crawley et al. 2010). Also, changes in fluorescent yield have been shown to correlate with changes in health status of sponges (Lesser 2001).

From the perspective of the sponge, biochemical profiling of ash, lipids, soluble protein, carbohydrates, and refractory material (insoluble protein) was quantified and total energetic composition was calculated. Biochemical composition is useful in understanding the energy flow in a community and total energetic costs for adaptation (Slattery & Bosch 1993, Lucas 1994, Slattery & McClintock 1995).

My research aims to further our currently limited knowledge of ocean acidification, especially on non-calcifying organisms. Furthermore, this research ventures into largely unknown and unstudied effects of climate change on marine sponges, vital components in many coral reef ecosystems.

CHAPTER II

ADAPTIVE TOLERANCE TO OCEAN ACIDIFICATION IN THE MARINE SPONGE, *CHONDRILLA NUCULA*

Sylvester J. Lee, Deborah J. Gochfeld and Marc Slattery

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Abstract

The dramatic increase in atmospheric carbon dioxide since the Industrial Revolution has led to a 30% increase in ocean acidification over pre-industrial levels. Although most ocean acidification research thus far has focused on calcifying organisms such as corals, the potential of this increase in acidity (H^+ ions) to cause acid-base imbalances in soft-bodied animals such as sponges has been grossly overlooked. Furthermore, many studies on ocean acidification have not considered the elevated temperatures that are predicted to accompany future climate change conditions. Sponges are crucial components to coral reef systems, providing food, nutrients, structure, and support. The sponge *Chondrilla nucula* is a common member of Caribbean coral reef communities, and is occasionally found in conditions exhibiting natural environmental hypercapnia, such as caves and dark portions of mangroves. We sought to test the hypothesis that such acclimation to acidic conditions *in situ* translates to a degree of tolerance to simulated near-future conditions of ocean acidification under laboratory conditions. In the summer of 2011, we conducted two experiments in the Exuma Cays, Bahamas, assessing the ability of *Chondrilla nucula* to adapt to "acidified" conditions. The first experiment examined sponges transplanted from a shallow reef site into a cave site ("Cave Hole" of variable pH (=8.2-7.7)), the reef immediately outside the cave ("Cave Reef " (pH=8.2)), and back-transplanted to the reef of origin ("Control Site" (pH=8.2)). Non-polar lipid fraction ratios increased significantly at the Cave Hole and Control sites, but not at the Cave Reef site. However, total lipids increased at the Cave Reef site, while remaining

unchanged at the Cave Hole and Control sites. Fluorescent yield, chlorophyll *a*, soluble protein, carbohydrate, refractory material, ash, and total energetic content were unchanged across the treatment sites, suggesting some acclimation to acidified conditions in the Cave Hole sponges after 2 months. In a second experiment, we utilized a subset of the sponges from the field experiment to examine simulated near-future climate change effects of low pH and high temperature under laboratory conditions. There were no significant effects of treatment across all biochemical constituents except for ash, which showed a significant site temperature interaction. The only other significant effects observed were site effects on the Cave Reef sponges, most likely due to elevated irradiance or other conditions in the field. These findings suggest that *Chondrilla nucula* is very tolerant of acidified conditions in the field and simulated near future conditions of ocean acidification and increased temperature.

1. Introduction

Since the Industrial Revolution (1850), oceanic uptake of anthropogenic carbon dioxide (CO_2) from the atmosphere has caused a 30% increase in acidity (8.18 to 8.10), a rate 100 times more rapid any other period in the past 300 million years (IPCC 2007). With current trends, ocean pH is predicted to reach 7.9 by 2050 and 7.8 by 2100 (IPCC 2007). Predicted to accompany this increase in acidity is a further increase in sea surface temperature (IPCC 2007). For the next two decades, a warming of about 0.2°C per decade is projected for a range of SRES (IPCC Special Report on Emission Scenarios) emission scenarios (IPCC 2007). This translates to a rise in sea surface temperatures of 1.5-2.6°C by end of the century relative to 1980-1999 temperatures (IPCC 2007).

Much of the research on the impacts of climate change conditions has focused on calcifying organisms such as corals. The loss of photosymbiotic zooxanthellae, a phenomena termed "bleaching", has been well documented due to the increase in sea surface temperatures (Porter et al. 1989, Glynn 1993, Goreau & Hayes 1994, Lesser 1996, Brown 1997, Hoegh-Guldberg 1999) and mass coral-bleaching events have greatly increased in frequency and intensity over the past 30 years (Wellington et al. 2001). Overall, it has been found that calcification rates are altered with increases in ocean acidification and the accompanying decreases in aragonite saturation (a metastable form of calcium carbonate), decreasing in most cases (Riebesell et al. 2000, Orr et al. 2005, Kuffner et al. 2007, Anthony et al. 2008), while a few studies have found an increase in calcification, although at a metabolic cost (Fine & Tchernov 2007, Wood et al. 2008). Ocean acidification

has also been implicated in causing many other stress responses in calcifying organisms, such as increasing the prevalence of bleaching events in coral (Anthony et al. 2008).

While ocean acidification has been demonstrated to impact calcifying organisms, this increase in acidity (H^+ ions) has the potential to cause many acid-base regulation imbalances in soft-bodied organisms such as sponges. Acid-base regulation occurs on systemic (extracellular), cellular, and subcellular levels (Pörtner et al. 2010). This regulation is an energy dependent process, as some acid-base equivalents are transported by H^+-ATPases or by processes exploiting the Na^+ -gradient established by Na^+/K^+-ATPases, such as Na^+/H^+ exchangers (Pörtner et al. 2010). The activities of many intracellular enzymes that are a part of cell metabolism are pH sensitive such as phosphofructokinase, the rate-limiting enzyme of glycolysis (Madshus 1988). Furthermore, soluble protein, DNA, and RNA synthesis are affected by pH oscillations. The synthesis of DNA and RNA increase with increasing intracellular pH within the physiological range (Gerson 1982). Low intracellular pH is common to both prokaryotic and eukaryotic resting cells; this is one reason these cells have low metabolic activities (Madshus 1988). A rapid increase in intracellular pH may be important to bring cells from G_o/G_1 into S phase (Madshus 1988).

Elevated sea surface temperatures can cause bleaching not only in corals, but also in sponges, which leads to stress, and occasionally death (Vicente 1990, Fromont & Garson 1999, Cowart et al. 2006, López-Legentil et al. 2008). Many sponges possess a wide range of photosynthetic endosymbionts, including cyanobacteria (Wilkinson 1987, Rützler 1990, Taylor et al. 2007), and bleaching in sponges is characterized by the loss of the photosynthetic endosymbionts and/or by the partial loss of endosymbiont pigments (Trench & Blank 1987, Hoegh-Guldberg & Smith 1989).

This study sought to analyze the impacts of climate change, which encompasses both ocean acidification and elevated temperature, on marine sponges, which play a crucial role in coral reef communities, providing food, nutrients, shelter, and structure (Diaz & Rutzler 2001, Wulff 2001, Bell 2008). To date, no studies have been published analyzing the combined impacts of ocean acidification and elevated temperature on these organisms. This study focused on the potential for adaptive tolerance to ocean acidification and elevated temperature in *Chondrilla nucula* (chicken liver sponge), an encrusting sponge common to Caribbean coral reef and mangrove communities. *Chondrilla nucula* contains photosynthetically active endosymbiotic cyanobacteria (Wilkinson & Vacelet 1979), but is occasionally found in marginal systems without light, such as marine caves, where it grows without such cyanobacteria (Gaino 1977, Swearingen 1998). Besides lack of light, marine caves exhibit natural hypercapnia due to high metabolic activities in a small, confined space, and dissolution of limestone (Barton & Northup 2007). Caves are also characterized by a myriad of additional stressors such as low light, low oxygen levels, low temperatures, and high levels of nutrients (Sket 1996, Lapointe et al. 2004).

Because *Chondrilla nucula* is occasionally found in these variable, acidified environments, this study aimed to assess *Chondrilla nucula*'s capacity to endure simulated near-future climate change conditions of ocean acidification and elevated temperature. First, we tested the hypothesis that *Chondrilla nucula* from the reef could acclimate to naturally acidified field conditions. Second, we assessed *Chondrilla nucula'*s capacity to adapt to simulated near-future climate conditions of low pH (7.9) and high temperature (31°C), based on IPCC predictions for 2050, under the "business as usual" IS92a scenario (IPCC 2007), in a controlled laboratory setting.

2. Materials and Methods

We conducted a field and laboratory experiment to examine the impacts of climate change stressors on *Chondrilla nucula.* Since *Chondrilla nucula* is not a calcifying sponge, these experiments were quantified through biochemical and physiological endpoints. The health of the endosymbiotic cyanobacteria and sponge were both analyzed. Fluorescent yield of the cyanobacteria was measured via Pulse Amplified Modulating (PAM) Fluorometry. Chlorophyll *a*, a major subunit of the photosystem II complex, is responsible for harvesting light for photosystem II usage, and also was quantified. Recent studies have found changes in chlorophyll *a* content in corals due to increases in CO_2 (Crawley et al. 2010). Also, changes in fluorescent yield have been shown to correlate with changes in health status of sponges (Lesser 2001).

From the perspective of the sponge, biochemical profiling of ash, lipids, soluble protein, carbohydrates, and refractory material (insoluble protein) was quantified and total energetic composition was calculated. Biochemical composition is useful in understanding the energy flow in a community and total energetic costs for adaptation (Slattery & Bosch 1993, Lucas 1994, Slattery & McClintock 1995).

2.1 Field Transplant Experiment

In the summer of 2011, working from the Perry Institute for Marine Science (PIMS) on Lee Stocking Island in the Exuma Cays, Bahamas, we utilized 3 sites: Norman's Pond Cave

(the Cave Hole) represented our naturally acidified site, the Cave Reef was the reef directly outside Norman's Pond Cave, and our Control Site was Shark Rock, a shallow reef site approximately 4 km south from Norman's Pond Cave (figure 2). Norman's Pond Cave fluctuated in pH from 7.7 to 8.2 on a semi-diurnal basis, while the Cave Reef and Control sites had a stable pH of 8.2, representing oceanic conditions (figure 3). pH levels were measured at the three sites for a period of approximately a week using water samples and a portable pH meter (Oakton® PCSTestr 35 Waterproof Multiparameter pocket tester). The Cave Hole and Control sites both had approximately equal average depths of 2m, while the Cave Reef site had a much shallower depth of approximately 0.5m, which equated to much higher irradiance conditions, but not higher temperatures (figure 4, figure 5). Over the course of the field experiment, continuously recording temperature loggers were deployed at each of the sites (HOBO Pro v2 Water Temperature Data Loggers, Onset Computer Corp). Temperature increased significantly (Multiple Regression, $P<0.0001$) at all three sites over the course of the field experiment.

Eight large pieces of *Chondrilla nucula* were collected from the Control site on SCUBA and transported back to PIMS for initial sampling and cloning. Cloning of sponges consisted of sectioning each sponge into ten pieces (ramets): one was taken as an initial sample, and the remaining nine left to be deployed in the field, three ramets at each of the sites. This cloning allowed us to control for any genotypic variation in the experiment, giving us the ability to track an individual genotype throughout our entire study. The ramets of all eight sponges were secured to plastic racks with zip-ties, and deployed to the three sites: the Cave Hole, the Cave Reef, and back transplanted to the Control site, to control for potential handling stress. All transplanted racks were secured to the substrate with waxed string and

fishing hooks. After two months of acclimation, all transplanted racks were collected and transported back to PIMS for sampling and preparation for the laboratory experiment.

Prior to the laboratory experiment, one ramet from each of the eight sponges at each of the three sites was sampled to characterize changes resulting from the field transplant experiment. A small (0.5 mm thick) slice was preserved in 4% paraformaldehyde (PFA) for cyanobacteria counts and the remaining portions of each sample were frozen for further analyses (chlorophyll *a*, soluble proteins, lipids, carbohydrates, and ash).

2.2 Laboratory Experiment

A subset of the remaining ramets (n=4) was kept in flow-through raceways for two days of acclimation and recovery before the laboratory experiment before being randomly assigned to treatments and placed in 2L tanks in a 0.5m x 5m raceway. The laboratory experiment consisted of four treatment combinations of pH (7.9 vs. 8.2), and temperature (29°C vs. 31°C), for a duration of 4 days. These levels (pH 7.9 and 31°C) were based on IPCC predictions for the year 2050, under the "business as usual" IS92a scenario (IPCC 2007). The four treatments were as follows: low temperature + ambient pH, low temperature + low pH, high temperature + ambient pH, high temperature + low pH. Since the seawater system temperature was at 31°, but ambient temperature on the reef was 29°C, temperature was controlled by a chiller (Neslab) for the low (29°C) treatment, and unaltered seawater was utilized as the high temperature treatment (31°C). The shade cloth used to cover the raceways provided irradiance conditions of approximately 10m underwater. The flow through system set up consisted of large header tanks of acidified

water, that was maintained using a control system (D207 pH and pCO2 Regulation System, Qubit systems), by feed-back loops between pH readings in the CO_2 header tanks and the open/close status of a solenoid valve.

Total alkalinity (TA) was calculated using the program CO2SYS (Lewis and Wallace 1998), with the dissociation constants of Mehrbach et al (1973) modified by Dickson and Millero (1987) (Table 1). During the daytime, the power requirements for maintaining daily facilities and this laboratory experiment flow-through system were too much for the main generator on the island. Because of this, the flow-through system would malfunction during the day. During the daytime, static water exchanges were performed on a two-hour basis. Under close watch, we allowed the CO_2 bubbling system to adjust the pH to the correct levels in the header tanks. After verifying the pH of the header tanks, water flow was provided to all treatments for fifteen minutes every two hours. Additionally, pH and temperature readings were taken every hour during the day. At night, as the power load on the main generator decreased, mechanical issues were resolved and the flow-through system provided adjusted water to the tanks until the following morning, when the static water changes resumed. At the end of the experiment, portions of each sample were frozen for further analyses (chlorophyll *a*, soluble proteins, lipids, carbohydrates, and ash), and preserved in PFA (cyanobacteria counts). Final sample sizes were n=4 for all treatments except for the Cave Reef low temperature + ambient pH, high temperature + ambient pH, and high temperature + acidified water treatments, which had n=3. The Cave Reef low temperature + acidified water treatment had n=2.

