The Genetic Landscape of Caribbean Acropora Corals

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Dedication

This dissertation is dedicated to my husband, Raşit Bilgin.
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Finally, I want to acknowledge the support of my family, who always encouraged me to pursue my passion in biology. Most of all, I dedicate this work to my husband, Raşit Bilgin, who had faith in my ability to succeed and encouraged me throughout this long-term commitment, even from half-way across the world.
Abstract of Dissertation

This dissertation explores multiple genetic aspects of Caribbean *Acropora* biology with a focus on transcriptomic regulation of growth and symbiosis, in addition to population genetics. These topics are unified in the application of genetics to understanding the evolution and biology of important endangered scleractinian coral species that have historically been the foundation of many Caribbean reef ecosystems.

The two true Caribbean *Acropora* coral species, *Acropora cervicornis* and *A. palmata*, provide an important and useful system to study coral growth because they exhibit within-colony differentiation of polyps, they have rapid growth rates, and they are sister species with distinctive growth morphologies. With an emphasis on *A. cervicornis* in Florida, Caribbean *Acropora* s are also the focus of ongoing restoration efforts to replenish their populations. This dissertation investigates: (1) gene expression patterns involved in growth, calcification and division of labor within the coral colony, (2 & 3) differences in gene expression between day and night for both the coral host and their endosymbiotic *Symbiodinium*, and (4) population genetic structure and viability of *A. cervicornis* in a highly disturbed reef system, the Florida Keys.

The first three studies employ next-generation RNA sequencing (RNA-seq) analyses to evaluate gene expression profiles in Caribbean *Acropora* corals and their *Symbiodinium*. These three studies also explore the differences in gene expression due to division of labor within the colony, using samples from coral colony branch bases and tips. The comparison of functionally different regions of the colony for the two species revealed that adult coral *Acropora* colonies have a high degree of genetic division of labor (> 2200 differentially expressed transcripts) within the colony and up-regulate numerous genes involved in developmental signaling among
other processes in the growing region of the colony. Both *Acroporas* appear to use similar genes for growth, but differences in growth form between species may be related to developmental signaling pathways, bicarbonate transport and extracellular matrix proteins. The comparison of coral gene expression in *A. cervicornis* samples between day and night revealed a potential influence of symbiont photosynthesis on coral circadian gene expression, as well as the potential control of *Symbiodinium* populations by nitrogen regulation and the glutamine metabolic pathway. Taking advantage of the same samples and RNA-seq data to evaluate *Symbiodinium* gene expression between day and night and within the coral colony revealed a diel aspect of transcription-driven photoacclimation in *Symbiodinium*, but low magnitude gene expression changes suggest that some photosynthesis proteins may be post-transcriptionally regulated. Additionally, expression of some *Symbiodinium* genes differed between regions of the colony, suggesting that coral-driven regulation of symbiont populations may affect symbiont transcription.

The final study utilized a mitochondrial DNA marker to characterize the genetic diversity of *A. cervicornis* in the Florida Keys and to understand relationships among Florida Keys and Caribbean populations. The results indicate high genetic diversity and connectivity within Florida with limited genetic exchange between Florida and other reefs in the Caribbean. This information can be used to support conservation policies for this species.
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Chapter 1
General Introduction

Overview

Coral reefs, the largest biogenic structures in the ocean, are largely constructed by the tiny polyps of scleractinian corals (Cnidaria: Anthozoa: Scleractinia), which build colonies atop a skeleton made of calcium carbonate (CaCO$_3$). The three-dimensional architecture of hermatypic (reef building) coral colonies, in addition to gorgonians, coralline algae and sponges, provides habitat for the most biodiverse communities in the ocean, rivaling tropical rainforests for species richness (Connell 1978; Jackson 1991). These ecosystems provide numerous services for humans, including supporting fisheries and ecotourism, and protecting coastal areas from storms (Moberg & Folke 1998; Principe et al. 2011). In addition to the economic benefits provided by corals, these unique marine organisms have long been of interest to naturalists, including Darwin (Darwin 1842), due to their ability to form reefs and their biology that hovers between plant and animal, both in growth form and in function as a result of the coral-algal symbiosis. However, it was only in the 1950s, with the availability of scuba equipment, that coral biology began to be studied in detail, and the ecology, physiology, and nature of the symbiosis began to be elucidated. Coral research has recently entered a new phase in which both long-standing questions, as well as new urgent questions, are being addressed with new technologies like next-generation RNA sequencing.

As with many of the earth’s ecosystems, modern reefs face growing threats associated directly and indirectly with human activities, including overfishing (Hughes
c 1994), coral disease (Harvell et al. 2004; 2007), and global climate change in the form of increased sea surface temperatures and ocean acidification (Hughes et al. 2003; Hoegh-Guldberg et al. 2007). When the severe decline of Caribbean reefs was noted in the 1980s and 90s following destruction caused by Hurricane Allen in 1980 in conjunction with the disease-based die off of the important grazing urchin Diadema antillarum (Lessios et al. 1984; Jackson 1991; Hughes 1994), it prompted a surge in coral reef research aimed at understanding the ecology of reefs. Around the same time, coral disease outbreaks became more prevalent, either due to increased pathogen load and virulence or increased susceptibility of corals due to stresses (Harvell et al. 2007). And since the mid-1990s, additional global threats to corals have surfaced. Of particular concern are the increased rate of mass bleaching episodes related to higher ocean temperatures that have decimated some reefs (Hoegh-Guldberg 1999), as well as the expectation that calcifying organisms will suffer in the face of increased carbon dioxide (CO₂) and lower oceanic pH (Hoegh-Guldberg et al. 2007). These relatively recent physiological challenges to corals have prompted a renewed interest in studying the cellular and molecular basis of coral development, growth, calcification, and immunity.

Recent technological advances in next-generation DNA and RNA sequencing provide a new approach to studying the genetic response of corals with unprecedented depth and detail. The first ‘next-generation’ sequencers became commercially available in 2004 (Roche 454 GS FLX) and 2006 (Illumina Genome Analyzer) (Mardis 2008), transforming the field of genetics and making detailed genetic analyses of non-model organisms possible. Since then, three anthozoan genomes, including Nematostella vectensis (Putnam et al. 2007) and two coral genomes, Acropora digitifera (Shinzato et al.
2011) and A. millepora (D. Miller, unpublished), plus at least six coral/holobiont transcriptomes (including the one used herein, Libro et al. 2013) (Traylor-Knowles et al. 2011; Polato et al. 2011; Moya et al. 2012; Mehr et al. 2013; Shinzato et al. 2014) and four Symbiodinium transcriptomes (Bayer et al. 2012; Ladner et al. 2012) have been sequenced and made publicly accessible. With this wealth of genetic information available, a next step is to decipher how these genes function and respond in the host and its algae in order to inform our understanding of coral biology and multicellular animal life in general.

A major question relates to how the diversity of coral growth forms are produced. According to the World Register of Marine Species, there are over four thousand species of scleractinian corals comprising 29 families across the globe (marinespecies.org); however, research into how corals have evolved to produce such a wide range of morphologies is limited. Research on the evolution of growth in corals has both theoretical and practical applications. First, because corals are primitive organisms within the eumetazoa, they can provide insights into the developmental biology and evolution of animals prior to the origin of bilaterians (Miller & Ball 2000; Ball et al. 2002), which have been the traditional animal research models. Second, coral growth is linked to environmental conditions, such as oceanic CO$_2$ and temperature, so knowledge about coral growth can inform our understanding of the past climate as well as help us to predict potential future outcomes for coral reefs (Kleypas et al. 1999; 2001; Hoegh-Guldberg et al. 2007; Pandolfi et al. 2011; De’ath et al. 2013).
The evolution of varied colony growth forms has enabled hard corals to colonize a broad range of tropical benthic habitats, from high-energy, high-light reef crests to areas with little water movement, high sedimentation and low-light availability (Chappell 1980). A coral species’ growth form can influence its habitat range, resulting in a characteristic species zonation pattern along many reefs (Goreau 1959a). However, some coral species can adapt to a wide range of habitats and exhibit a high degree of phenotypic plasticity in colony growth form (reviewed by Todd 2008). In many species, coral growth responds to light (Jaubert 1977; Hidaka & Shirasaka 1992; Kaniewska et al. 2009), water movement (Bottjer 1980; Oliver 1984; Bruno & Edmunds 1998) and interspecific (Goreau & Hartman 1966) or intraspecific biological interactions (Rinkevich & Loya 1985; Raymundo 2001). In general, coral colonies in high light environments take on a more highly-branched or vertically-oriented morphology, while those in lower light tend to be flatter (Dustan 1975; Jaubert 1977; Chappell 1980). Corals in high water movement areas often have more robust skeletons, while those in low flow areas have more delicate skeletons with more widely spaced branches and higher surface area to volume ratios (Lesser et al. 1994). The coral’s photosynthetic dinoflagellate endosymbionts, Symbiodinium spp (aka zooxanthellae), also play an important role in growth, particularly calcification, which is enhanced in light during the daytime (Goreau 1959b; Goreau & Goreau 1959; Chalker & Taylor 1975).

Despite this morphological plasticity in colony form in some corals, the skeletal characteristics of the corallite (skeleton of an individual polyp) and colony are often conserved within species and serve as the basis for taxonomic classification (Veron 2000), indicating heritable genetic regulation of growth and skeleton formation (Todd 2008).
However, the relationship between genes and coral growth is poorly understood, both in terms of colony morphology and diel patterns of calcification.

One genus of corals in particular, *Acropora*, has dominated tropical reefs through rapid growth rate and, in the Pacific, through a high diversity of growth forms that may have arisen through hybridization (Willis *et al.* 2006). While there are over a hundred species of *Acropora* in the Indo-Pacific, there are only three in the Caribbean. Two of these, *A. cervicornis* and *A. palmata*, have highly distinct growth forms, while the third species is a hybrid with an intermediate morphology (van Oppen *et al.* 2000; Vollmer & Palumbi 2002). The evolutionary proximity of the two main Caribbean *Acropora* species sets them up as an ideal system in which to study evolution of coral growth form.

In the Caribbean, the two true species of *Acropora* were once the primary component of many reefs (Goreau 1959a; Farrell *et al.* 1983; Knowlton *et al.* 1990), but healthy *Acropora* reefs are now uncommon largely due to mass die-offs because of white-band disease (Gladfelter 1982b; Aronson & Precht 2001). These two species are now a high priority for conservation and restoration efforts, and rehabilitation of reefs using farmed *Acropora* are currently underway (Johnson *et al.* 2011; Young *et al.* 2012). Information on how *Acroporas* grow and calcify can inform restoration efforts, but in this perspective, it is also important to determine the population genetic status of the reefs where such efforts are being applied.
Coral physiology and biochemistry

Coral body plan & tissue layers

The majority of reef-building corals are colonial and grow through asexual budding of polyps. Healthy reef-building corals live in association with photosynthetic dinoflagellate endosymbionts in the genus *Symbiodinium* that occupy vacuoles called symbiosomes within the endodermal cells (gastrodermis) adjacent to the gastrovascular cavity (Trench 1987). As cnidarians, corals are comprised of two tissue layers (endoderm and ectoderm) separated by a layer of gelatinous mesoglea (Figure 1). The skeleton is formed by deposition of the aragonite form of CaCO$_3$ at the base of the calicoblastic epithelium (aboral ectoderm). Polyps are connected through tissue overlying the skeleton (the coenosarc) and, in some corals such as *A. cervicornis*, by coelenteric canals within the skeleton (Gladfelter 1982a; 1983a).
Figure 1. Anatomy of an *A. cervicornis* apical polyp and two radial polyps showing magnified views of the coral tentacle (left) and coenosarc (right). Tissue layers are labeled as follows: oral (free) ectoderm (OEc, containing nematocysts), mesoglea (M), gastrovascular canal (coelenteron, GC), oral endoderm (gastrodermis, OEn, containing *Symbiodinium*), skeleton (S), calicoblastic ectoderm (CEc) and aboral endoderm (gastrodermis, AEn). Illustration adapted from Fowler 1896 (as cited in Wallace 1999), Johnston 1980, Gladfelter 1983a and Veron 2000.

Calcification of the skeleton

The CaCO$_3$ skeleton is produced from calcium ions (Ca$^{2+}$) and bicarbonate (HCO$_3^-$) or carbonate (CO$_3^{2-}$). Equations 1-3 demonstrate the basic reactions for inorganic carbon from CO$_2$ entering seawater through CaCO$_3$ production. Because most inorganic carbon in the marine environment (at pH~8.1) is in the form of bicarbonate, the overall equation representing the reaction is equation 4 (Tambutté *et al.* 2011).
Equation 1. The reversible reaction of carbon dioxide and water produces bicarbonate ($\text{HCO}_3^-$) and a proton. Carbonic acid ($\text{H}_2\text{CO}_3$) is an intermediate molecule that dissociates easily in seawater. In living systems the interconversion of these inorganic carbon species is facilitated by the enzyme carbonic anhydrase.

Equation 2. In solution, bicarbonate acts as a buffer. The direction of the reaction will depend on the pH of the solution. When pH decreases ($\text{H}^+$ increases), the reaction proceeds toward the left.

Equation 3. Carbonate ($\text{CO}_3^{2-}$) reacts with calcium cations to precipitate $\text{CaCO}_3$. When pH decreases, carbonate is depleted by conversion to bicarbonate (eq. 2), and $\text{CaCO}_3$ will dissolve.

Equation 4. The overall reaction of bicarbonate with calcium ions to form calcium carbonate and a proton as it would occur in seawater at pH ~8.1.

Carbonic anhydrase (CA) activity (interconversion of $\text{CO}_2 + \text{H}_2\text{O}$ and $\text{HCO}_3^- + \text{H}^+$) and *Symbiodinium* photosynthesis both have dramatic effects on rates of skeletogenesis (Goreau 1959b), but the coral also appears to actively regulate the concentration of protons (i.e. pH) (Al-Horani *et al.* 2003; Venn *et al.* 2009; 2011) and calcium ions (Chalker 1976; Al-Horani *et al.* 2003; Marshall *et al.* 2007) within and beneath the calicoblastic epithelium (Figure 2). In *Acropora*, the process of skeleton formation varies throughout the day, with more $\text{CaCO}_3$ deposition occurring during the day, but greater linear branch extension at night (Barnes & Crossland 1980; Gladfelter 1983b). The increased $\text{CaCO}_3$ deposition during the day is referred to as light-enhanced calcification (LEC) (Goreau 1959b; Goreau & Goreau 1959; Chalker & Taylor 1975), and is thought to be at least partially driven by the increase in available fixed carbon from
*Symbiodinium* photosynthesis available during the day (Pearse & Muscatine 1971). Alternative explanations for the influence of photosynthesis on calcification include (1) decreased CO$_2$ reduces H$^+$, increasing pH and CO$_3^{2-}$ concentration (Goreau 1959, Erez *et al.* 2011), (2) production of O$_2$ stimulates coral metabolism (Rinkevich & Loya 1984), (3) organic matrix from *Symbiodinium* is transferred to the coral (Muscatine & Cernichiari 1969), and (4) photosynthesis removes calcification inhibitors, such as phosphates (Simkiss 1964). Yet, it is still not known how the corals regulate growth and CaCO$_3$ deposition or why calcification is enhanced during the day and in light.

The resemblance of coral skeletal CaCO$_3$ to abiotically precipitated CaCO$_3$ mineral suggests that corals maintain an environment conducive to aragonite precipitation by transporting ions, particularly protons, across the calicoblastic epithelium (Holcomb & Cohen 2009; Holcomb *et al.* 2009). A number of genes thought to be involved with the process of calcification include CA (*Moya et al.* 2008a), a bone morphogenic protein (BMP2/4) ortholog (*Zoccola et al.* 2009), a calcium transport pump/ATPase (*Zoccola et al.* 2004) and possible organic matrix proteins (*Cuif et al.* 1999; *Fukuda et al.* 2003; Watanabe 2003; Puverel *et al.* 2004; 2005; *Helman et al.* 2008; *Ramos-Silva et al.* 2013). In addition, micro-array studies directed towards understanding the gene expression of coral development in *A. millepora* (*Grasso et al.* 2008; 2011) and *Montastraea* (*Orbicella*) faveolata (*Reyes-Bermudez et al.* 2009a) have shed some light on genetic regulation at the onset of calcification; including differential expression of two alpha-type CAs, calmodulin and putative skeletal organic matrix (SOM) proteins. In chapter two of this dissertation, I examine genes that may be responsible for the rapid growth rate found
at the tips of *Acropora* branches. In chapter three, I also examine genes that may play a role in LEC.