Table 1. Laboratory experiment water parameters at 25°C, based on calculations using the CO2SYS program

pH	Total alkalinity (µmol kg^{-1})	pCO_2 (µatm)	HCO_3^- (µmol kg^{-1-})	CO_3^{2-}	CO_2 (µmol kg^{-1-})
7.9-8.0	1568-1611	461-572	1322-1335	96-117	12-15
8.1-8.2	1540-1601	240-351	1131-1254	138-160	6-9

2.3 Endosymbiont analyses

Fluorescent yield

A Diving-PAM (Pulse Amplified Modulating Fluorometer; Walz, Germany) was used to quantify the fluorescent yield of the photosystem II of the cyanobacteria in each sponge sample. Fluorescent measurements were taken at the beginning of the field transplant experiment (May), after the field transplant experiment (July), during the two days of acclimation before the laboratory experiment, and every night throughout the duration of the laboratory experiment (4 days). Sponges were allowed to dark-adapt for 30 minutes after sunset before readings. Three readings were taken from each sponge and the calculated mean maximal fluorescent yield was used for quantitative analysis. If any one reading deviated by +/- 200Y from the other readings in a given sponge, a fourth reading was taken. Care was taken to assure that the locations of the readings did not overlap on a sponge.

Chlorophyll a analysis

Chlorophyll *a* quantification was determined using the methods of Parsons et al. (1984) and Erwin and Thacker (2008). Sponge samples were wrapped in aluminum foil and preserved at -20°C to prevent degradation of chlorophyll *a*. For analysis, 30-50mg of dried, ground sponge was weighed and placed in a scintillation vial. Then, 10mL of 90% acetone was added to each vial, placed in a dark box to eliminate light exposure, and

preserved at -20°C for 20 hours. Prior to sampling, each vial was inverted twice to mix the contents, and 1.5mL was removed to a centrifuge tube. After a short 5 second spin in a centrifuge, absorbances were read in quartz cuvettes at 750nm, 664nm, 647nm, and 630nm on a spectrophotometer (Agilent 8453). Chlorophyll a concentrations were estimated using equations of Parsons et al. (1984), and standardized to sponge mass.

2.4 Biochemical profile of sponge samples

Protein analysis

Soluble protein concentration was determined using the methods of Bradford (1976). First, 5mL of 1M NaOH were added to 10mg of dried sponge sample, and allowed to extract for 18 hours. Then, 100uL of standard and sample were pipetted into clean, dry test tubes with 5 mL of dye reagent (Quick start Bio-Rad Bradford Protein Assay kit 500-0202), and vortexed. After a 5 -minute incubation, absorbances were read using a microplate spectrophotometer (Eppendorf Biophotometer) set to 595nm.

Lipids: Lipid fraction ratio and total lipids

Non-polar lipid fraction ratios were determined using thin layer chromatography (TLC) (Yamashiro et al. 1999, Saunders et al. 2005). First, 100-200mg of dried sponge tissue was extracted with 3mL of chloroform: methanol (2:1 by volume) and sonicated for 15 minutes. The extract was then filtered and the process was repeated. After drying the extracts under vacuum, total lipid content was determined gravimetrically. Extracts were

dissolved at a concentration of 10µg/µL, and stored at -20°C when not in use. A glass-spotting rod was used to transfer 5µL of dissolved sample and standards (cholesteryl oleate and cholesterol) onto a 10 cm x 10 cm TLC plate (EMD silica gel 60, Germany). The chromatogram for each plate was made by carrying out elution in a hexane:ether:acetic acid (14 mL:6 mL:0.5 mL) mixture to a distance of 8 cm. After drying in air, the plates were immersed in a phosphoric acid/33% acetic acid/sulphuric acid/0.5% copper sulphate (5: 5 : 0.5 : 90, by volume) solution for 30 seconds. The acid-treated plates were initially dried under warm air for 1 min to prevent spotting and then left to air dry thoroughly before placing in a drying oven at 110–115°C for 15 min. The resulting chromatogram was scanned (Canon Pixma MX700; 600 dpi, brightness 100, contrast 135), within 5 minutes of removal from the oven, to generate greyscale images. Relative concentrations of the different lipid fractions were estimated from the peak-greyscale intensity of each lipid band (Image J software, RSB, Bethesda, MD). Neutral/nonpolar (wax ester) to polar (sterol) lipid ratios were then calculated as the mean of three replicate TLC runs for each sample.

Carbohydrate analysis

Carbohydrate content in sponges was measured in microplate format (Trevelyan 1952, Slattery & McClintock 1995, Masuko et al. 2005). First, 10-15 mg of dried sponge tissue was extracted with 5% trichloroacetic acid (TCA) (2mg/mL) for 3 hours. In a 96-well microplate, 50 µl of sample and glucose standards were added to wells in triplicate. Following this, 150 µl of concentrated sulfuric acid was added, followed immediately by 30 µl of 5% phenol. After incubating for 5 minutes at 90 °C in a static water bath by carefully floating the microplate, the plate was cooled to room temperature for 5 minutes in another

water bath, wiped dry, then measured at A490nm by a microplate reader (Biotek Synergy HT).

Ash analysis

Percent ash was determined gravimetrically by drying pre-weighed tissue samples in a muffle furnace at 500°C for 4 hours (Slattery & McClintock 1995).

Refractory calculations

The refractory material of each sample was determined by subtracting the percent composition of soluble protein, lipid, carbohydrate, and ash percent composition from 100%, which was assumed to represent insoluble protein (Lawrence & Kafri 1979, Slattery & McClintock 1995).

Energetic content

The energetic composition of each sample was calculated utilizing coefficients for lipid, soluble protein, and carbohydrates: 9.46, 5.65, 4.10 cal g^{-1}, respectively (Fruton & Simmonds 1953), and converted to joules. The total energetic content of each sample was then calculated as the sum of all components.

2.4 Data Analysis

Statistical analyses were conducted using repeated measures ANOVA, using $\alpha=0.05$ and individual differences were assessed using Tukey's HSD test. When there was no significant effect of temperature or acidification treatments, the treatments were collapsed in order to test for site effects. For fractions and percentages, statistical analysis was carried out after arcsine-transformation to satisfy the conditions of normality and equal variance. Variation around means is given as standard error (SE).

3. Results

Fluorescent yield

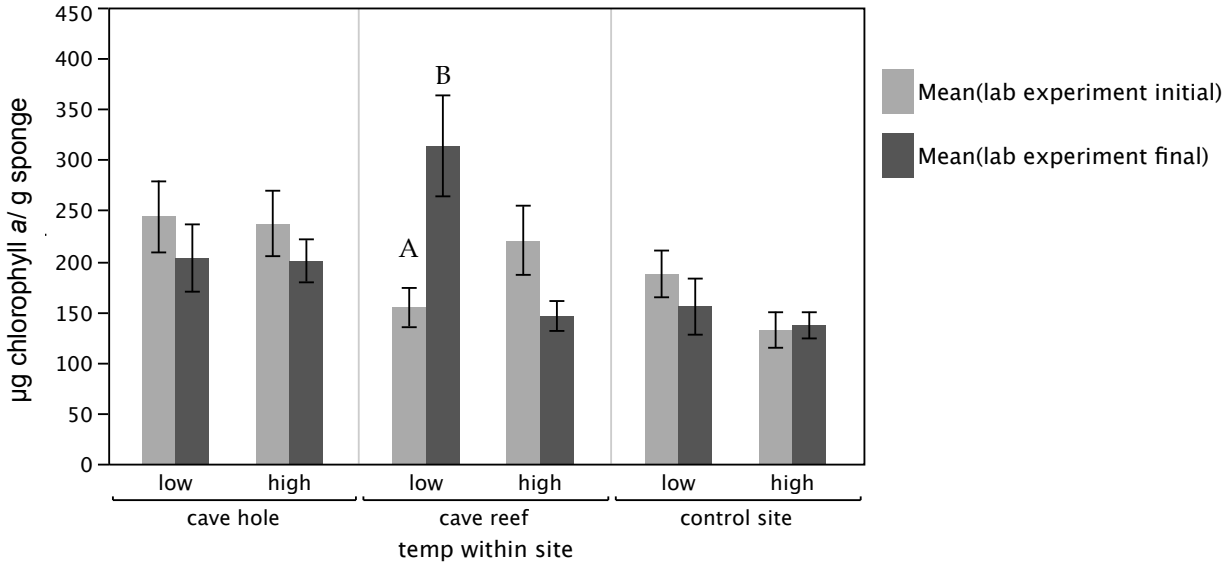
There were no significant differences in fluorescent yield among the three sites (Cave Hole, Cave Reef, Control site) after the field experiment (repeated measures ANOVA, $P>0.05$). Additionally there were no significant differences among treatments after the laboratory experiment (repeated measures ANOVA, $P>0.05$).

Chlorophyll a analysis

There were no significant differences in sponge chlorophyll *a* concentration among the three sites after the field experiment (repeated measures ANOVA, $P>0.05$). In the laboratory experiment, when the data from the two acidification levels were combined, the chlorophyll *a* in the Cave Reef, low temperature treatment increased significantly after the

4-day experiment (repeated measures ANOVA ($P=0.0044$), Tukey's HSD test ($P<0.05$)) (figure 6).

Figure 6. Chlorophyll *a* concentration (mean ± SE; μg/g sponge) in *Chondrilla nucula* in low and high temperature treatments in the laboratory experiment for all three sites. Change in chlorophyll *a* concentration increased significantly in the Cave Reef- low temperature treatment (Repeated Measures ANOVA (P=0.0044), Tukey's HSD test (P<0.05))



Protein analysis

There were no significant differences in soluble sponge protein concentration among the three sites after the field experiment (repeated measures ANOVA, $P>0.05$). Additionally there were no significant differences in soluble protein concentration among treatments after the laboratory experiment (repeated measures ANOVA, $P>0.05$).

Non-polar lipid fraction ratio analysis

Non-polar lipid fraction ratios of sponges from the Cave Hole and Control sites increased significantly in after the field experiment, while Cave Reef sponges remained unchanged (repeated measures ANOVA ($P=0.0008$), Tukey's HSD test ($P<0.05$)). In the laboratory experiment, there was no significant effect of treatment, but there was a significant effect of site: non-polar lipid fraction content increased significantly in Cave Reef samples, while it decreased in Cave Hole and Control sites samples (repeated measures ANOVA ($P=0.0008$), Tukey's HSD test ($P<0.05$)) (figure 7).

Total Lipids

Sponges from the Cave Reef site increased significantly after the field experiment, while the Cave Hole and Control sites sponges remain unchanged (repeated measures ANOVA ($P<0.0001$), Tukey's HSD test ($P<0.05$)). In the laboratory experiment, there was no significant effect of treatment, but there was a significant effect of site: total lipid content increased significantly in Cave Hole and Control sites samples, while it remained

constant in Cave Reef samples (repeated measures ANOVA ($P<0.0001$), Tukey's HSD test ($P<0.05$)) (figure 8).

Figure 7. Non-polar lipid fraction ratio (mean ± SE) in *Chondrilla nucula* over the course of both experiments. All laboratory experiment final bars contain all 4 treatments pooled, as there were no significant effects of treatments. Means with different letters are significantly different (Repeated Measures ANOVA (P=0.0008), Tukey's HSD test (P<0.05))

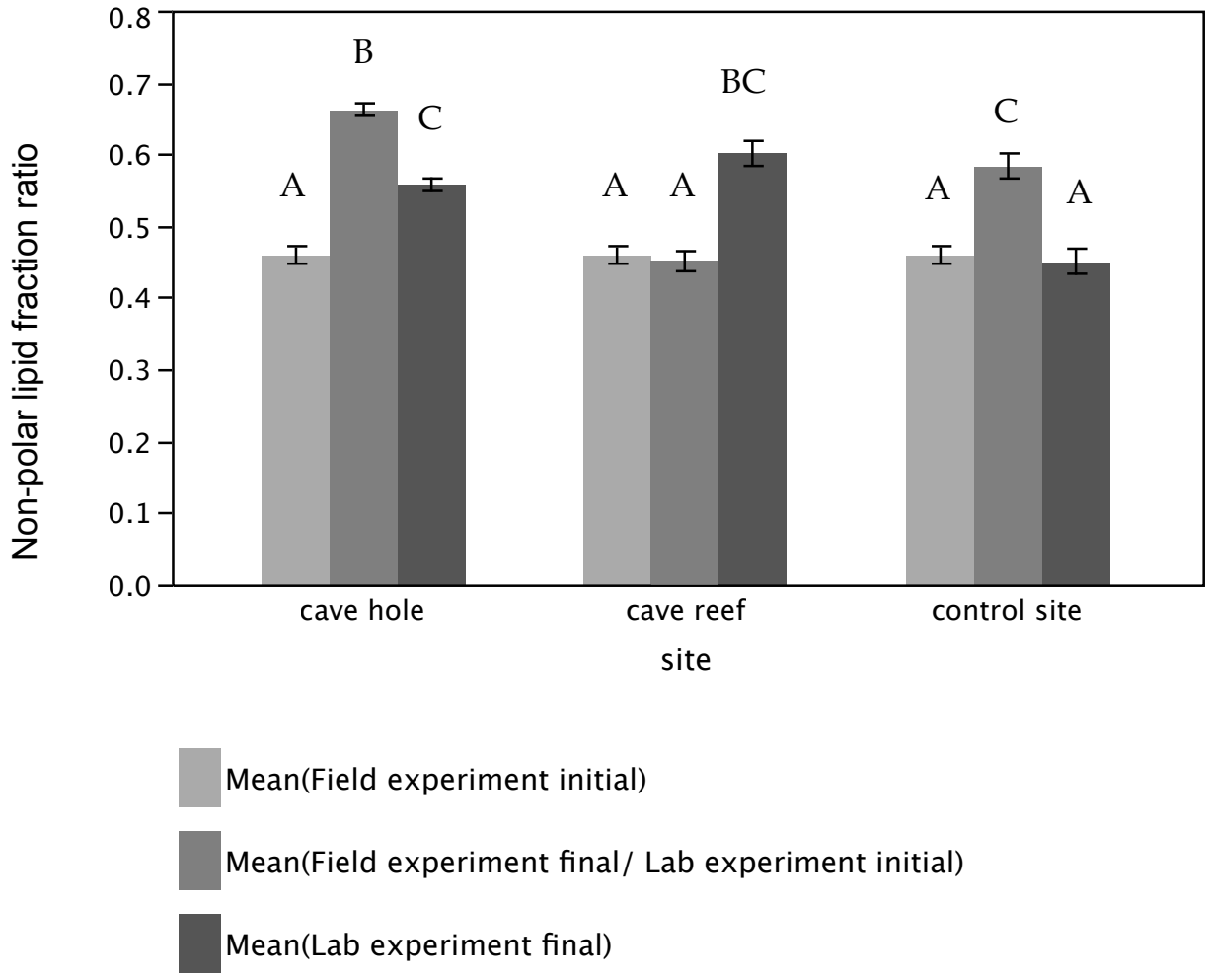
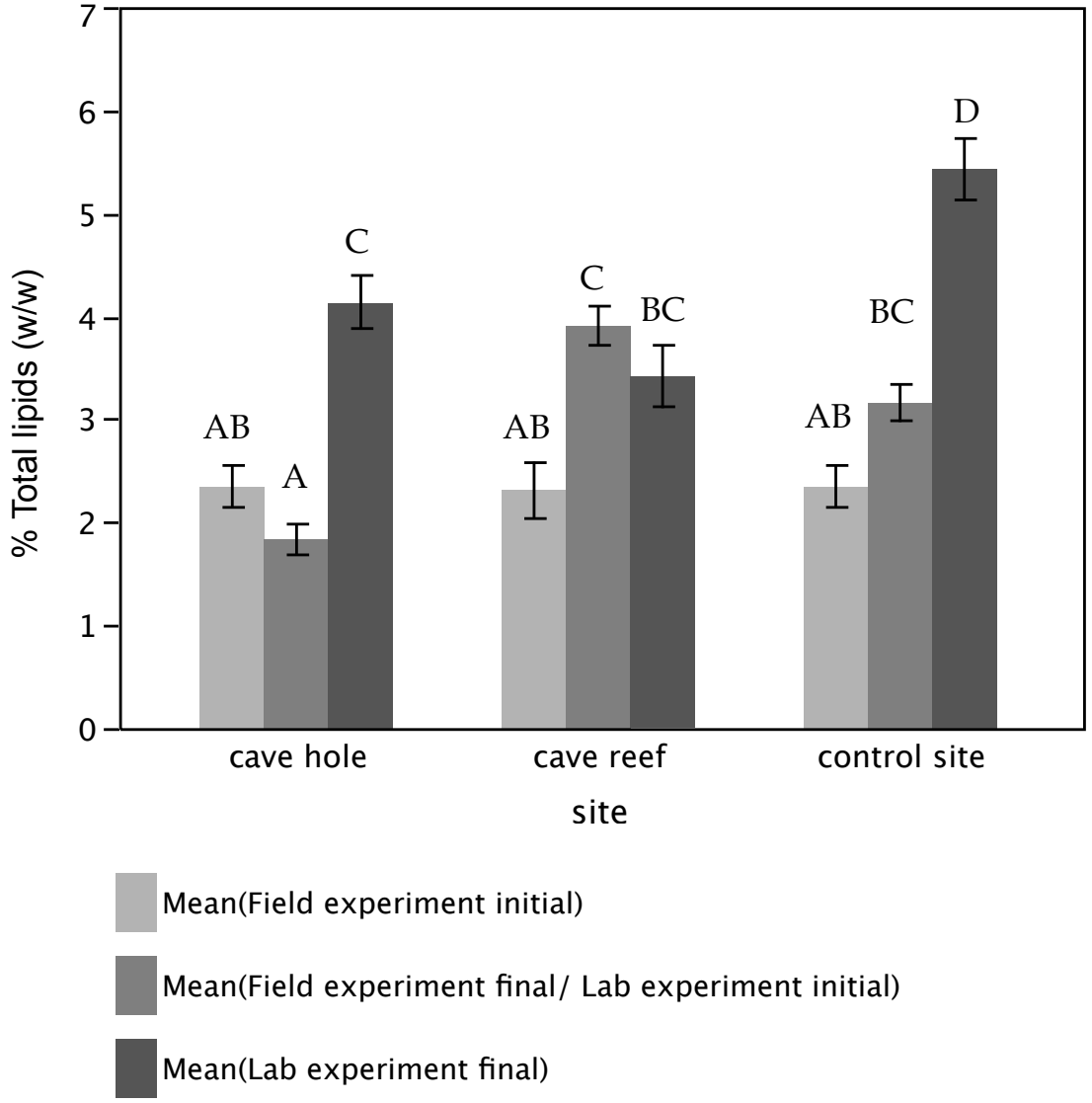