Figure 2. Model of biological regulation of calcification, modified from (Allemand et al. 2004; Moya et al. 2008a). Z = *Symbiodinium*, CA = carbonic anhydrase. Molecules may use paracellular pathways (between cells) or transcellular pathways (through cells), but charged molecules require transport proteins. CO₂ can diffuse through cell membranes and be used either for photosynthesis by the *Symbiodinium*, or converted to HCO₃⁻ by CA and used by the coral for calcification. Carbohydrate from photosynthesis may be transferred to the coral, transported to the calicoblastic epithelium and used to drive energy-demanding processes, such as ATP-driven Ca²⁺ and H⁺ transporters. HCO₃⁻, which may be produced from respired CO₂ through conversion by CA, cannot passively diffuse through the cell membrane and may require an uncharacterized anion transporter to pass from the calicoblastic epithelium to the calcifying space.
Cnidarian development and colony coordination

Gene expression of cnidarian development

Most animals have determinate growth, and their body plan is established during the stages of embryonic development. However, colonial corals have indeterminate growth, as they generate new polyps through asexual reproduction and live to an unknown age. Therefore, in studying the genetics of coral growth, it is necessary to consider the genetics of polyp development and tissue growth. Compared to research in corals, tissue growth and development have been studied in much greater depth in non-calcifying and non-colonial cnidarian models, particularly the freshwater cnidarian *Hydra* and the anthozoan model *Nematostella vectensis*. Signaling pathways, including wnt/β-catenin (Lee et al. 2006; Momose & Houliston 2007; Lee et al. 2007; Duffy et al. 2010; Trevino et al. 2011), Hox-like (Grens et al. 1996), BMP/TGFβ and others (Steele 2002) appear to be important in axial patterning and polarity determination of these cnidarians. Yet, it is hard to predict how the results of studies on early development or growth in solitary, non-calcifying organisms will translate to growth in colonial, skeleton-building corals.

Research on the genetic regulation of tissue growth in corals has been limited to comparisons of gene expression at different times during development, including larvae and newly calcifying polyps (Hayward et al. 2001; 2002; 2004; Lee et al. 2007; Grasso et al. 2008; Reyes-Bermudez et al. 2009b; a; Grasso et al. 2011). These studies indicate that developmental signaling in corals involves many of the same genes involved in bilaterian development, such as disheveled (Lee et al. 2007), lectins (Grasso et al. 2008), BMP2/4
(Hayward et al. 2002) and snail (Hayward et al. 2004), and some cnidarian-specific genes such as minicollagen (Grasso et al. 2008) and cnox-2Am, a Hox-like gene (Hayward et al. 2001). At a descriptive level, transcriptome analysis of A. millepora showed that hard corals express genes involved in many of the major signaling pathways (Meyer et al. 2009; Ryu et al. 2011); however, regulation of expression within colonies and between species has received little attention.

Division of labor in colonial cnidarians

Division of labor (DOL) by way of zooid polymorphism is fairly common among colonial cnidarians in the class Hydrozoa, but is more unusual among anthozoans. In hydrozoans, such as Hydractinia and siphonophores, DOL is highly specialized with each zooid performing a function such as eating (gastrozooid), defense (dactylozooid), or sexual reproduction (gonozooid) on behalf of the colony. Gene expression studies have found that these zooid types show strikingly different transcriptome profiles (Siebert et al. 2011; Sanders et al. 2014). Work by Cartwright and colleagues (Cartwright et al. 1999) has shown that expression of a Hox protein may direct the morphology of each zooid by defining oral ‘head’ versus body column identity within each polyp. Within the Anthozoa, DOL is less common, but acroporid corals do display distinct DOL among polyp types (axial vs. radial). Using a microarray-based approach, Bay et al. (Bay et al. 2009b) examined transcriptomic differences between different parts of the A. millepora colony finding only two apparent functional differences between branch bases and tips, including up-regulation of lysosomal lipase and fluorescent protein in tips. Yet these differences do not seem to fully explain the functional differences within Acropora colonies. In chapter
2. I examine genes that appear to contribute to DOL and polyp development in both *A. cervicorns* and *A. palmata*.

**Symbiosis with Symbiodinium spp.**

All hermatypic corals depend on a symbiosis with the photosynthetic dinoflagellates of the genus *Symbiodinium* (Chromalveolata: Dinoflagellata: Dinophyceae), often called zooxanthellae. Described in more detail in chapter 4, the symbiosis benefits the coral through transfer of fixed carbon from the *Symbiodinium*, while the algae receive nutrients and shelter from the coral (Muscatine & Cernichiari 1969; Yellowlees *et al.* 2008). Corals acquire their symbionts either directly from their parent colony or through ingestion of *Symbiodinium* suspended in the water column (Stat *et al.* 2006). The symbionts reside in the gastrodermis (endoderm) inside of vacuoles, called symbiosomes, comprised of the dinoflagellate enclosed in multiple algal-derived membranes and an outer host-derived membrane (Wakefield *et al.* 2000; Kazandjian *et al.* 2008). A functioning relationship between host coral and endosymbiont requires that the coral recognize and tolerate these foreign organisms within its tissues (reviewed in Davy *et al.* 2012). In addition, the coral likely regulates population growth of the symbiont to prevent overgrowth, and is also thought to induce transfer of photosynthetic products from *Symbiodinium* to coral. In *Acropora* corals, *Symbiodinium* occur in much lower densities in the rapidly growing tips of the branches, compared to the rest of the colony. This suggests that the relationship between host and symbiont may vary within the colony.
Differences in ribosomal DNA sequence have been used to identify nine clades of *Symbiodinium* (A-I), six of which (A-D, F & G) form symbiotic relationships with scleractinian corals (Stat *et al.* 2006; Pochon & Gates 2010). Most *Symbiodinium* clades contain a number of sub-clades (aka “types”) and have distinct local (Ulstrup & van Oppen 2003) and global geographic distributions (Baker & Rowan 1997; Glynn *et al.* 2001; LaJeunesse 2002; 2005). Clade distribution also varies by depth (Rowan *et al.* 1997; Baker 2001; Warner *et al.* 2006), host species (Rowan & Powers 1991; LaJeunesse 2002) and location within colonies of some species (Rowan & Knowlton 1995; Rowan *et al.* 1997; Ulstrup & van Oppen 2003; Ulstrup *et al.* 2007). In many cases, these distributions appear to be regulated by adaptations to tolerate solar irradiation and thermal stress (Rowan & Knowlton 1995; Rowan *et al.* 1997; Ulstrup & van Oppen 2003; Rowan 2004; Warner *et al.* 2006). The coral-algal relationship can breakdown under high light and thermal stress conditions, in an event called coral bleaching, which is discussed in the following section.

In chapters three and four, I evaluate gene expression of both symbiotic partners, which can be used as reference data when examining genetic responses of these organisms to experimentally manipulated conditions. For these chapters, I parse RNA-seq data from *A. cervicornis* samples collected during the day and night into coral and *Symbiodinium* data. In chapter 3, as one component of the diel gene expression variation, I investigate the coral host response to *Symbiodinium* activity, as well as ways in which coral may regulate symbiont population growth. In chapter 4, I evaluate the transcriptomic regulation by the *Symbiodinium* between diurnal and nocturnal activity, and between branch bases and tips.
Effects of climate change and increasing CO$_2$ on corals

Bleaching and thermal stress

The breakdown of the host-symbiont relationship can occur due to a number of stresses, but the most severe mass bleaching episodes have been related to exposure of corals to a combination of excessive heat and light stress (Hoegh-Guldberg 1999). Under these conditions, damage to the *Symbiodinium*’s chloroplast proteins leads to rapid production of reactive oxygen species (ROS), which can harm proteins, lipids and DNA (Lesser 2006). Both coral and symbiont produce antioxidant proteins, but if these are not sufficient to offset oxidative stress, the coral removes its endosymbionts through expulsion or degradation of the algal and/or host cells (Weis 2008). Extreme bleaching can result in mortality of the coral, and the increasing frequency and duration of extreme temperature events resulting from global climate change are expected to severely deplete coral reefs if corals and their symbionts cannot adapt quickly to changing thermal conditions (Hoegh-Guldberg 1999). Both coral host and *Symbiodinium* show variability in their tolerance of conditions that may induce bleaching, which suggests that the composition of reefs may change as more thermally tolerant species preferentially survive and reproduce (Stat & Gates 2011; Barshis et al. 2013). Susceptibility to bleaching appears to be related to photoinhibition of photosynthesis, a reduction in photosynthetic capacity, in the *Symbiodinium* when photosystem II is damaged. Three components of the photosynthetic complex have been implicated in susceptibility to bleaching: the photosystem II D1 reaction center protein and its repair mechanisms (Warner et al. 1999; Takahashi et al. 2004), Rubisco (Jones et al. 1998), and thylakoid
membrane lipids (Tchernov et al. 2004). Loss of function of any of these components ultimately leads to production of ROS within the chloroplast (Smith et al. 2004). This suggests that the genetic basis for thermal tolerance may lie in these photosynthesis genes or possibly in the antioxidant genes of *Symbiodinium*, which are described in greater detail in chapter 4.

Changing ocean chemistry

Atmospheric CO$_2$ partial pressure ($p$CO$_2$) levels recently exceeded 400 ppm, up from pre-industrial levels of ~280 ppm. Retention of heat within the earth’s atmosphere due to increased atmospheric $p$CO$_2$ and other greenhouse gasses has caused sea surface temperatures to rise by approximately 0.11°C per decade over at least the past 40 years, and probably longer. In addition, as a sink for atmospheric CO$_2$, seawater is becoming less basic, with pH decreasing by 0.1 since industrialization (from ~8.2 to ~8.1). As pH is on a log scale, this corresponds to an increase of 26% in hydrogen ion (proton) concentration (Rhein et al. 2013). Models project an additional decrease of pH (0.7 units) over the next several centuries, which is unprecedented in the past 300 million years (Caldeira & Wickett 2003). This rapid increase of oceanic $p$CO$_2$ reduces coral skeleton deposition by lowering the carbonate ion concentration and subsequently the aragonite saturation state (Kleypas et al. 1999; Ries et al. 2009), and it may also reduce the ability of corals to grow and reproduce (Albright & Mason 2013).

Under the projected conditions of ocean pH between 7.3-7.6, some corals can survive without a calcified skeleton (Fine & Tchernov 2007), yet corals without a skeleton are functionally like anemones and do not provide the habitat forming structure
needed to maintain the reef ecosystem or to keep up with sea level, which is rising at a
global mean rate of approximately 3.2 mm per year (Rhein et al. 2013). Food availability
appears to be important for skeletal deposition, with heterotrophic input contributing to
coral spat calcification even under high $p$CO$_2$ conditions (Drenkard et al. 2013). The
energy inputs from both heterotrophy and Symbiodinium probably contribute to the
coral’s ability to maintain a relatively high pH (Venn et al. 2011) and high concentration
of calcium ions (Al-Horani et al. 2003) within the calcifying space and to produce
proteins that facilitate calcification. With the increased frequency and duration of
bleaching events and reduced aragonite saturation state, corals face unfavorable
conditions for growth and survival. While this dissertation does not directly address the
effects of pH or thermal stress on corals, it provides data on the baseline genetic
functioning of corals and Symbiodinium in their native environments that can be used to
inform further stress-related studies.

**Caribbean Acropora corals**

Evolution and morphology

*Acropora* is the most specious genus of scleractinian corals, with at least 113
described species (Wallace 1999); however, only two well defined species, *A. cervicornis*
and *A. palmata*, plus their hybrid, *A. prolifera*, are found in the Caribbean (van Oppen et
al. 2000; Vollmer & Palumbi 2002). Based on the fossil record, *A. cervicornis* (first
occurrence 6.6 mya) (Budd & Johnson 1999) and *A. palmata* (3.6-2.6 mya) (McNeill et
al. 1997) are thought to have diverged approximately three million years ago. These
species generally occupy different habitats, with *A. cervicornis* found in deeper, low-
energy fore- and back-reef and *A. palmata* found in high-energy reef crest (Goreau 1959a). Both species are broadcast spawners and their spawning events occur in late summer (Szmant 1986). Due to weak prezygotic isolating mechanisms, occasional hybrids do occur, with the resulting hybrid morphology an intermediate between the two parental species (van Oppen *et al.* 2000; Vollmer & Palumbi 2002; Fogarty *et al.* 2012). The resemblance of the hybrid colony to *A. cervicornis* or *A. palmata* depends on the parental identity of the egg and sperm, which suggests maternal or cytoplasmic effects on morphology (Vollmer & Palumbi 2002).

*Acropora* species provide a good system in which to study growth due to their rapid growth rates and specialized axial polyps at the tips of branches (Ehrenberg 1834 as cited in Wallace 1999), which are the primary site of skeletal extension (Goreau & Goreau 1959) and new polyp development. These corals have a relatively well defined division of labor within the colony. Growth takes place in the apical regions of the colony, particularly the axial polyp, and gamete production occurs in the radial polyps (greater than 2 cm from branch tips) (Szmant 1986). Physically, axial polyps differ from radial polyps in that they are larger, have six tentacles (radial polyps have twelve), have a lower density of *Symbiodinium*, and their gastrovascular canal extends farther into the skeleton, allowing mesenterial connections with radial polyps (Gladfelter 1983a). The Caribbean *Acropora* group, comprised of two sister species and their hybrid is a much simpler system to study evolution of morphology compared to the species-rich Pacific *Acropora* complex, in which evolutionary relationships inferred based on morphology versus molecular data are conflicting (van Oppen *et al.* 2001).
Distribution & population genetics

The ranges of *A. cervicornis* and *A. palmata* are sympatric and extend throughout the Caribbean from Venezuela and Panama in the south to the Florida Keys and the Bahamas in the north. Across this range, declines of up to 95% of both species have been observed, largely due to outbreaks of white-band disease starting in the 1980s (Aronson & Precht 2001; Miller et al. 2002). Population densities vary greatly across the Caribbean with relatively healthy stands of *Acropora* in some parts of Panama and sparse populations in the Florida Keys. The loss of these habitat-forming species has prompted concern for their conservation, resulting in the listing of both species for protection as threatened under the Endangered Species Act (Hogarth 2006) and as critically endangered on the IUCN Red List (Carpenter et al. 2008), motivating a number of restoration efforts (Johnson et al. 2011; Young et al. 2012). Part of establishing an effective conservation plan for these species involves understanding their population dynamics and connectivity throughout their ranges. Population genetic analyses can identify source and sink populations, as well as evaluate standing levels of genetic diversity.

The Caribbean *Acroporas* are broadcast spawners of floating egg bundles, and their gametes and larvae have the potential to disperse over a period of approximately 3-5 days; yet previous work by Vollmer and Palumbi (2007) established that *A. cervicornis* populations are regionally restricted and can show differentiation over short distances. However, due to low sample size, this previous study could not thoroughly evaluate one of the most threatened populations of *A. cervicornis*, the Florida Keys. Additional
sampling and a total of 52 samples from across the Florida Keys Reef Tract facilitated the analysis of genetic diversity and connectivity of this population included in chapter 5 of this dissertation.

**Molecular techniques**

Gene expression, high-throughput sequencing and RNA-seq

Multiple recently developed high-throughput sequencing platforms have become available for generating massive amounts of genomic and transcriptomic data, allowing the sequencing and quantification of thousands of genes in a single sample. These techniques are based on simultaneous sequencing of millions of short read sequences and provide a number of novel opportunities, including gene expression analysis for non-model organisms. High-throughput sequencing data can also be used for cross-species comparisons and to simultaneously analyze multiple symbiotic partners. The reference transcriptome used for this dissertation was assembled from *A. cervicornis* and *A. palmata* samples, which permitted an unbiased reference to perform cross-species comparisons. To distinguish between coral and dinoflagellate transcripts, reference transcriptome sequences were compared to aposymbiotic coral larvae transcriptomes for *A. millepora* (D. Miller, unpublished) and *A. digitifera* (Shinzato et al. 2011) as well as to *Symbiodinium* transcriptomes for clades A, B, C and D (Bayer et al. 2012; Ladner et al. 2012).

The RNA-seq analyses used in this dissertation were carried out using single-end sequences generated by Illumina platform sequencing. The earlier study (chapter 2) used the Illumina GAII platform at the FAS Center for System Biology at Harvard University,
which produced 1.9-7.3 million 40 bp single-end reads per sample. The later studies (chapters 3 and 4) used the Illumina Hi-Seq 2000 platform, also at Harvard, which produced 8.4-22.5 million 50 bp single end reads. The workflow to produce the data is described in figure 3.

Figure 3. RNA-seq workflow for all samples in chapters 2-4.