Figure 8. Total Lipids (mean ± SE; μg/g sponge) in *Chondrilla nucula* over the course of both experiments. All laboratory experiment final bars contain all 4 treatments pooled, as there were no significant effects of treatments. Means with different letters are significantly different (Repeated Measures ANOVA (P<0.0001), Tukey's HSD test (P<0.05))



Carbohydrate analysis

There were no significant differences in carbohydrate concentration among sponges from the three sites after the field experiment (repeated measures ANOVA, $P<0.05$). In the laboratory experiment, there was no significant effect of treatment, but there was a significant effect of site: carbohydrate concentration significantly decreased in Cave Reef samples, while Cave Hole and Control sites samples remained constant (repeated measures ANOVA ($P=0.0008$), Tukey's HSD test ($P<0.05$)) (figure 9).

Ash analysis

There were no significant differences in ash concentration among the three sites after the field experiment (repeated measures ANOVA, $P>0.05$). In the laboratory experiment, the Cave Reef low temperature/ambient pH treatment had significantly higher ash content than the Cave Reef high temperature/ambient pH treatment (repeated measures ANOVA ($P=0.0455$), Tukey's HSD test ($P<0.05$)) (figure 10).

Figure 9. Percent carbohydrate (mean ± SE) in *Chondrilla nucula* over the course of both experiments. All laboratory experiment final bars contain all 4 treatments pooled, as there were no significant effects of treatments. Means with different letters are significantly different (Repeated Measures ANOVA (P<0.0008), Tukey's HSD test (P<0.05))

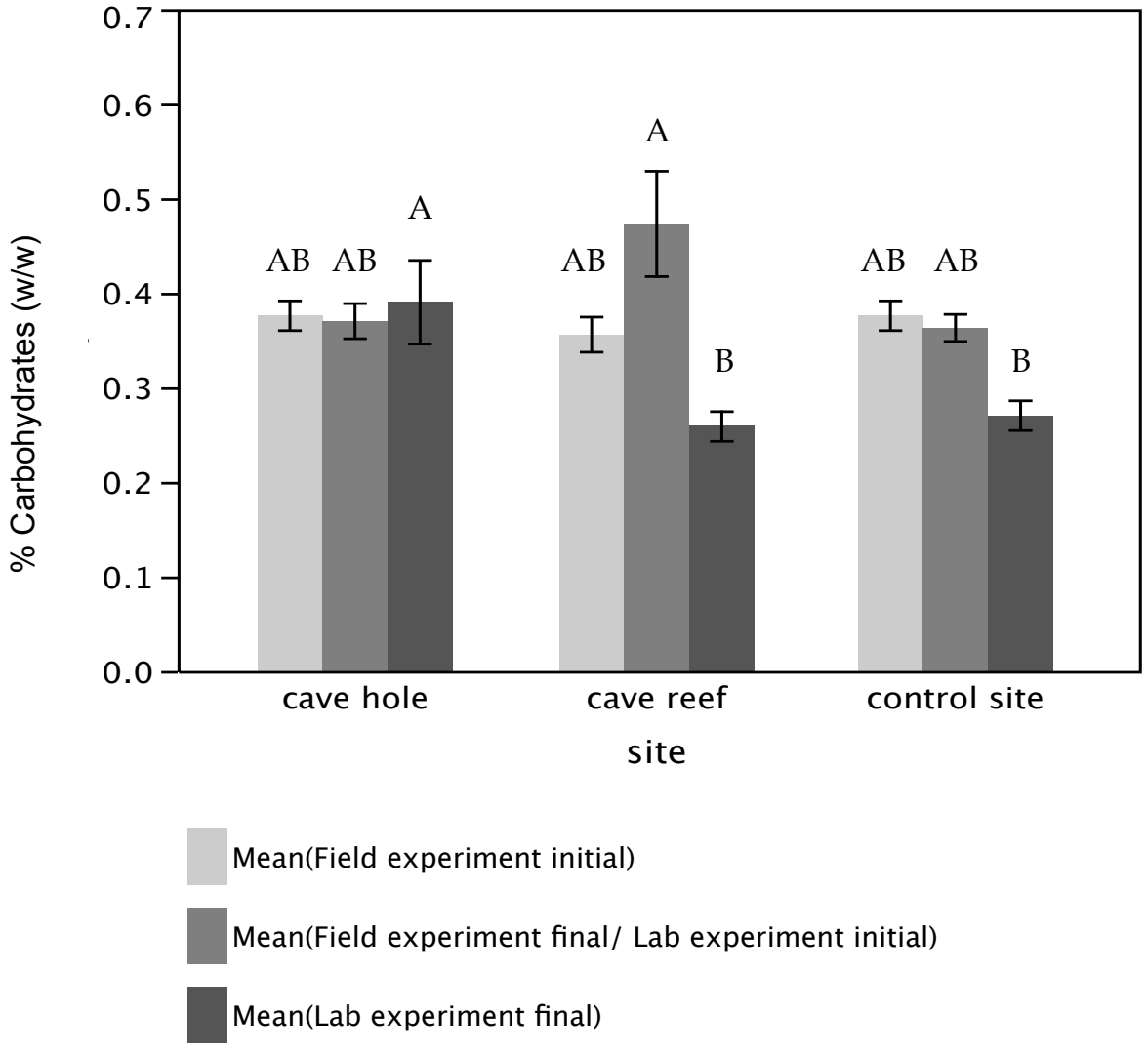
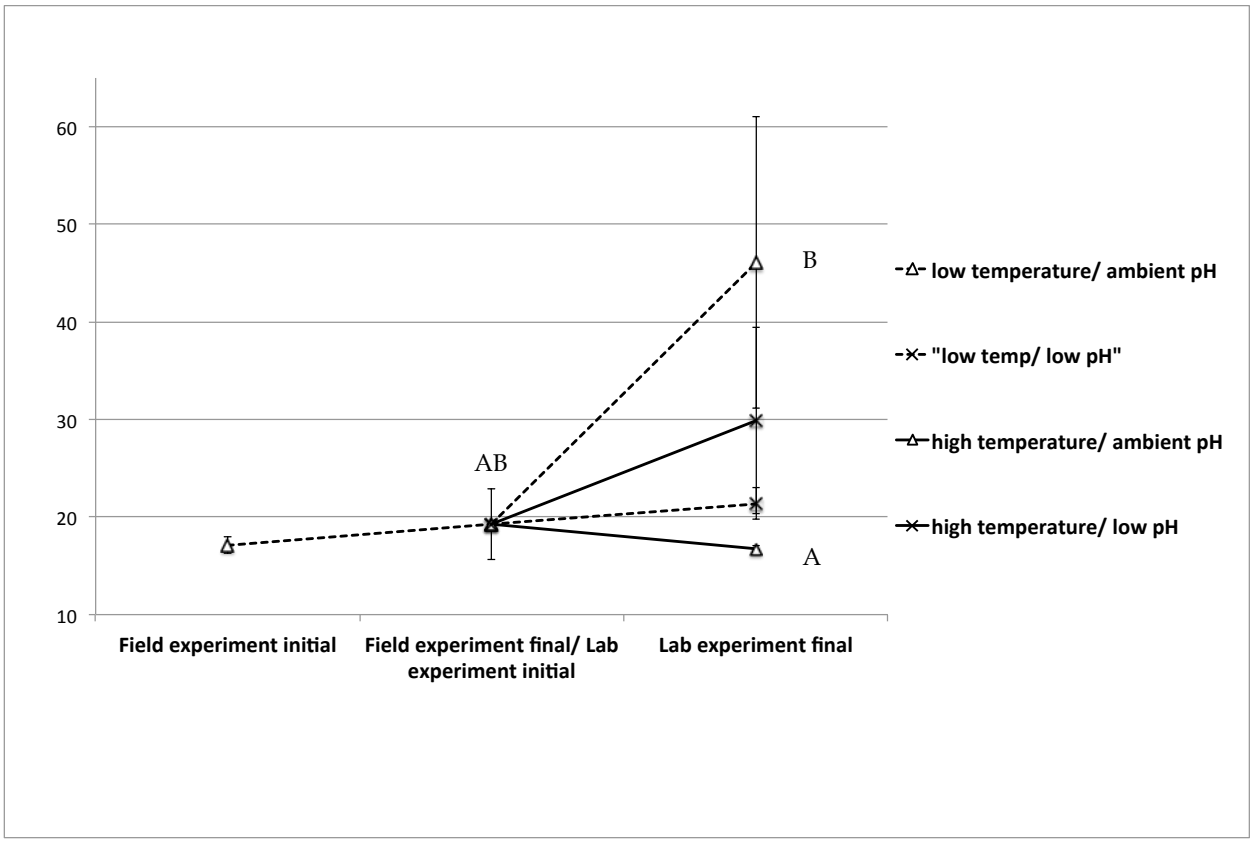


Figure 10. Percent ash (mean ± SE) in *Chondrilla nucula* over the course of both experiments for the Cave Reef site. Means with different letters are significantly different (Repeated Measures ANOVA (P=0.0455), Tukey's HSD test (P<0.05))



Refractory Material

There were no significant differences in sponge refractory material concentration among the three sites after the field experiment (repeated measures ANOVA, $P>0.05$), nor after the laboratory experiment (repeated measures ANOVA, $P>0.05$).

Energetic content

There were no significant differences in percent change in energetic content among the three sites after the field experiment (repeated measures ANOVA, $P>0.05$). In the laboratory experiment, there was no significant effect of temperature or acidification treatment, but there was a significant effect of site: energetic content significantly decreased in the Cave Reef site (repeated measures ANOVA ($P=0.0343$), Tukey's HSD test ($P<0.05$)) compared to the Cave Hole site, but not compared to the Control site (figure 11). Figures 12 and 13 depict the percent composition of each of the biochemical components in *Chondrilla nucula*, and the energetic contributions of each.