Mitochondrial DNA sequencing

The second technique I used in this dissertation was mitochondrial DNA sequencing of the putative mitochondrial control region, which was previously used to resolve population structure in Caribbean Acroporas (Vollmer & Palumbi 2007). It is necessary to use mitochondrial DNA, rather than nuclear genes or microsatellites, when
evaluating the population genetics of Caribbean Acropora due to the incidence of introgression of nuclear genes from A. palmata to A. cervicornis (Vollmer & Palumbi 2007). mtDNA is haploid and the sequences native to A. cervicornis are readily identified so that introgressed haplotypes can be removed from population genetic analyses. Introgressed alleles cause at least two problems for population genetic analyses (1) A. palmata alleles are more divergent (see Figure 2 in chapter 5), which can interfere with comparisons based on nucleotide diversity, and (2) A. palmata alleles that are shared by A. cervicornis individuals at different sampling sites may not represent gene flow among A. cervicornis, but rather separate hybridization events at the different locations.

**Dissertation objectives**

To summarize, the primary focus of this dissertation is identifying genes that regulate coral growth, calcification and morphology using the Caribbean acroporid model. In chapter 2, I compare RNA-seq gene expression profiles of apical branch tip (including axial polyp) and branch base samples in both Caribbean Acropora species. Genes that were significantly up-regulated (expressed more) in the rapidly growing and calcifying parts of the colony may play a central role in growth, either in calcification or polyp generation. Genes with significant differences between branch tips and bases as well as between species may be involved in generating the morphological differences between species, such as single versus fused axial polyps.

The second and third objectives of this dissertation are to investigate how the genetic functioning of the coral animal and the endosymbiotic Symbiodinium differ between day and night. I use RNA-seq libraries prepared from A. cervicornis at mid-day
and mid-night to evaluate transcriptomes for both organisms. In chapter 3, I focus on how DOL within the colony may be affected by (or affect) diurnal versus nocturnal gene expression. In chapter 4, I investigate the differential expression of the *Symbiodinium* between regions of the colony and between day and night.

The final objective of this dissertation is to evaluate the genetic connectivity and diversity of a highly threatened population of *A. cervicornis* in the Florida Keys using an mtDNA marker, which is carried out in chapter 5.
Chapter 2

The Genetics of Colony Form and Function in Caribbean Acropora Corals

Abstract

Background

Colonial reef-building corals have evolved a broad spectrum of colony morphologies based on coordinated asexual reproduction of polyps on a secreted calcium carbonate skeleton. Though cnidarians have been shown to possess and use similar developmental genes to bilaterians during larval development and polyp formation, little is known about genetic regulation of colony morphology in hard corals. I used RNA-seq to evaluate transcriptomic differences between functionally distinct regions of the coral (apical branch tips and branch bases) in two species of Caribbean Acropora, the staghorn coral, A. cervicornis, and the elkhorn coral, A. palmata.

Results

Transcriptome-wide gene profiles differed significantly between different parts of the coral colony as well as between species. Genes showing differential expression between branch tips and bases were involved in developmental signaling pathways, such as Wnt, Notch, and BMP, as well as pH regulation, ion transport, extracellular matrix production and other processes. Differences both within colonies and between species identify a relatively small number of genes that may contribute to the distinct “staghorn” versus “elkhorn” morphologies of these two sister species.
Conclusions

The large number of differentially expressed genes supports a strong division of labor between coral branch tips and branch bases. Genes involved in growth of mature *Acropora* colonies include the classical signaling pathways associated with development of cnidarian larvae and polyps as well as morphological determination in higher metazoans.
Background

Colonial modular organisms, such as corals, bryozoans, and tunicates, are formed from groups of asexually produced, genetically identical modules (‘polyps’ or ‘zooids’) that are interconnected to produce an integrated super-organism (Mackie 1986). Modules are connected by living tissue, which allows intra-colony communication, resource sharing (Pearse & Muscatine 1971), and in some cases a coordinated division of labor (DOL) permits specific modules to specialize in feeding, reproduction or defense (Cartwright 2004; Siebert et al. 2011). The coordination of growth among modules can create diverse colony morphologies, such as those in tropical reef-building corals ranging from simple hemispherical and plating colonies to complex branching colonies.

Scleractinian (hard) coral colonies have a simple body plan of polyps and connecting tissue overlaid on top of a secreted aragonite (calcium carbonate) skeleton. The coral animal is comprised of anemone-like polyps with a gastro-vascular cavity formed by two cell layers (ectoderm and endoderm) separated by a layer of mesogloea. The polyps are interconnected by a layer of tissue (coenosarc) overlaying the skeleton, but may also share coelenteric canals within the skeleton (Gladfelter 1982a). Colony integration and inter-polyp communication is evident in the elaborate colony morphologies formed by corals, which often serve as key identifying characteristics of species (Rinkevich 2002). However, within species some morphological variation may also occur due to environmentally-induced phenotypic plasticity (Shaish et al. 2007; Todd 2008) or genetic polymorphism.
The genetic mechanisms underlying colony growth are poorly understood, but cnidarian genomes are known to contain many of the key genes expressed during coordinated development of bilaterians, such as Hox/ParaHox (Chiori et al. 2009), Hedgehog (Matus et al. 2008), Wnt (Kusserow et al. 2005; Lee et al. 2006; Guder et al. 2006), TGFβ/BMP (Samuel et al. 2001; Zoccola et al. 2009), Notch (Käsmbauer et al. 2007; Marlow et al. 2012) and other developmental signaling pathways (Steele 2002; Ball et al. 2004; Martindale 2005; Meyer et al. 2009; Steele et al. 2011). Some of these developmental genes, including Hox/ParaHox and Wnt genes, have been shown to function in axial patterning of the solitary freshwater hydrozoan Hydra (Hobmayer et al. 2000; Bode 2001; Müller et al. 2004; Broun et al. 2005), the colonial marine hydrozoan Hydractinia echinata (Plickert et al. 2006; Duffy et al. 2010), the solitary marine anthozoan Nematostella vectensis (Wikramanayake et al. 2003; Kusserow et al. 2005; Trevino et al. 2011), as well as in the early developmental stages of scleractinian corals (Hayward et al. 2001; de Jong et al. 2006). A ParaHox gene, cnox-2, has also been associated with DOL among zooids in hydrozoans (Cartwright et al. 1999; 2006), likely relating to the development or exclusion of oral structures. Additionally, characteristic gene expression differences have been observed between swimming (nectophore) and feeding (gastrozooid) zooids in siphonophores (Hydrozoa) (Siebert et al. 2011). These findings suggest that other colonial cnidarians, including some corals, may show polyp-specific transcription. The genetics of coral development has been examined through post-larval settlement (Grasso et al. 2008; 2011), yet little is known about the basis of either colony coordination or DOL in mature colonies.
Branching *Acropora* corals are a good system for studying genetic regulation of growth form and colony coordination in anthozoans, because corals in this genus (with the exception of the subgenus *Isopora*) exhibit dimorphic polyp types. *Acropora* corals have axial polyps with six tentacles located at the apical tip of their branches and radial polyps with twelve tentacles located along the sides of the branches. Axial polyps are typically the site of rapid growth and have lighter coloration due to a lower concentration of symbiont algae (i.e. *Symbiodinium* or zooxanthellae) (Goreau 1959b; Goreau & Goreau 1959; Goreau 1963; Pearse & Muscatine 1971; Oliver 1984; Gladfelter *et al.* 1989). In contrast, radial polyps are generally smaller and have darker pigmentation corresponding to higher symbiont densities. Actively growing branch tips in *Acropora* corals are usually sterile, and gamete production occurs only in mature radial polyps (Oliver 1984; Wallace 1985; Szmant 1986; Oliver 1987). *Acropora* have diversified into the most specious genus of scleractinian corals with over 120 described species and growth forms ranging from arborescent, such as *A. cervicornis*, to more tabulate forms, such as *A. palmata*. The success of these growth forms has enabled *Acropora* corals to colonize a wide range of habitats and become dominant reef-building corals both in the Indo-Pacific and the Caribbean. However, the genetic basis of this DOL within the colony remains unknown. One microarray study examined differential gene expression within *A. millepora* and found few differences between bases and tips of the branches. Differences were limited to lysosome lipase activity and fluorescence (Bay *et al.* 2009a), which do not explain the large functional differences within the colony.

Despite the extensive species diversity globally, the Caribbean has only two *Acropora* species (Figure 1), the staghorn coral, *A. cervicornis*, and the elkhorn coral, *A.
_palmata_, which are known to hybridize, generating an intermediate morphology hybrid called _A. prolifera_ (Vollmer & Palumbi 2002). These two sister species, which have distinct morphologies allowing them to occupy different habitats, are thought to have diverged over three million years ago, when _A. palmata_ first appears in the fossil record (McNeill et al. 1997). _A. palmata_, which has robust branches with fused axial polyps, inhabits the shallow, high-energy reef crest, whereas _A. cervicornis_, which has thin branches generally dominated by a single axial polyp, tends to inhabit the fore-reef and back-reef habitats.