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Figure 11. Percent change in total energetic content (mean ± SE) in *Chondrilla nucula* after the laboratory experiment. Means with different letters are significantly different (One way ANOVA (P=0.0343), Tukey's HSD test (P<0.05))

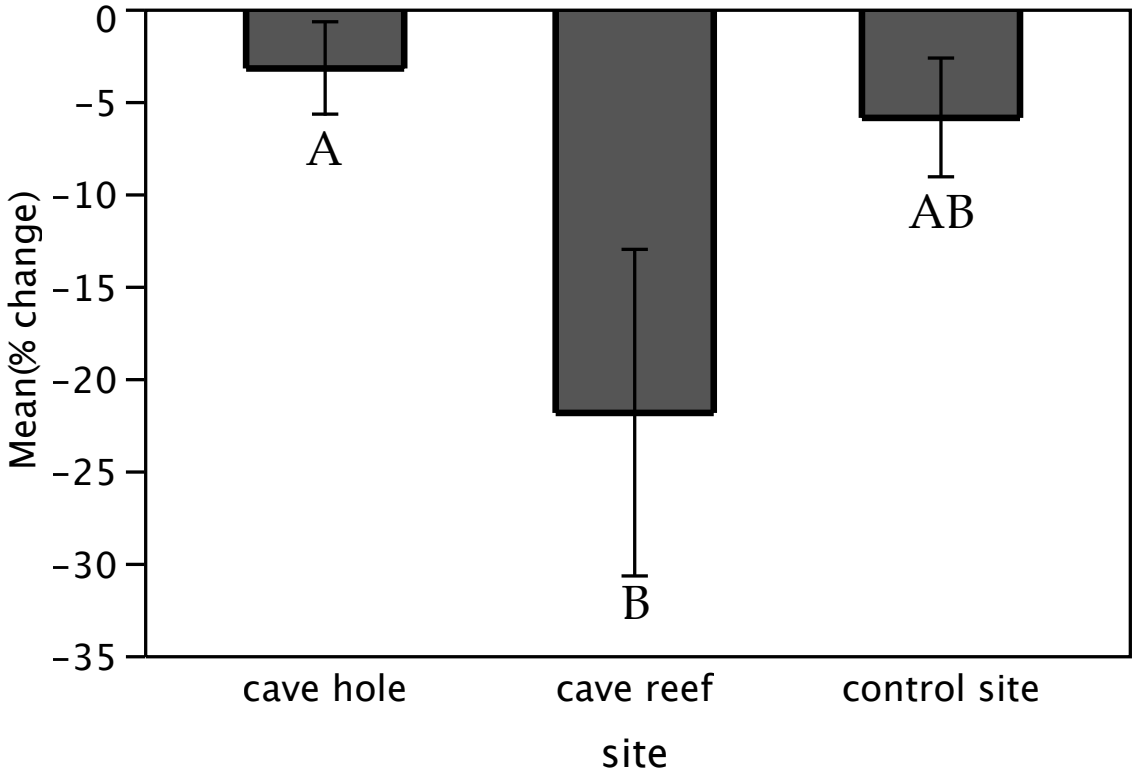
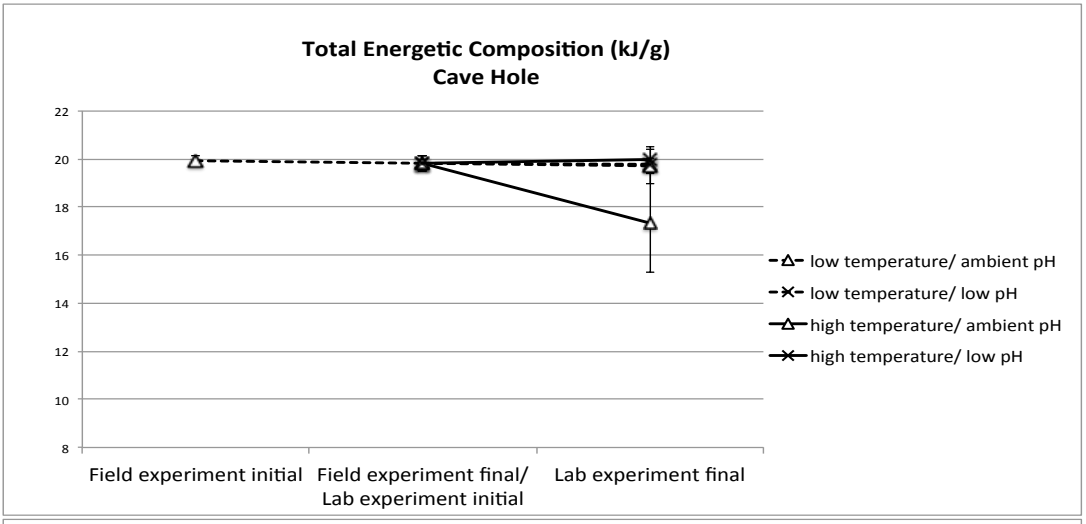
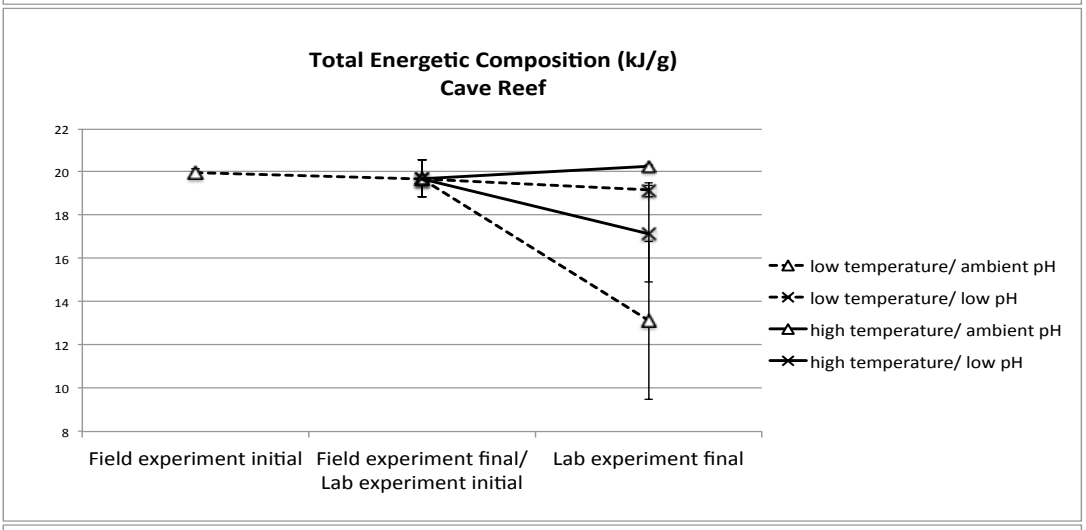


Figure 12. Total energetic composition of refractory material, soluble protein, ash, lipid, and carbohydrate (mean ± SE; w/w sponge) in *Chondrilla nucula* from both experiments. No significance was observed (Repeated measures ANOVA, P>0.05)





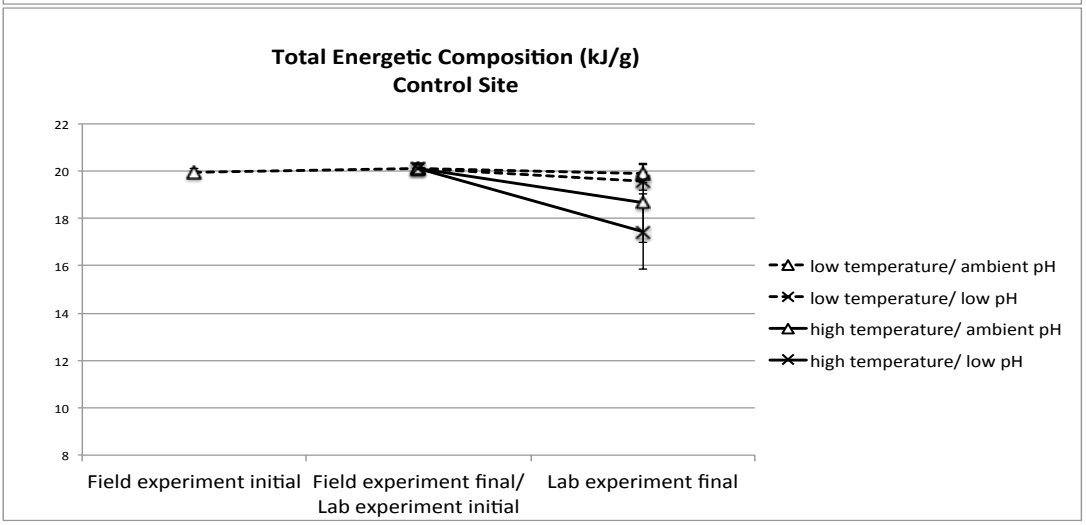
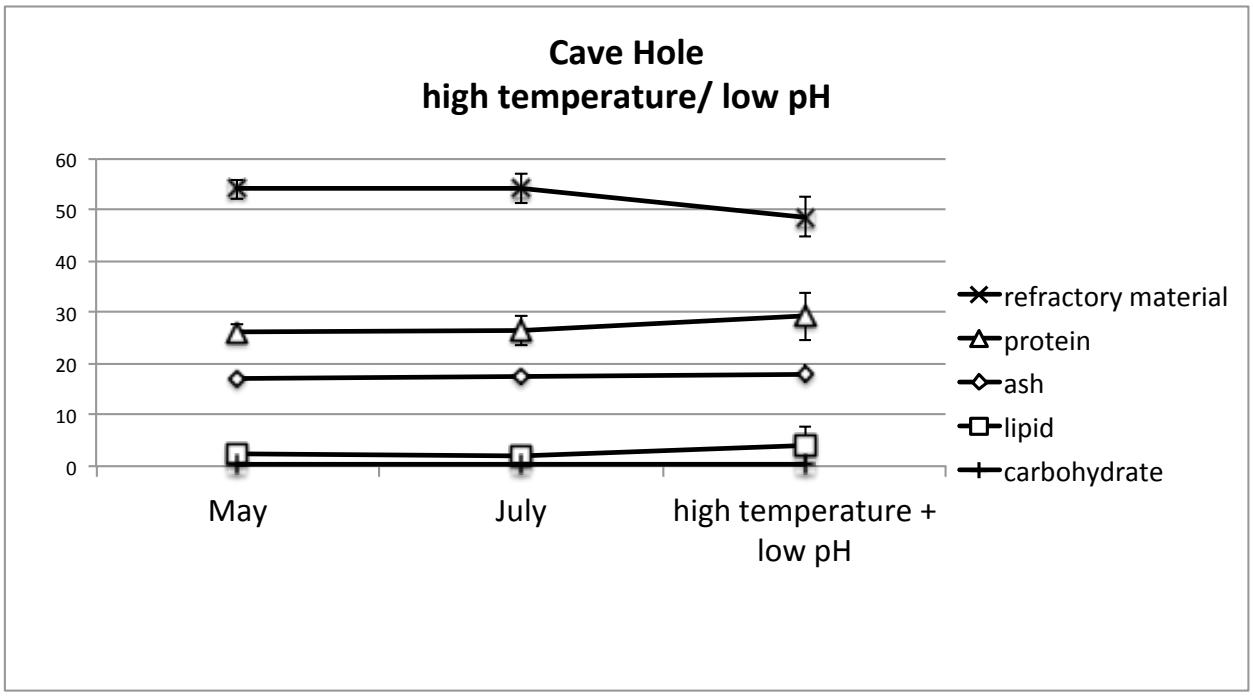
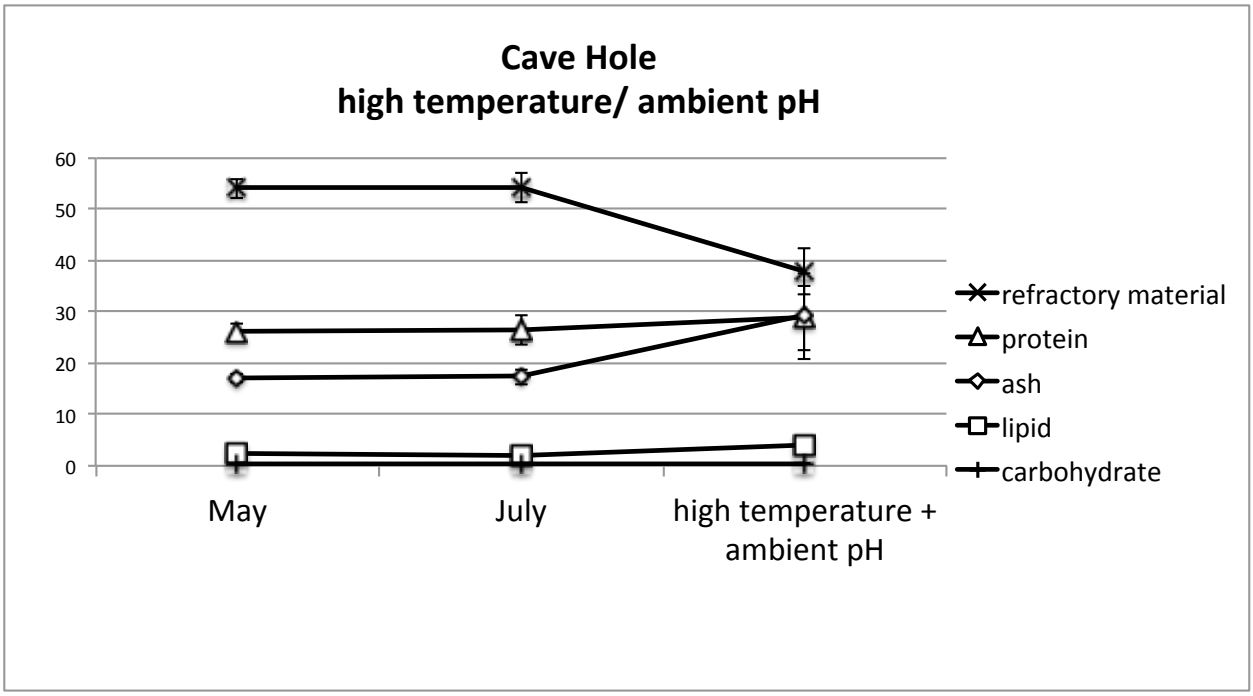
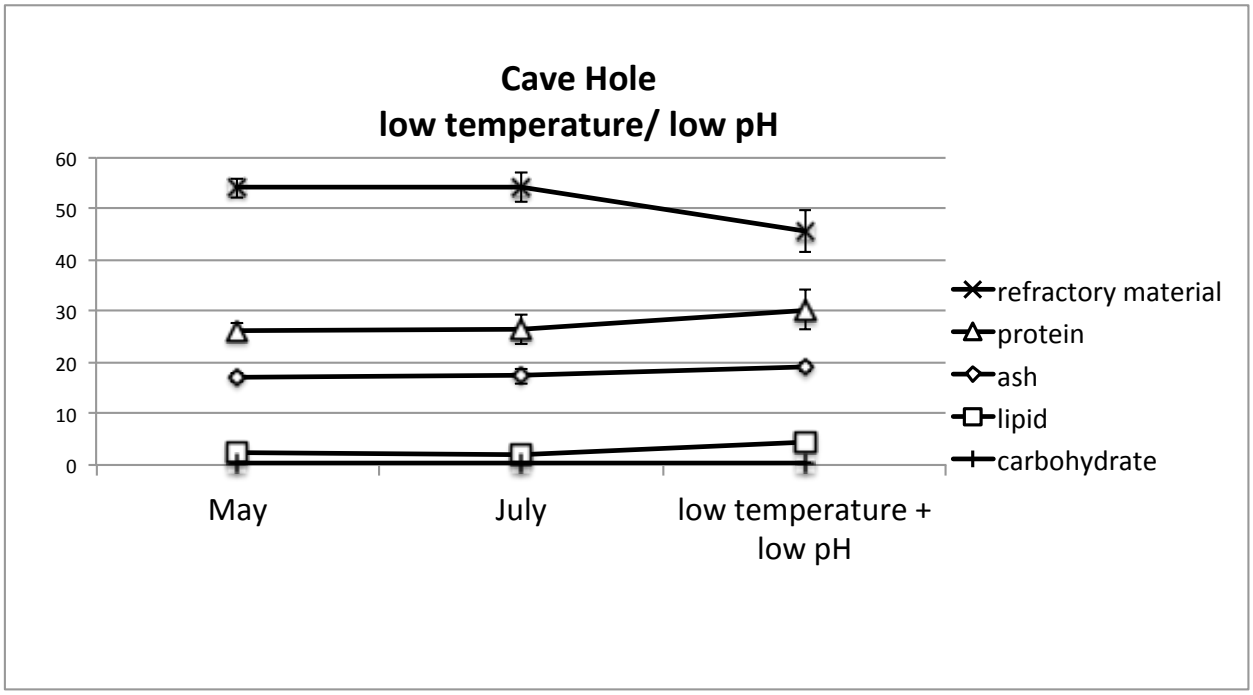