High-throughput sequencing facilitates the characterization and quantification of whole transcriptomes in non-model organisms and is a powerful new tool for studying species of conservation concern, such as _Acropora_ corals. This technology is already being used to study issues such as coral response to climate change (Meyer et al. 2011; Polato et al. 2011; Moya et al. 2012) and disease (Libro et al. 2013), and is the best currently available method to comprehensively study total transcriptomic variation. In this study, I used Illumina RNA sequencing (RNA-seq) to examine within-colony gene expression differences in the two Caribbean _Acropora_ species, comparing actively growing apical branch tips to less actively growing branch bases. I examined overall gene expression differences within the coral colonies and between species, and I evaluated the differentially expressed (DE) genes for patterns relating to growth, polyp development, and deposition of a mineralized skeleton. Here I describe and focus on (1) genes that are DE between branch bases and branch tips for both species (i.e. DE by colony position), which indicate processes that contribute to or arise from DOL within the colony, and (2)
genes that are DE by both colony position and species, which indicate processes that may be involved in facilitating morphological differences between species.

Methods

Sample collection & RNA extraction

*Acropora cervicornis* and *A. palmata* samples were collected in August 2009 from Crawl Cay, Bocas del Toro, Panama. Paired apical tip (top 2 cm of branch) and base (25-30 cm from the branch tip in *A. cervicornis* and >10 cm from the branch tip in *A. palmata*) samples were collected for four colonies of *A. cervicornis* and three colonies of *A. palmata*. *A. cervicornis* samples were collected from colonies at least 10 m. apart at 5-6 m. depth, and *A. palmata* samples were collected from colonies 4-5 m. apart at 1-2 m. depth. Samples were flash frozen, placed in TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) and stored at -80°C. Total RNA extraction was conducted using the TRI Reagent manufacturer’s protocol with an additional 75% ethanol wash step. Total RNA quality was assessed using Agilent Bioanalyzer 2100 RNA Pico Chips, and only extractions with clear distinct 18S and 28S ribosomal RNA peaks were used (RIN 5.3-8.3).

Illumina RNA-seq library preparation

mRNA was isolated using Dynabeads® oligo(dT) mRNA isolation beads (Life Technologies, Grand Island, NY) to exclude non-protein coding RNAs and non-eukaryotic mRNAs from the samples. cDNA was produced using random hexamer primers and SuperScript® II reverse transcriptase (Life Technologies) for first strand synthesis and DNA polymerase I (New England BioLabs, Ipswich, MA) for second
strand synthesis. RNA-seq libraries were prepared by fragmenting the double stranded cDNA with dsDNA fragmentase (NEB) followed by library preparation using NEBNext® reagents. The cDNA transcripts with barcoded adapters were then size selected at 250 bp by gel extraction and 15 rounds of PCR amplification. RNA-seq libraries were sequenced (3 samples per lane) using single-end 40 bp sequencing on the Illumina GAII platform (Illumina, Inc., San Diego, CA, USA) at the FAS Center for System Biology at Harvard University.

Bioinformatics

Read quality control and barcode removal were conducted with custom Perl scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). RNA-seq reads were mapped against our published de novo transcriptome for A. cervicornis and A. palmata (Libro et al. 2013) using CLC Genomics Workbench (CLC bio). Reference transcriptome annotation was conducted using translated nucleotide queries against the curated UniProt/Swiss-Prot database. Reference transcriptome sequences and RNA-seq reads are available on BioProject (accession number PRJNA222758). Coral genes were identified by aligning the de novo reference transcriptome contigs to the genomes of congeners A. digitifera (Shinzato et al. 2011) and A. millepora (D. Miller, unpublished). Transcripts with a significant BLAST hit (E-value < 10^{-10}) were identified as coral and retained for the analyses. Putative non-coral transcripts were removed.

Because galaxin and SCRiP genes may be important in coral calcification and development, I performed additional BLAST for multiple galaxin proteins (UniProt: D9IQ16, D9IQ17, D9IQ18, B8UU51, Q8I6S1, A8C9K2) and SCRiP translated nucleotide (GenBank: FJ842102-FJ842109, EU659816, BK006534-BK006538).
sequences obtained from NCBI against translated queries for the full reference transcriptome. RNA-seq quantification of gene expression was conducted in CLC Genomics Workbench (CLC bio, Aarhus, Denmark) using local alignments, including non-specific mappings across multiple contigs via random assignment. Default parameters were changed by lowering the length fraction to 0.4 and increasing the similarity to 0.9 to account for potential transcriptome fragmentation resulting from the short length (36 bp) of the reads.

Statistical analysis of differential expression

To determine whether sample groups shared similar expression profiles, clustering of samples using nMDS and the Bray-Curtis similarity index were conducted in Primer v6 (Clarke & Gorley 2006) using coral-only count data for transcripts with greater than 100 total normalized counts. Count data were normalized by library size using the DESeq package (Anders & Huber 2010) in R (R Development Core Team, 2012). Permutational multivariate analysis of variance (PERMANOVA) (Anderson 2001) was conducted using Bray-Curtis similarity with permutation of residuals under a reduced model in Primer v6 to test the effect of the two random independent variables, species and colony position, as well as their interaction. A significant interaction indicates that the level of expression for one factor is dependent on the level of the other factor.

DE genes were identified using a two-factor negative binomial GLM test implemented using DESeq in R. This tests the effect of two independent factors, species (i.e. A. cervicornis vs. A. palmata) and colony position (i.e. branch base vs. branch tip) on the dependent variable (gene expression); the interaction between factors was also tested.
Transcripts expressed at normalized counts less than 100 (sum for all 14 samples) were excluded from the analyses to prevent bias (i.e. genes expressed at a low concentration for which a small absolute change in expression would appear to be a large fold change). Transcripts with greater standard deviation than mean within any sample group (e.g. A. cervicornis tips) were also excluded (after Pespeni et al. 2013). An adjusted p-value < 0.05 was used to evaluate significance (Benjamini & Hochberg 1995). I further narrowed my list of important significant DE genes by applying a threshold of 2-fold (i.e. |log₂-fold change| > 1) difference in gene expression, except for transcripts with a significant interaction effect. “Up-regulated” indicates a higher expression in the stated treatment, e.g. up-regulated in tips indicates higher expression in tips than in bases. Heat maps of selected genes were created using log-transformed normalized count data in GENE-E (Gould).

Functional genetic pathway analysis

GO terms were obtained from the GO database (Ashburner et al. 2000) for transcripts annotated with UniProt protein IDs (BLAST e-value < 10⁻⁵). Functional GO categories (for Biological Process) were further identified for DE genes using ErmineJ v. 3.0 (Lee et al. 2005), which classifies genes by GO category, identifies categories with many ‘multifunctional’ genes (which may be involved in many processes other than that specified by the current GO term), and determines if a category is ‘enriched’ for DE genes. The depth of GO annotation varied among transcripts; therefore, for functional definitions described in the results I conducted additional classification of genes based on UniProt annotations and a review of the literature.
For the DE Wnt genes, an additional BLAST (blastx) was conducted against the NCBI non-redundant protein sequence database to identify the closest cnidarian homolog to more precisely describe the putative functions of these genes.

Results and discussion

Transcriptome-wide RNA-seq profiles

The RNA-seq libraries contained an average of 4.7 million reads, with 3.6 million mapped reads (Table 1). The combined A. cervicornis and A. palmata coral-only dataset included 47,748 transcripts, of which 23,554 transcripts were expressed at least 100 normalized counts, and 22,320 transcripts remained after removing those with group SD>mean. Of the 22,320 transcripts in the coral dataset, nearly 15,000 were annotated with known or predicted proteins at an e-value < 10^{-5} in the UniProt database.

Non-metric multidimensional scaling (nMDS) analyses of the gene expression profiles show that the samples form distinct groups by species (A. cervicornis and A. palmata) and by colony position (tip and base) (Figure 2). PERMANOVA analyses indicate that polyps sampled from different locations along the colony branch have highly different gene expression profiles (d.f.=1, Pseudo-F=4.4169, P=0.001), as do polyps sampled from the two species (d.f.=1, Pseudo-F=5.8765, P=0.001). However, there was no significant interaction between these factors for the transcription profiles as a whole (d.f.=1, Pseudo-F=0.9161, P=0.480).

Differentially expressed genes

A two-factor, negative binomial generalized linear model (GLM) was used to identify differentially expressed (DE) genes that differed significantly due to colony position and species or that had an interaction effect between factors (Figure 3; for
annotated DE genes see Additional file 1). Out of the 2288 transcripts DE by species, 50% were up-regulated in *A. cervicornis* and 50% were up-regulated in *A. palmata* (Figure 4A). Out of the 2215 transcripts DE by colony position, 60% were up-regulated in branch tips and 40% were up-regulated in bases (Figure 4B). To elucidate the differences associated with DOL between branch base and branch tips consistent for both species, I focus on the 679 annotated transcripts DE with > 2-fold change in gene expression as a function of colony position only. To understand the genetic underpinnings of differences in growth morphology between *A. cervicornis* and *A. palmata*, I focus on transcripts that were DE for both colony position and species with > 2-fold change in gene expression or were significant for the interaction between factors. A total of 315 transcripts were DE for both factors and nine transcripts showed a significant interaction between factors. Of these, 69 transcripts were annotated with known or predicted proteins (Figure 5).

Biological function of DE genes

Within an *Acropora* colony, branch tips are the site of rapid growth, a process including both asexual reproduction of polyps and skeleton deposition, each regulated by a number of biological functions. The basal/radial polyps of the colony are the site of gamete synthesis and energy production via the photosynthesis carried out by their higher concentration of *Symbiodinium*. Asexual reproduction of polyps requires mitotic cell proliferation as well as regulation of cell identity through developmental signaling pathways. Production of the calcium carbonate skeleton is dependent on maintaining a high aragonite saturation state at the site of calcification (Cohen & McConnaughey 2003)
and controlling the shape of the precipitated biomineral, most likely through extracellular matrix (ECM) proteins. As expected, genes associated with these processes were significantly DE.

Some Gene Ontology (GO) Biological Process categories contained a relatively high number of DE genes for the colony position factor (Figure 6). Relevant categories include those involved in signaling and pattern development, metabolic processes, transport, and ECM. Categories containing many more genes up-regulated in tips include regulation of Wnt Signaling, translation, electron transport chain, ATP biosynthesis, ECM organization and collagen fibril organization. Cell-cell adhesion and calcium ion transport showed greater up-regulation in bases. To evaluate a broader number of biological functions, additional analyses were conducted based on UniProt annotation information and a review of the literature for DE genes showing greater than 2-fold difference in gene expression.

Signaling and development

There were pronounced patterns of DE signaling genes between branch tips and bases, particularly associated with three major signaling pathways, Wnt, Notch and Bone Morphogenetic Protein (BMP) (Table 2, Figure 7). These pathways are involved in pattern specification, cell fate commitment, establishment of tissue polarity, regeneration, and biomineralization and have previously been identified in cnidarians, such as Hydra, N. vectensis, and A. millepora (Ball et al. 2004; Technau & Steele 2011). Differential expression of these signaling pathways within the coral colony supports their roles in reef
coral development, not only during embryonic and early polyp stages, but also throughout the life of the colony.

In addition, I observed a small number of genes associated with these signaling pathways that were DE for both colony position and species factors. I propose that these intercellular signaling pathways, particularly Wnt signaling, may play an important role in organizing the asexual development of new polyps at branch tips and in regulating the branching patterns of *A. cervicornis* versus *A. palmata*.

Wnt signaling pathway

Wnt signaling functions via the interaction of Wnt ligands and two types of receptor molecules, Frizzled (Fzd) receptors and low-density lipoprotein co-receptors. Wnt pathway inhibitors include Dickkopf proteins (Dkk), Wnt inhibitory factor and secreted Frizzled-Related Proteins. Studies in multiple cnidarian species, including *Hydra*, *N. vectensis*, *Clytia hemisphaerica* and *Hydractinia echinata*, indicate that expression of Wnt and Dkk proteins are involved in oral/aboral axis formation, head regeneration and tentacle formation (Broun *et al.* 2005; Guder 2006; Plickert *et al.* 2006; Momose & Houliston 2007; Duffy *et al.* 2010; Trevino *et al.* 2011). Wnt signaling also appears to direct axial patterning in cnidarian larva, playing a similar role to that of Hox signaling in bilaterians (Kusserow *et al.* 2005). A number of Wnt genes DE in this dataset are either associated with developmental regulation in cnidarians or have been implicated in regulating biomineralization or cartilage formation in vertebrates (Table 3), suggesting that in calcifying corals Wnt signaling may also participate in skeleton formation.
Fourteen Wnt-related genes were DE between colony bases and tips for both species. The majority of these transcripts were more highly expressed in branch tips (wnts 2, 2b, 5a, 5b, 8a & 8b, lrp1, krem1, and dkk3), but five genes showed higher expression in colony bases (wnts 7a & 7b, lrp6, sox17 and casein kinase I). One putative Wnt regulatory gene, dickkopf-related protein 3 (dkk3), was represented by four transcripts exhibiting high up-regulation in branch tips (3.4-7.5 fold change). Although dkk3 has shown some Wnt-related activity in bilaterians, in cnidarians there is evidence that dkk3 facilitates migration of mature cnidocytes from the gastric region towards the tentacles (Fedders et al. 2004).

Four putative Wnt pathway transcripts were significantly DE for both species and colony position, all of which were up-regulated in branch tips. Two transcripts were significantly up-regulated in A. cervicornis branch tips, collagen triple helix repeat-containing protein 1 (cthrc1) and apolipoporphins, and two were up-regulated in A. palmata branch tips, fzd6 and sox9. Another gene, low-density lipoprotein receptor-related protein, lrp1b, which was DE by colony position and significant for the interaction between factors, is closely related to LRP1, a Wnt regulator (Zilberberg et al. 2004). lrp1b was more highly expressed in tips of both species but to a much greater degree in A. cervicornis (110-fold) than A. palmata (8-fold change).

Notch signaling pathway

Canonical Notch signaling occurs between adjacent cells and involves a transmembrane surface receptor (Notch) that interacts with membrane-bound ligands (Delta-Serrate-LAG2) on neighboring cells (Koch et al. 2013). Activation of Notch is
associated with maintaining the undifferentiated state of cells, while suppression of Notch is required for cells to progress toward a specific cell fate (Koch et al. 2013). In cnidarians, Notch signaling is involved in asexual budding and tentacle formation, as well as development of neural cells, oocytes and cnidocytes, as shown in developing Hydra (Käsbauer et al. 2007; Münder et al. 2010) and N. vectensis (Marlow et al. 2012).

However, Hydra and N. vectensis are both non-calcifying cnidarians and Notch may have additional roles in calcifying corals. The effect of Notch signaling may also be influenced by interactions with Wnt and TGFβ/BMP signaling pathways (Lin & Hankenson 2011; Collu et al. 2012).

Eighteen transcripts in the Notch signaling pathway were DE by colony position and were usually up-regulated in tips. In this dataset, notch1 and notch2 transmembrane protein genes and a regulatory gene, GDP-mannose 4,6, dehydratase, were consistently up-regulated in tips, while one Notch regulatory gene, E3 ubiquitin-protein ligase MIB2, was up-regulated in bases. One Notch transcript (notch1), which was up-regulated in A. cervicornis base samples, was significantly DE for both factors.

Bone Morphogenetic Protein signaling pathway

BMPs are secreted signaling molecules that bind to transmembrane BMP receptors (I & II) and initiate a downstream signaling cascade regulating the expression of target genes. BMP inhibitors include chordin, noggin, and intracellular inhibitory proteins, while tolloid-like proteins may cleave chordin to enhance signaling (Matus et al. 2006). All six BMP-associated DE genes for colony position showed higher expression in branch bases (bmp3b, follistatin-related protein 5, kielin/chordin-like protein, tolloid-like
protein (Ill) 1 & 2, and transcription factor scleraxis), revealing the opposite pattern from Wnt and Notch signaling pathways. One potential BMP-related gene, follistatin-related protein 4 (fstl4), which shares similarities with follistatin, a BMP inhibitor (Iemura et al. 1998), was DE for both factors and was up-regulated in A. cervicornis bases.