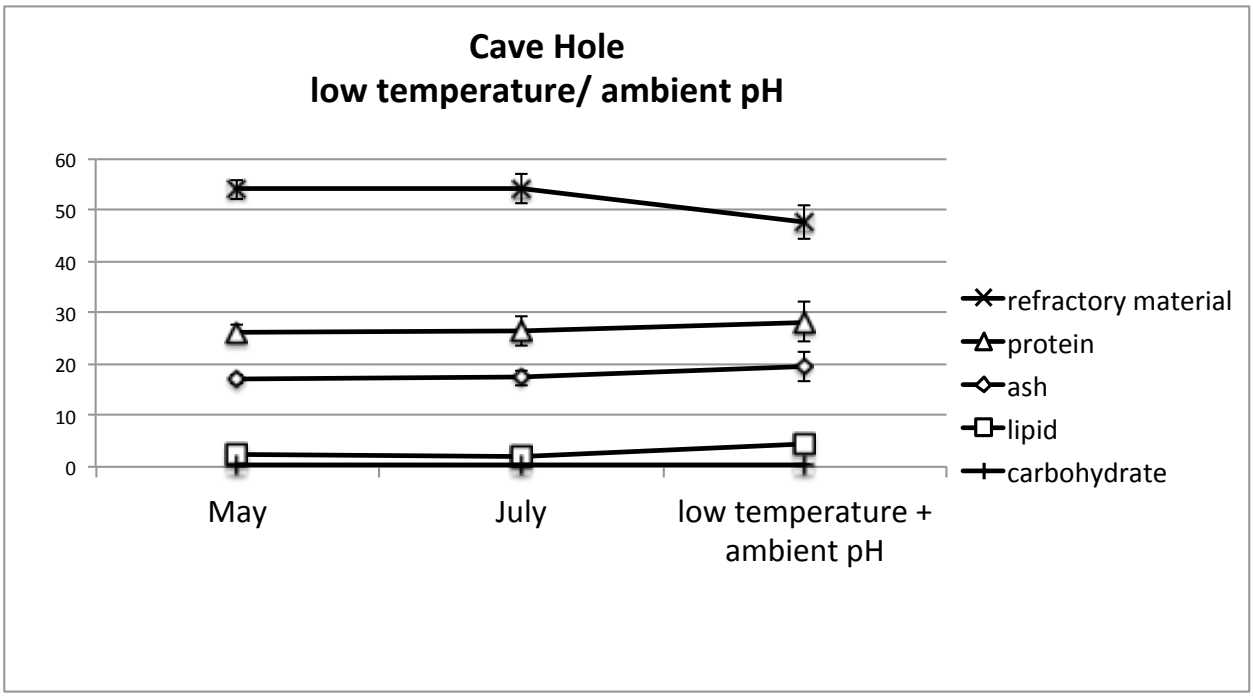
Figure 13. Percent composition: refractory material, soluble protein, ash, lipid, and carbohydrate (mean ± SE) in *Chondrilla nucula* from both experiments. A). Cave Hole. B). Cave Reef. C). Control site

A). Cave Hole

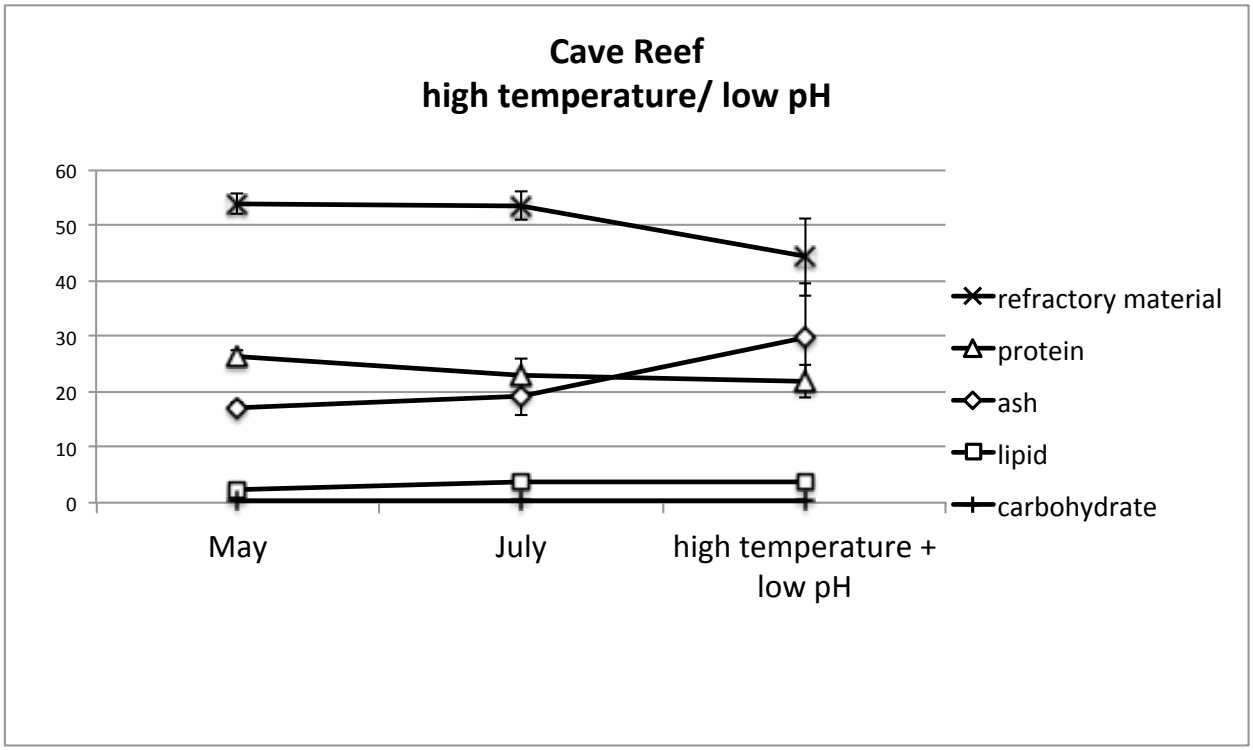





Cave Hole
low temperature/ low pH
60
50
40
30
20
10
0
May
July
low temperature + low pH
refractory material
protein
ash
lipid
carbohydrate


Cave Hole
low temperature/ ambient pH
60
50
40
30
20
10
0
May
July
low temperature + ambient pH
refractory material
protein
ash
lipid
carbohydrate

B). Cave Reef


Cave Reef
high temperature/ low pH
60
50
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30
20
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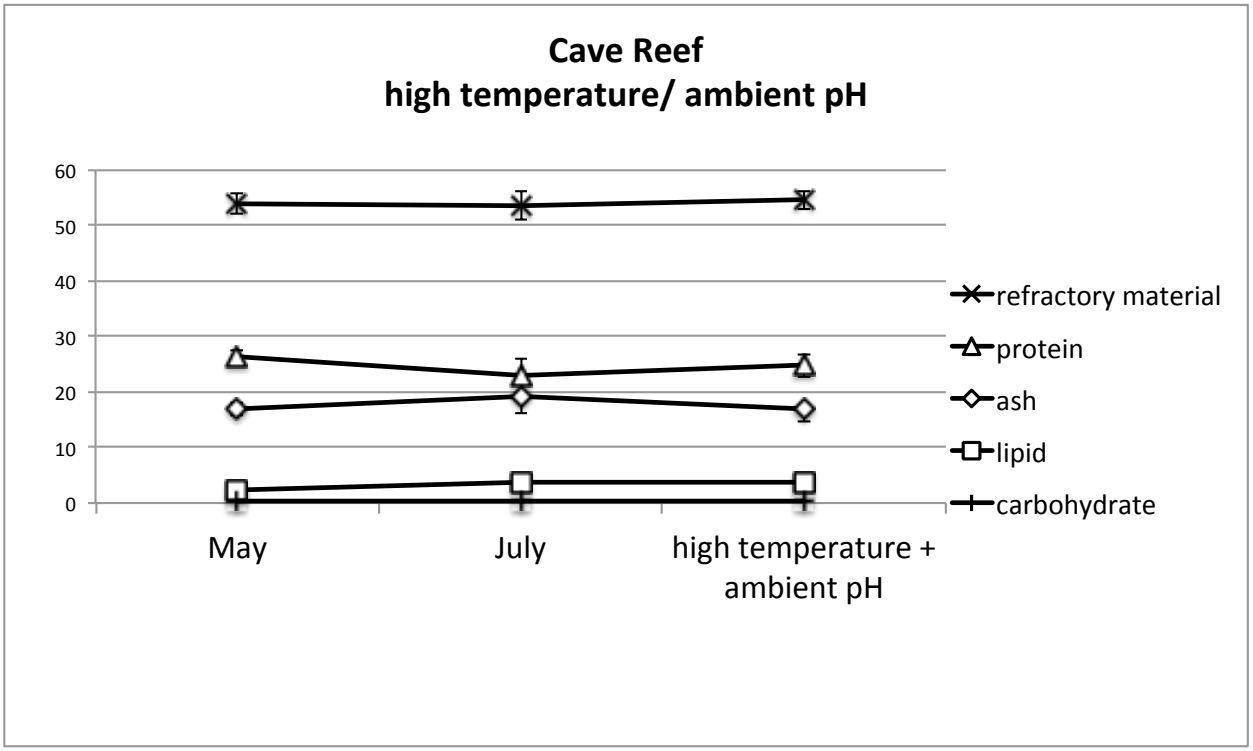
0
May
July
high temperature + low pH
refractory material
protein
ash
lipid
carbohydrate


Cave Reef
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May
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high temperature + ambient pH
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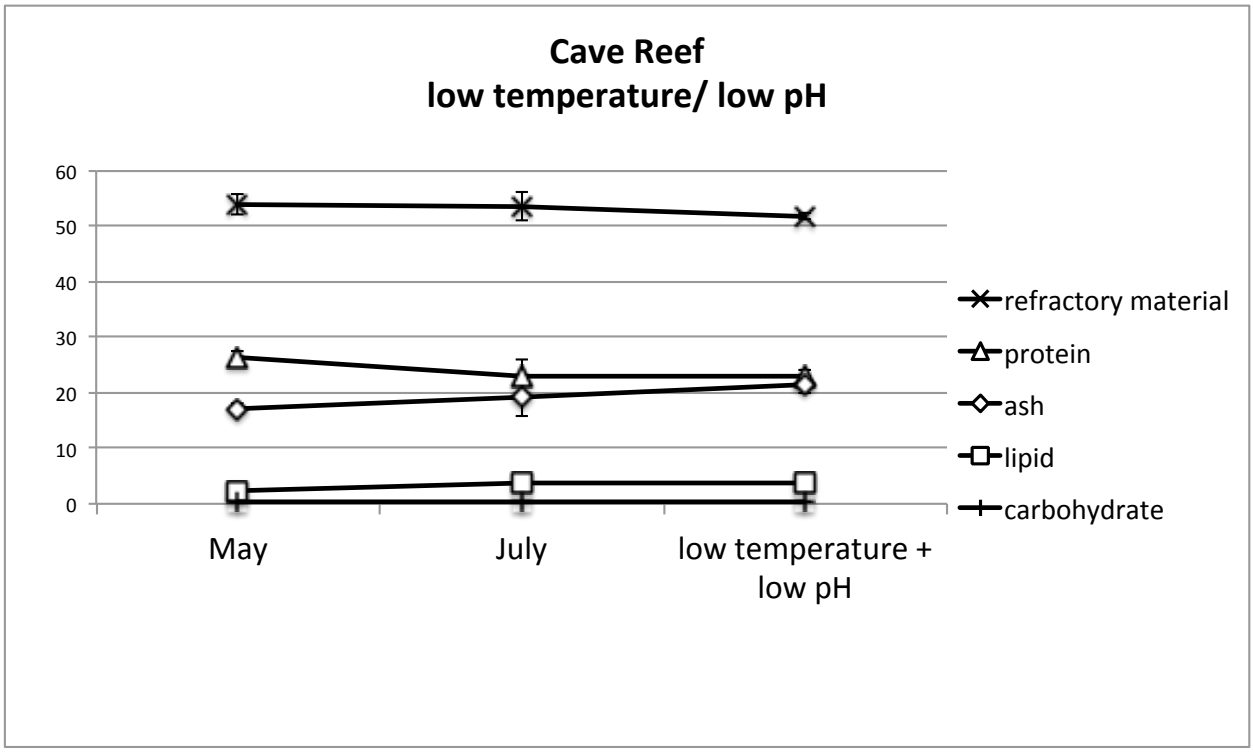
protein
ash
lipid
carbohydrate


Cave Reef
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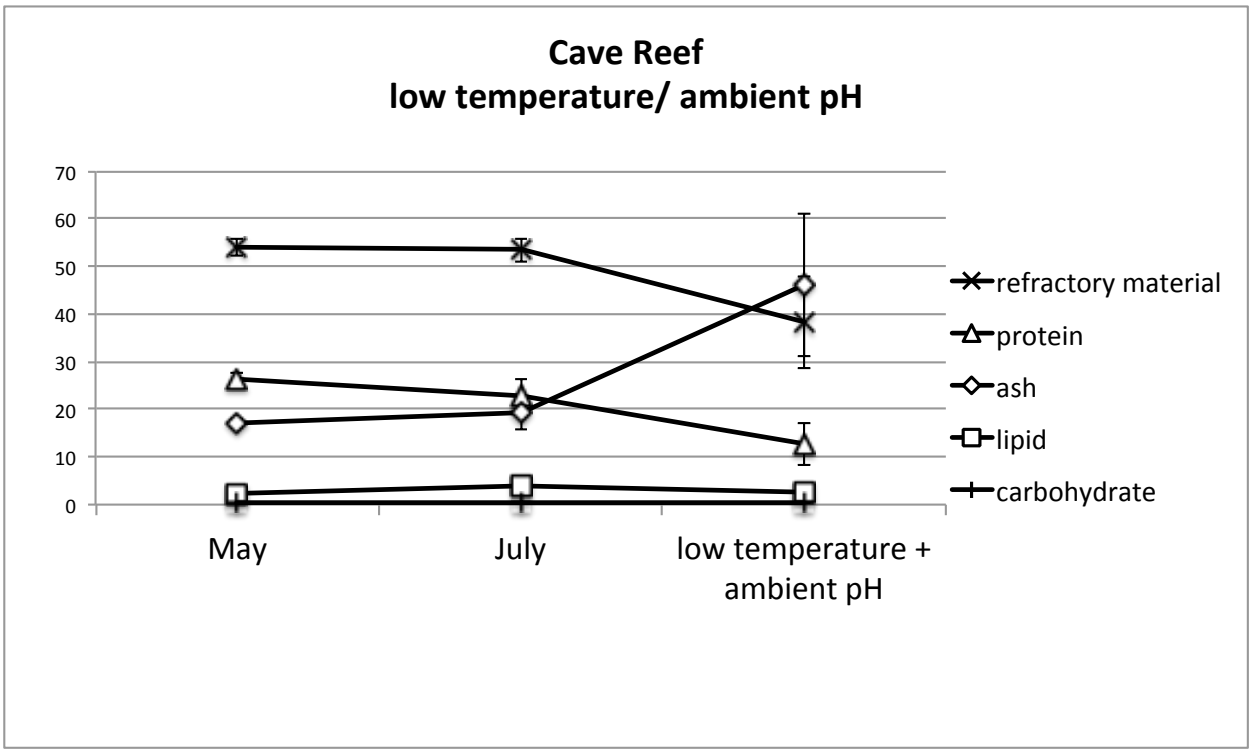
May
July
low temperature + low pH
refractory material
protein
ash
lipid
carbohydrate


Cave Reef
low temperature/ ambient pH
70
60
50
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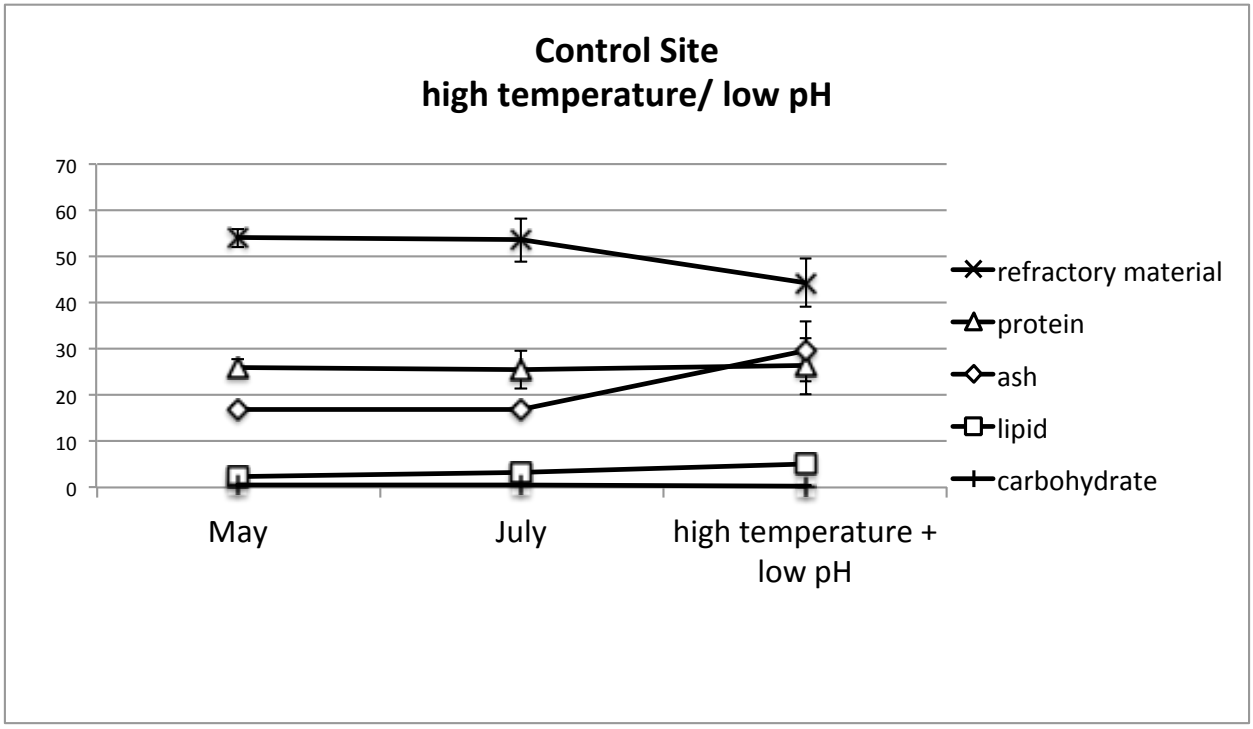
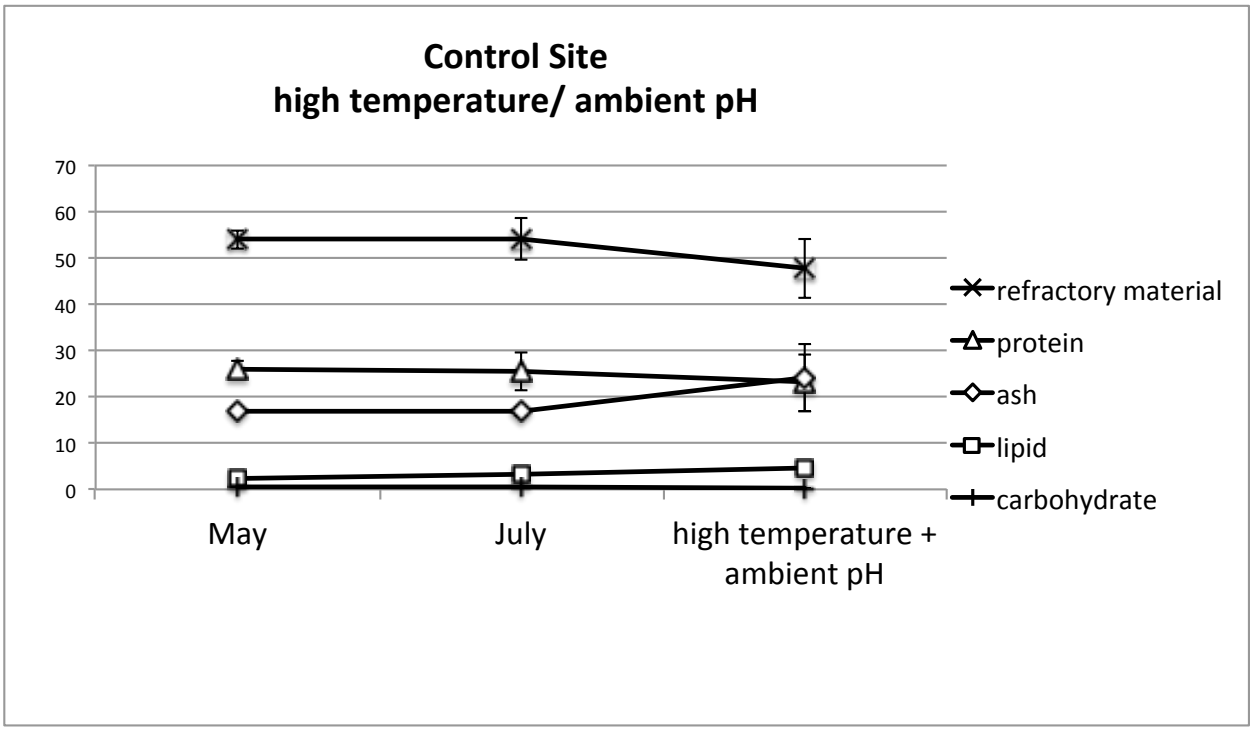
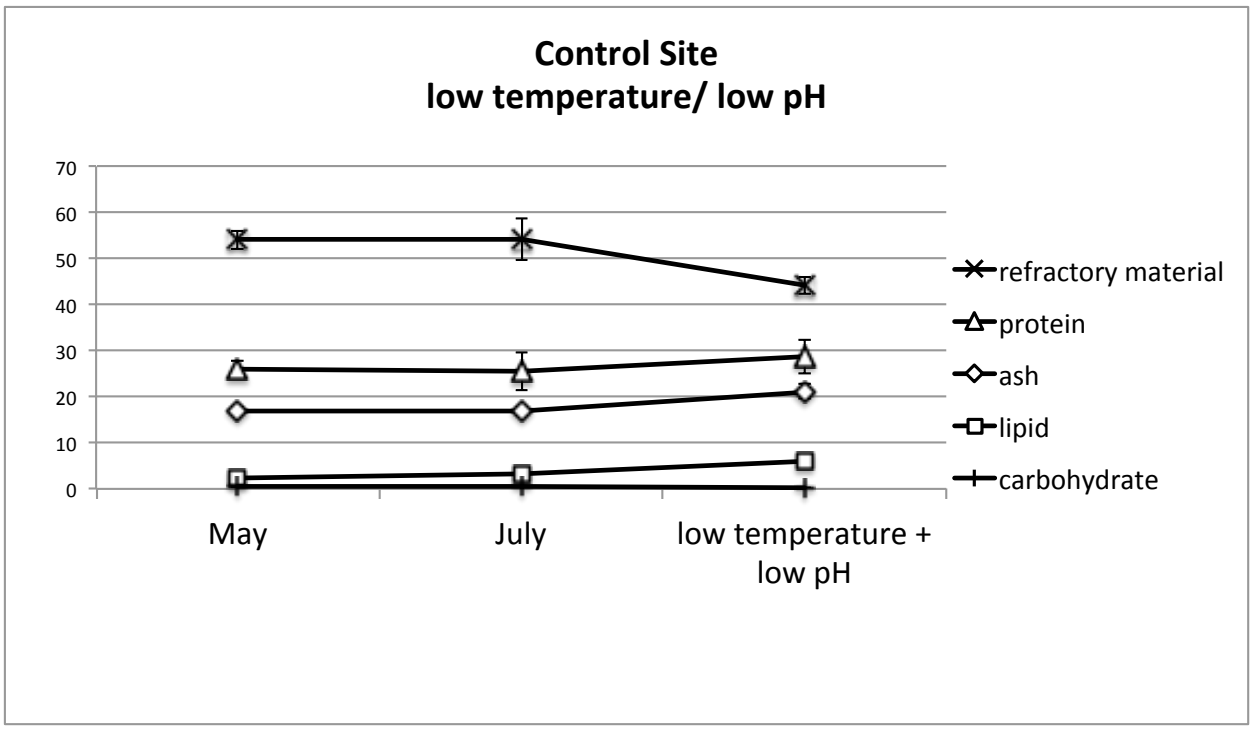
30
20
10
0
May
July
low temperature + ambient pH
refractory material
protein
ash
lipid
carbohydrate

C). Control Site






Control Site
low temperature/ low pH
70
60
50
40
30
20
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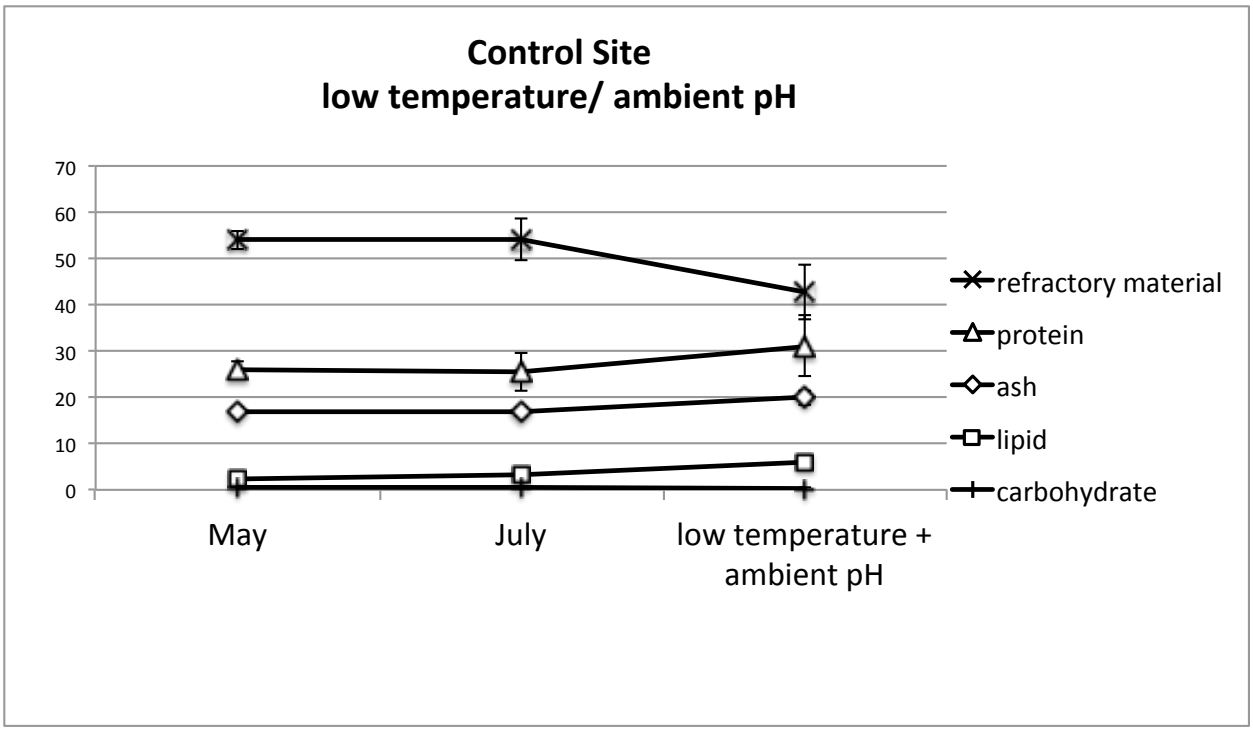
May
July
low temperature +
low pH
refractory material
protein
ash
lipid
carbohydrate


Control Site
low temperature/ ambient pH
70
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May
July
low temperature +
ambient pH
refractory material
protein
ash
lipid
carbohydrate

4. Discussion

1. Chondrilla nucula's acclimation in the field

Chondrilla nucula is occasionally found in some marginal systems such as marine caves, which are subjected to a myriad of variable conditions such as low light, low oxygen levels, low temperatures, high levels of nutrients and environmental hypercapnia (Sket 1996, Lapointe et al. 2003). In the field transplant experiment, no significant changes occurred in fluorescent yield or chlorophyll *a* concentration, indicators of cyanobacterial abundance or condition, although some studies have shown photoacclimation can influence chlorophyll *a* concentrations within cyanobacterial cells (MacIntyre et al 2002). This absence of change even under variable acidic conditions in the Cave Hole signifies a degree of acclimation of the photosynthetic cyanobacteria in the sponge tissue. The lack of significant changes in carbohydrate, soluble protein, refractory material content at for any of the sites also signals a degree of acclimation of the sponge tissue, as sponges from all sites maintained energetic yields from respective biochemical constituents.

In contrast to the cyanobacterial symbionts, there were significant changes in the lipid components in the sponge itself. Numerous studies have found lipids in corals to be influenced by various environmental stressors including thermal stress, bleaching, and irradiance (Harland et al. 1992, Oku et al. 2003, Grottoli et al. 2004, Imbs & Yakovleva 2011). Fewer studies have examined lipid content in sponges, but Chanas & Pawlik (1995) documented higher levels of total lipid content in chemically defended sponges compared

to undefended sponges. Polar lipids and sterols are the structural basis of cell membranes, whereas non-polar lipids (triacylglycerols, wax, and sterol esters) serve as storage lipids and determine energy balance for many marine invertebrates (Imbs & Yakovleva 2011). Because significant increases in non-polar lipid fraction ratios were observed in the Cave Hole as well as the Control site, and because both sites had approximately the same depth, this change can probably be attributed to seasonality, as opposed to a site effect (Ben-David-Zaslow & Benayahu 1999). In fact, linear regression shows that over the course of the field experiment, temperatures did significantly increase at all of the sites (figure 5). Thus, the question is why an increase in non-polar lipid fraction ratio was not observed in sponges at the Cave Reef site as well. The Cave Reef site was subjected to many of the same conditions as the Cave Hole site, as it was directly outside the mouth of the cave. However, the acidic water of the Cave Hole diluted rapidly upon exiting the cave, and the Cave Reef site maintained a constant oceanic pH levels of 8.2, which was also the same pH as the Control site (figure 3). A significant factor may be the shallow depth of the Cave Reef site, which equated to approximately twice the irradiance exposure than the Cave Hole and Control sites (data from Lesser et al. 2010) (figure 4), even though temperature was comparable to the control site (figure 5). Another possible explanation is that the Cave Reef site did not receive as much flow as the other two sites, and instead exhibited a pooling effect with the Cave Hole flushing water out onto the Cave Reef, and the open ocean pushing water toward shore. Alternatively, a combination of these or other unmeasured variables may explain this result.

Chondrilla nucula contains photosynthetically active endosymbiotic cyanobacteria, that have a flexible photosynthetic apparatus that enables them to thrive in a wide range of

irradiances, including ocean depths receiving only 1% of surface irradiance, as well as in full sunlight (Furnas & Crosbie 1999). Phycobilisomes are the light harvesting antennae of the Photosystem II in the cyanobacteria. In addition to changing the relative ratios of phycobiliproteins, adaptation may be achieved by changing the concentration and size of light-harvesting complexes (Bennett & Bogorad 1973, Wyman & Fay 1986, Golubic et al. 1999, Moore & Chisholm 1999). Cyanobacteria avoid photoinhibition by moving phycobilisomes around on the thykaloid membrane, frequently detaching and re-associating with reaction centers, changing their distribution between Photosystem II and Photosystem I (Josua & Mullineaux 2004). In very intense light these state transitions allow cyanobacteria to maximize their use of available energy, and reduce photodamage to the Photosystem II (Usher 2008). Lipids have been implicated in playing a role in controlling phycobilisome reaction center interactions (Sarcina et al. 2001). Thus the lack of increase in non-polar lipid fraction ratio in the Cave Reef sponges may be attributed to the activities of the cyanobacteria maintaining a level of health status, as there were no changes in chlorophyll *a* or fluorescent yield. In actuality, there must have been an increase in both non-polar lipids and polar lipids in the Cave Reef sponges, as the total lipids increased; this helps explain why the ratio did not change. In contrast, there were no changes in total lipid content at the Cave Hole and Control sites. Therefore, the fundamental difference of the Cave Reef site actually may lie in the increase of polar lipids, as opposed to a lack of increase in non-polar lipid fraction ratio. Phycobilisomes have been suggested to lack any integral membrane domain, and instead interact with polar lipid head-groups at the membrane surface (Yu et al. 1999, O'Toole et al. 1999, Sarcina et al. 2001, Sarcina et al. 2003). This interaction with the polar lipid head groups may partially

explain the increase in polar lipids in the Cave Reef site. Sarcina et al. (2003) demonstrated that modification of membrane lipid composition by increasing diunsaturated fatty acids strongly influences ability to recover from photoinhibition. Research on the influence of polar lipids on membrane fluidity is lacking, so it is difficult to attribute any increase in ability to prevent or recover from photoinhibition on polar lipids, but perhaps this may partially explain the increase in polar lipids in the Cave Reef sponges.

2. Chondrilla nucula's response in the laboratory experiment

In the laboratory experiment, temperature was a greater stressor for *Chondrilla nucula* than acidification. Chlorophyll *a* is a major subunit of the photosystem II in cyanobacteria. Measurement of chlorophyll *a* is an indicator of how well the cyanobacteria can absorb light for photosynthetic processes (Duysens & Sweers 1963), and chlorophyll *a* concentrations are directly correlated with the abundance of cyanobacteria within a sponge (Wilkinson 1983, Rai 1990, Thacker et al. 2007). Thus, the increase in chlorophyll *a* that was observed in the Cave Reef low temperature + ambient pH treatment may indicate an increase in cyanobacteria, although at the moment this is unknown, as the cyanobacteria analysis has not been completed. Such an increase may be due to the transition of high irradiance conditions at the Cave Reef site in the field to much lower irradiance conditions in the laboratory experiment. As irradiance decreased, there may have been a need to increase chlorophyll *a*, as the amount of light available to each cell decreased. It is interesting that an increase was not seen in the Cave Reef high temperature treatment, perhaps suggesting that high temperature was able to inhibit the cyanobacteria from

producing more chlorophyll *a.* The fact that any acidification treatments did not significantly alter chlorophyll *a* concentration implies that these cyanobacteria were not stressed to the point of decreased efficacy, but instead were tolerant to the acidification. The lack of significant change in fluorescent yield across all treatments and sites suggests the photosystem II was able to cope and maintain normal functionality throughout the experiment.

The increase in percent ash that was observed in the Cave Reef low temperature + ambient pH treatment was only significant compared to the Cave Reef high temperature + ambient pH treatment (figure 10). In sponges, ash is a measure of structural elements, largely represented by glass spicules or carbonate sand in a few species such as *Dysidea janiae* (Chanas & Pawlik 1995). It has been suggested that spicules may serve a defensive function in sponges (Randall & Hartman 1968, Birenheide et al. 1993, Hill & Hill 2002), although many studies have found spicules to be non deterrent (Chanas & Pawlik 1995, 1996, Waddell & Pawlik 2000a,b). Spicules of *Chondrilla* spp. are round spherasters, and studies have demonstrated them to be non deterrent to predators (Ferguson & Davis 2008). Thus the increase in ash most likely does not represent increase in defense condition.

For carbohydrates, non-polar lipids, and total lipids, there were no significant effects of treatment, but there were significant effects of site. The decrease in carbohydrates in the Cave Reef site (all treatments pooled) suggests a decrease in energetic status in those sponges (Slattery & McClintock 1995, Ben-David-Zaslow & Benayahu 1999). However, the increase in non-polar lipid fraction ratio in the Cave Reef site (all treatments pooled) suggests an increase in energy stores (Imbs & Yakovleva 2011). Perhaps these

seemingly contradictory results can be once again attributed to the cyanobacteria and interactions of the phycobilisomes with polar lipids. The transition of high irradiance conditions in the field to moderate irradiance conditions in the laboratory perhaps may have caused a decrease in the amount of polar lipids necessary for phycobilisome mobility, thus resulting in an increase non-polar to polar lipid fraction ratio. In fact, there was no significant change in the total lipids of the Cave Reef site, indicating that the increase in non-polar lipid fraction ratio was probably due to a decrease in polar lipids, not an increase in non-polar lipids. Thus, lipid data does not contradict the significant decreases seen in carbohydrates. This transition from the field to laboratory irradiance conditions perhaps can also explain the results in the Cave Hole (all treatments pooled) and Control sites (all treatments pooled) sponges. For both sites, the non-polar lipid fraction ratio significantly decreased, while total lipids increased for both sites. This indicates a net increase in polar lipids in both sites. Additionally, there may be other unmeasured variables that can explain these changes from field to laboratory conditions.

Although there was no treatment effect in total energetic composition, there was a site effect: the Cave Reef sponges (all treatments pooled) significantly decreased compared to the Cave Hole sponges (all treatments pooled) after the laboratory experiment. This is most likely due to the decrease of refractory material, which is assumed to be insoluble protein (Lawrence & Kafri 1979).

Overall, biochemical composition *Chondrilla nucula* was not altered by effects of acidification in the field or by acidification and temperature treatments in the laboratory experiment. The significant biochemical effects that were observed were due to site, most likely due to irradiance, reduced water flow, or other unknown conditions at the Cave Reef

site. The tolerance of *Chondrilla nucula* to acidified conditions projected for the near future, even under the added stress of elevated seawater temperature, may indicate a potential for a shift from coral dominated reefs to sponge dominated reefs, assuming that *Chondrilla nucula* is a reliable model sponge species. This study predicates a good framework and stepping off point into the assessment of sponges and future climate change conditions.

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CHAPTER III

SUMMARY AND CONCLUSIONS

1. Environmental Hypercapnia

While a majority of research on ocean acidification has been conducted in laboratory settings, it is worth noting that there are a few marine environments that are naturally acidified. Such systems are usually characterized by enclosed spaces, with high metabolic activities. Examples of such systems include marine caves (Baldini et al. 2006) and tide pools (Truchot 1986). Marine caves are naturally acidified environments due to high metabolic activities in small, confined spaces, and dissolution of limestone (Barton & Northup 2007). Previous studies have largely found that sponges and algae are the major inhabitants in and around marine caves (Corriero et al. 2000, Bell 2002). Algae cover decreases as the light gradient decreases, while the number of species and abundance of sponges are greatest in the first part of the caves (Corriero et al. 2000, Bell 2002).

In tidepools, where plant life is abundant, photosynthesis usually surpasses respiratory activity during the day, and when tidepools are isolated from open sea at low tide, this leads to elevated pO_2 and decreased pCO_2 levels. At night, when photosynthesis is absent, respiratory activity causes the opposite effect: elevated pCO_2 and decreased pO_2 levels (Truchot 1986).

Other naturally acidified marine environments exist that are not restricted to confined spaces. Submarine volcanic carbon dioxide vents exhibit naturally high levels of pCO_2 (Hall-Spencer et al. 2008, Cigliano et al. 2010). Natural CO_2 flux from submarine volcanic vents and high heat flow areas amounts to less than 0.5% of anthropogenic emissions to the global carbon budget, but can alter local ocean chemistry (Dando et al. 1999, Hall-Spencer 2008). Studies on volcanic CO_2 vents have shown a decrease in the

abundance of calcifying organisms such as scleractinian corals, sea urchins, and coralline algae (Hall-Spencer et al. 2008), but an increase in some non-calcifying organisms such as polychaetes (Cigliano et al. 2010). Other studies have found that these vents caused a decrease in coral diversity, recruitment and abundances of structurally complex framework builders, but no change in coral cover, until conditions fell below pH 7.7, at which point all reef development ceased (Fabricius et al. 2011).

Deep reefs have also been documented in exhibiting decreased pH levels compared to oceanic conditions (Slattery & Lesser 2012). High concentrations of macro and microorganisms in deep reefs, combined with the lack of water exchange at those depths, contribute to environmental hypercapnia (Slattery & Lesser 2012).

According to the IPCC "business as usual" IS92a scenario, oceanic pH is expected to reach 7.9 by 2050, and 7.8 by 2100. The pH of the study site, Norman's Pond Cave, fluctuated on a semi-diurnal basis, reaching 7.7 at low tide and rebounding to 8.2 at high tide (figure 3). Studies on volcanic vents are usually conducted at pH levels of approximately 7.8, but can be as low as 7.4 (Hall-Spencer et al. 2008, Cigliano et al. 2010, Fabricius et al. 2011). These systems that exhibit environmental hypercapnia may be a comparable model for future ocean acidification conditions, but they do contain additional, potentially confounding variables. For example, besides acidified conditions, marine caves are also characterized by a myriad of additional variables such as low light levels, low oxygen levels, low temperatures, and high levels of nutrients (Sket 1996, Lapointe et al. 2003). Furthermore, marine caves, such as Norman's Pond Cave, exhibit a tidally driven variability in pH, as opposed to predicted conditions of ocean acidification (i.e., constant, low pH). Volcanic vents also have natural limitations. Fabricius et al. (2011) found that

volcanic vents in Papua New Guinea are surrounded by areas with ambient pH, supplying larvae of sensitive taxa for re-colonization, and hence partly offsetting the negative effects of ocean acidification on recruitment. Additionally, these vents exhibit a temporal variability in pH, partly due to wave mixing (Cigliano et al. 2010, Fabricius et al. 2011). Although not completely analogous to global-scale ocean acidification, these systems are useful in examining the long-term effects of ocean acidification on marine ecosystems (Cigliano et al. 2010, Fabricius et al. 2011).

2. Acid-Base Regulation and Metabolic Depression

Many studies have concluded that ocean acidification inhibits calcification in many organisms (Riebesell et al. 2000, Orr et al. 2005, Kuffner et al. 2007, Anthony et al. 2008). However, ocean acidification may have detrimental impacts on non-calcifying organisms as well. Cellular processes mediating metabolic depression have been extensively studied (Hand & Hardewig 1996, Guppy & Withers 1999, Storey & Storey 2007, Gutowska et al. 2008). Hypercapnia has been demonstrated to induce metabolic depression (Barnhart 1989, Rees & Hand 1990, Pörtner et al 2004), and numerous studies have supported this finding in marine organisms (Pörtner et al. 1998, Pörtner et al. 2000, Michaelidis et al. 2005). Although this process is not entirely understood, Pörtner et al. (2000) suggests that decreasing extracellular pH slows down the rate of H^+ equivalent ion exchange between the extracellular and intracellular space, which in turn decreases the work load of Na^+/K^+ ATPase in maintaining the electrochemical gradient across epithelial membranes. This could effectively lower energy requirements of cellular acid-base regulation, however, it

could also reduce rates of protein synthesis and eventual depression of somatic growth rates has been demonstrated under low pH conditions (Smith et al. 1996, Reid et al. 1997, Langenbuch & Pörtner 2003). This is perhaps due to changes in amino acid catabolism that may result from new steady-state levels of decreased extracellular pH, elevated pCO_2, and HCO_3^- (Langenbuch & Pörtner 2002, Reipschläger & Pörtner 1996).

Despite these studies, some model marine invertebrates have failed to exhibit any decrease in metabolism due to ocean acidification (Gutowska et al. 2008, Wood et al. 2008), such as *Chondrilla nucula* in this study. In both the field experiment and the laboratory experiment, *Chondrilla nucula* maintained metabolic rates of fluorescent yield and chlorophyll *a* in its endosymbiotic cyanobacteria. Additionally, in both experiments, *Chondrilla nucula* maintained metabolic rates of all biochemical constituents, as well as total energetic content, regardless of treatment. There were significant changes in ash, carbohydrates, non polar lipid fraction ratio, and total lipids, but these were significant site effects, largely due to the Cave Reef site, which exhibited differing responses than the Cave Hole and Control Site most likely due to field conditions of high irradiance or UV, reduced flow due to a potential "pooling" effect, or a combination of factors.

There are other studies that have demonstrated similar results (Gutowska et al. 2008, Wood et al. 2008). Gutowska et al. (2008) demonstrated that the cuttlefish, *Sepia officinalis*, did not exhibit metabolic depression in response to acute, elevated CO_2 exposure. Wood et al. (2008) demonstrated an increased metabolic rate in the brittle star, *Amphiura filiformis*, under chronic exposure to elevated CO_2 levels. However, in order to support the significant metabolic increase, significant arm muscle catabolism was observed, ultimately compromising the organism's long-term fitness (Wood et al. 2008).

Marine teleosts are also capable of maintaining metabolic rates in conditions of reduced CO_2 due to their high ion transport and acid-base regulatory abilities; in conditions of hypercapnia, they rapidly increase HCO_3^- levels in their blood, and are able to fully compensate for the decrease in extracellular pH (*Conger conger*: Toews et al. 1983, *Squalus acanthias*: Claiborne & Evans 1992, *Gadus morhua*: Larsen et al. 1997, *Paralichthys olivaceus, Seriola quinqueradiata, Mustelus manazo*: Hayashi et al. 2004, *Zoarces viviparous*: Deigweiher et al. 2008). This is achieved by net importation of HCO_3^- from the environment via epithelial ion transporters (Heisler 1993). Calcifying organisms also have the ability to rapidly increase HCO_3^- levels in hypercapnic situations (Michaelidis et al. 2007). In the mussel, *Mytilus galloprovincialis*, the increases in HCO_3^- levels are derived mainly from the dissolution of shell $CaCO_3$ (Michaelidis et al. 2007). However, this compensation to acidosis leads to decreased metabolic rates and the eventual demise of these organisms (Michaelidis et al. 2007). Elevation of HCO_3^- levels in response to hypercapnia-induced acidification is actually a common response in most organisms (Heisler 1989), but the efficacy of these ion transport and acid-base regulatory abilities species specific (Gutowska et al. 2008), which may help explain *Chondrilla nucula*'s ability to maintain metabolic rates in acidified conditions in this study.

3. Increased Sea Surface Temperature and Bleaching

Bleaching has been well documented due to the increases in seawater temperature in both corals and sponges (Porter et al. 1989, Vicente 1990, Glynn 1993, Goreau & Hayes 1994, Lesser 1996, Brown 1997, Fromont & Garson 1999, Hoegh-Guldberg 1999, Cowart et

al. 2006, López-Legentil et al. 2008). Mass coral-bleaching events have greatly increased in frequency and intensity over the past 30 years (Wellington et al. 2001).

Bleaching in sponges can be cyclic, which is characterized by a loss of coloration and eventual recovery, or fatal, which results in a total whitening of sponge tissue, and consequently the complete disintegration of tissue, followed by mortality (Cowart et al. 2006). In this study, thermal stress failed to cause any significant decline in health in the endosymbiotic cyanobacteria within *Chondrilla nucula.* No significance changes due to treatment were observed in fluorescent yield or chlorophyll *a* content, suggesting that metabolic rates were maintained in spite of elevated acidity and temperature treatments. Other studies have produced similar studies (Vicente 1990, Schönberg & Wilkinson 2001). Bioeroding sponges, such as *Cliona* sp., have been observed to be more bleaching-resistant than corals (Vicente 1990, Schönberg & Wilkinson 2001). Such resistance may be attributed to the thermal stress-tolerant *Symbiodinium* clade D that *Cliona* sp. harbors (Baker et al. 2004, Fabricius et al. 2004, Rowan 2004). Studies on cyanobacteria have found that phycobilisomes found that experimentally induced bleaching caused virtually no alteration in chlorophyll or carotenoid absorption (Zhao & Brand 1989). Thus, if bleaching did occur in the cyanobacteria in *Chondrilla nucula* from this study, it did not have any deleterious effects on the health and functionality of the endosymbiont.

4. Conclusions and the future of coral reefs

Coral reefs arguably harbor the most biodiversity on the planet, providing a myriad of ecosystem services including fisheries, coastal protection, biotechnologies, and tourism (Moberg & Folke 1999). Unfortunately, the warming and acidification of the oceans

threaten these invaluable systems. During the 20th century, the increase in atmospheric CO_2 has driven an increase in the ocean's average temperature by 0.74°C, increased sea level by 17 cm, depleted seawater carbonate concentrations by ~30 μmol kg^{-1} seawater, and increased acidity by 0.1 pH unit (IPCC 2007). Approximately 25% of the CO_2 emitted from anthropogenic sources currently enters the ocean (Canadell et al. 2007).

Studies on climate change have produced varying results, although most have shown that the current rate of climate change will cause deleterious effects for a majority of marine organisms. Many studies have demonstrated that a doubling of pre-industrial atmospheric CO_2 by the mid-century, from 280ppm to 560 ppm, decreases coral calcification and growth by up to 40% through the inhibition of aragonite formation as carbonate-ion concentrations decrease (Kleypas & Langdon 2006). Ocean acidification has also been shown to negatively affect the olfactory sensory and homing ability of marine fish (Munday et al. 2008). In other organisms, such as sea urchin larvae, the increase in temperature has been shown to negate the negative effects of acidification (Brennand et al. 2010). In this study, *Chondrilla nucula* was not significantly altered by acidification either in the field, or by the acidification and temperature treatments in the laboratory. Because *Chondrilla nucula* is widespread in Caribbean marine habitats and appears to be relatively plastic in its tolerance to environmental conditions, one might argue that it is not the ideal model organism for research on climate change. Nonetheless, *Chondrilla nucula* is not completely adaptable to all stressors in the field; in previous studies, reef *Chondrilla nucula* that were transplanted to dark caves underwent metabolic collapse, presumably from the absence of light (Arillo 1993). We conducted a pilot study in which we also transplanted reef *Chondrilla nucula* to dark portions of the Cave, the Cave Hole, and Cave Reef. After a

period of 3 days, the sponges inside the cave perished and eroded away. A follow-up shading experiment, which subjected all sponges at all sites (dark portion of the Cave, Cave Hole, and Cave Reef) to a complete lack of light, suggested that this mortality was most likely not due to lack of light, but to another environmental factor in the cave, perhaps low pH, or to a combination of cave-associated conditions. Thus, reef *Chondrilla nucula* are not able to adapt to all extreme conditions in the field, and this study predicates a good framework and basis of assessment for a crucial organism in coral reef communities. Supporting results from Stubler & Peterson (2012) demonstrated that the boring sponge, *Cliona* sp., is also relatively unaffected by elevated CO_2 conditions, perhaps indicating that sponges as a taxon are metabolically tolerant to acidified conditions. This tolerance to acidified conditions projected for the near future, even under the added stress of elevated seawater temperature, may indicate a potential for a shift from coral dominated reefs to sponge dominated reefs. In fact, coral reefs are already undergoing phase shifts, morphing from coral dominated reefs to fleshy macroalgae dominated reefs (Hughes 1994, Nyström et al. 2000, Hughes et al. 2007), and in the Florida Keys National Marine Sanctuary, coral to sponge and algal reefs are being observed (Maliao et al. 2008). Based on results of Stubler & Peterson (2012) and the results of this study, it is plausible that marine sponges may join macroalgae as a dominant organism on coral reef systems in the near future.

5. Future directions

As this is one of the first studies on the effects of ocean acidification and elevated temperature on marine sponges, there is much room for future work. The laboratory experiment portion of this study lasted for 4 days, which may not have been long enough to see changes under realistic time scales. Longer experimental durations will be useful in providing more comprehensive results, and better frame the true long-term responses of such organisms. Additionally, taking samples at multiple time points along the duration of an experiment can track responses over a time scale, providing additional insight. Testing the independent and interactive impacts of multiple stressors, as was done in this study, more realistically models real-world scenarios than testing single stressors alone.

Protein and RNA expression can change rapidly depending on surrounding conditions, and thus both proteomics and transcriptomics are very powerful tools that have the sensitivity to detect rapid, minute changes in metabolic health, that might not be observable in other biochemical and physiological analyses (Slattery et al. submitted, Hoover et al. 2007).

Experiments utilizing multiple species rather than single species will provide a better model for predicting community responses. Furthermore, organisms on coral reefs are not isolated individuals, and therefore experiments assessing interactions among organisms in response to climate change can provide invaluable insights into the responses of such dynamic relationships. Interactions of sponges with corals, algae, or other sponges, and combinations of these can provide invaluable information to the future of reef communities. A recent study on boring sponges, *Cliona* sp., and two coral species, *Porites*

sp. and *Montastraea faveolata,* demonstrated that species interactions may amplify effects of ocean acidification on coral reefs (Stubler & Peterson 2012).

Additionally, *in situ* experiments have the potential to provide more realistic results. While laboratory experiments are very useful in controlling conditions and focusing on one or a few variables at a time, they may not always represent true, heterogeneous field conditions.

Lastly, studies that measure intracellular and extracellular pH can provide meaningful insights into the physiology of an entire organism, as acid-base regulation occurs on both extracellular and intracellular levels. Capillary pH electrodes, microelectrodes, pH optodes, magnetic resonance imaging, spectroscopy, and fluorescent pH indicators, all have their strengths and weaknesses, but can provide understanding on the response of both intracellular and extracellular reactions (Pörtner et al. 2010).

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APPENDIX

APPENDIX: CYANOBACTERIA ANALYSIS

1. Introduction

It was once commonly thought that cyanobacteria could be classified simply as phototrophic or heterotrophic through observation of sponge morphology alone (Usher 2008). Flat, encrusting sponges were commonly assumed to harbor phototrophic cyanobacteria, simply due to large surface area relative to mass (Usher 2008). However, preliminary data from sections of *Chondrilla* nucula in this study have suggested some contrasting results to old modes of thoughts.

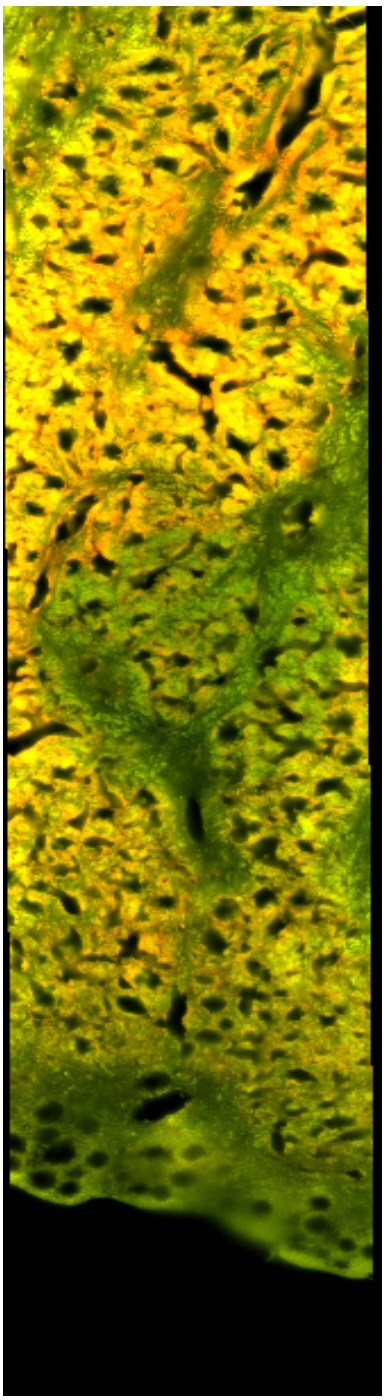
2. Methods

Small pieces (5mm x 2mm) of *Chondrilla nucula* were cut cross-sectioned and preserved in 4% paraformaldehyde (PFA). Samples were kept for two days at -20°C. After two days, PFA was removed and replaced with 70% ethanol. For embedding, samples were embedded in increasing concentrations of paraffin wax: citrisolv solvent (0%, 10%, 20%, 40%, 50%, 80%, and 100%) for at least an hour at each concentration. Then samples were secured in paraffin wax using twelve-well plates. After hardening, waxed samples were sectioned using a microtome at 20-micron thick sections and placed on microscope slides. Slides were left in a 57°C oven overnight, and then soaked in citrisolv solvent for at least 1 hour to dissolve the paraffin wax. Slides were removed from solvent and permount was added to fix the cyanobacteria. After adding a coverslip, slides were left to dry for at least 2 weeks before viewing under a fluorescent microscope. Pictures of slides were taken under a red and green fluorescent filter.

3. Preliminary Results

Chondrilla nucula grows in flat, amorphous sheets over the benthos of coral reef communities and is known to harbor phototrophic cyanobacteria (Usher 2004). The results from the present study, however, suggest that endosymbiotic bacteria in this species may serve multiple functional roles. Preliminary examination of histological sections of *Chondrilla nucula* from the present study indicate that cyanobacteria, which fluoresce orange, are actually clustered around the oscules of the sponge in the choanosome, rather than in the ectosome (Figure 14), as one might expect if their role was purely phototrophic (Usher 2008). Visual examination of these cyanobacteria may indicate that they are mixotrophic rather than phototrophic, utilizing both light and heterotrophic feeding. It is plausible that this distribution could be explained by the phenomenon of spicules conducting light to the inner portions of the sponge tissue, as was demonstrated by Brümmer et al. (2008). However, this is unlikely, as the sponge in their study, *Tethya aurantium*, has visible funnel-like spicule/ tissue structure, while the sponge in this study, *Chondrilla nucula,* has a flat, amorphous structure. I plan to analyze both the cyanobacteria density and any changes in their distribution within the study samples to assess any effects of temperature and/or pH treatment.

Figure 14. Histological 20µm section of *Chondrilla nucula* under a fluorescent microscope; red and green filter; cyanobacteria fluoresce orange.



VITA

Sylvester Jack Lee was born in Oxford, MS on January 24, 1989, the eldest of three boys. He attended grade school in Oxford, MS and graduated from Oxford High School in 2007. The following August he attended the University of Mississippi and in May 2010, received a Bachelor of Science in Pharmaceutical Sciences. After a NOAA funded study abroad program at the Perry Institute for Marine Science on Lee Stocking Island, Bahamas, he decided to stray from the path of a Doctorate of Pharmacy to pursue a life of research. In August of 2010 he re-entered as a graduate student at the University of Mississippi, and in May 2012, earned his Master of Science degree in Pharmaceutical Sciences with emphasis in Environmental Toxicology.