BMP signaling, specifically via BMP2/4, chordin and tolloid, is important in the dorsal-ventral (D-V) axis determination of bilaterians (Holley et al. 1995; Niehrs 2010). bmp2/4 and chordin show localized expression during cnidarian larval development (Hayward et al. 2002; Rentzsch et al. 2006; Saina et al. 2009), and BMP2/4 has also been localized to the calicoblastic epithelium of mature corals, suggesting involvement in skeleton formation (Zoccola et al. 2009). As its name suggests, BMP is often associated with bone growth and biomineralization, and the combined effects of BMP4 and calmodulin have been proposed to determine the overall length and width of bird beaks and fish jaws (Parsons & Albertson 2009). The differential expression of BMP signaling pathway (up-regulated in bases) and calmodulin (two transcripts up in tips, one up in bases; see calcium signaling) may be involved in coral biomineralization as well.

Interestingly, the only BMP protein DE by colony position was bmp3b, which was up-regulated in bases. In vertebrate models, BMP3 and BMP3b function differently than other BMP proteins and are antagonists of osteogenic BMP2 (Daluiski et al. 2001; Hino et al. 2003; 2004). Consequently, lower bmp3b expression in branch tips may enhance activity of other BMP proteins that were not DE by colony position, including BMP2/4.

Other developmental signaling pathways
Hox genes encode homeodomain-containing proteins, regulatory proteins that
direct patterning and identity of embryonic regions in animals. In cnidarians, some
Hox/ParaHox genes appear to be involved in anterior/posterior patterning during larval
development, development of sensory cells (Chiori et al. 2009), and determination of
polyp morphotype (cnox2) (Cartwright et al. 1999). Most of the Hox/ParaHox genes DE
by colony position, including Hox/LIM proteins, goosecoid, paired-like, and six3/6
homeobox genes (Gauchat et al. 1998; Broun et al. 1999; Srivastava et al. 2010;
Sinigaglia et al. 2013), have likely roles in larval or polyp development. Some transcripts
annotating to Hox or Hox-like genes were DE in this dataset, yet none were DE for both
factors with >2-fold change, suggesting that these genes function similarly in both A.
cervicornis and A. palmata.

Two forkhead domain containing proteins and a Hedgehog pathway receptor were
DE by colony position. Some forkhead box proteins are involved in embryonic
development of Hydra (Martinez et al. 1997) and N. vectensis (Magie et al. 2005), and
many forkhead transcription factors interact with other signaling pathways, including
Wnt, TGF-β, and Hedgehog (Benayoun et al. 2011). patched homolog 1, a Hedgehog
pathway receptor associated with the divergence of jaw morphologies in cichlid fishes
(Roberts et al. 2011), was up-regulated in branch bases.

Calcium signaling

Calcium signaling relies on the gradient of calcium ions, rather than biomolecules,
and is found in both prokaryotes and eukaryotes (Case et al. 2007). In cnidarians calcium
signaling may affect multiple functions including reproduction (Hilton et al. 2012),
nematocyst regulation (Russell & Watson 1995) and biomineralization (Reyes-Bermudez et al. 2009a). Eight calcium signaling genes were DE by colony position. While GO term analysis indicated up-regulation of calcium signaling in bases, this is misleading because a number of calcium signaling-related genes up-regulated in tips were not annotated with this GO category, including calmodulin, dysferlin and delta-latroinsectotoxin-Lt1a. Both delta-latroinsectotoxin-Lt1a, a putative toxin (Dunlap et al. 2013), and its likely receptor, latrophilin-1, were up-regulated in branch tips. Five calcium signaling genes up-regulated in bases included a transcript of calmodulin, metabotropic glutamate receptor 1, extracellular calcium sensing receptor, calcium/calmodulin dependent protein kinase, and E-selectin. Calmodulin is a highly-conserved calcium-binding protein that interacts with other proteins to facilitate calcium signaling and is associated with shaping craniofacial morphology of some bird and fish species (Abzhanov et al. 2006; Parsons & Albertson 2009; Mallarino et al. 2012).

Skeleton deposition

Skeletal growth in *Acropora* proceeds by a lattice-like arrangement of extending parallel ‘rods’ and reinforcing perpendicular ‘bars’ that construct the corallite (Gladfelter 1982a; 1983b; 2007). Corallites of *A. cervicornis* are comprised of four concentric rings of skeleton (Gladfelter 1982a), while those of *A. palmata* contain three concentric rings (Gladfelter, E. H., personal communication). The linear extension growth rates of these two species have been estimated at 6.5-20 cm/yr for *A. cervicornis* and 5-10 cm/yr for *A. palmata* (Shinn 1966; Gladfelter et al. 1978; Bak 1976), with *A. cervicornis* also demonstrating a faster rate of calcium deposition (µg Ca/mg N/hr) (Goreau & Goreau...
1959). Despite the broader branches of *A. palmata* and its ability to withstand higher wave energy, skeletal construction in *A. cervicornis* is stronger and slightly less porous (Schuhmacher & Plewka 1981). My results, described below, suggest that many of the putative calcification genes are similarly expressed between species, such as carbonic anhydrase (CA), calcium ion transport proteins and ECM proteins like galaxin. Differences between species may be related to bicarbonate transport or ECM proteins that have been linked to coral skeleton or biomineralization in other organisms.

Regulation of pH, carbonate and calcium

In scleractinian corals, mineralization of calcium carbonate occurs beneath the calicoblastic epithelium as the conversion of calcium ions and carbonate to the aragonite form of calcium carbonate (\(\text{Ca}^{2+} + \text{CO}_3^{2-} \rightarrow \text{CaCO}_3\)) (Tambutté et al. 2011). In practice the source of inorganic carbon for calcification may also be bicarbonate (\(\text{Ca}^{2+} + \text{HCO}_3^- \rightarrow \text{CaCO}_3 + \text{H}^+\)) or carbon dioxide (\(\text{Ca}^{2+} + \text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{CaCO}_3 + 2\text{H}^+\)), both of which produce protons that must be removed from the site of calcification (Tambutté et al. 2011). Corals actively contribute to calcification through CA activity (interconversion of \(\text{CO}_2 + \text{H}_2\text{O}\) and \(\text{HCO}_3^- + \text{H}^+\)), and by regulating proton (i.e. pH) (Al-Horani et al. 2003; Venn et al. 2009) and calcium ion concentrations (Chalker 1976; Al-Horani et al. 2003; Marshall et al. 2007) within the calicoblastic epithelium and in the sub-epithelial space. Previous work has confirmed the presence and importance of CAs, anion channel, and calcium channel proteins in coral calcification and within the calcifying tissues (Allemand et al. 2004; Tambutté et al. 2006; Grasso et al. 2008; Moya et al. 2008a). Though calcium ion transport across the oral epithelial layers occurs via passive diffusion
in some species (Tambutté et al. 1996), energy-driven calcium transport across the calicoblastic epithelium is believed to involve an L-type Ca\(^{2+}\) channel protein and Ca\(^{2+}\)-ATPase coupled with an anion carrier to transport calcium ions across the oral calicoblastic epithelial membrane (Tambutté et al. 1996; Zoccola et al. 1999; Al-Horani et al. 2003) and a PMCA-type calcium pump (Zoccola et al. 2004) to transport calcium to the extracellular calcifying site. I found that CA, bicarbonate transport, and calcium ion transport transcripts were DE by colony position.

As expected for the location of rapid calcification, CA activity (CAs 2, 3 & 7) was highly up-regulated in branch tips, though one transcript (CA2) was up-regulated in bases. No CA transcripts were DE for both factors. In corals, it has been proposed that CAs function to provide increased inorganic substrates for both skeleton formation (bicarbonate) (Moya et al. 2008a) and symbiont photosynthesis (carbon dioxide) (Furla et al. 2000), which may explain up-regulation of CA2 in branch bases.

One inorganic carbon transport protein, *electrogenic sodium bicarbonate transporter 1* (*SLC4A4*), was up-regulated in tips, while one, *sodium-driven chloride bicarbonate exchanger* (*SLC4A10*), was DE for both factors. *SLC4A10*, which was up-regulated in *A. palmata* tips, imports bicarbonate and sodium into the cell. In corals sodium-driven bicarbonate exchangers may regulate pH and supply bicarbonate for calcification (Polato et al. 2011). A number of other ion transporters were DE by colony position. Those up-regulated in tips included anion exchange proteins, potassium channels and sodium/potassium-transporting ATPases. Those up-regulated in bases include an organic cation transporter, four solute carriers, and a cation channel.
Calcium transport may be involved directly in transporting calcium ions to the site of skeleton deposition, but may also be related to calcium signaling. A voltage-dependent L-type calcium channel protein was DE by colony position, but was up-regulated in branch bases. Three transcripts of \textit{VWFA and cache domain containing protein (cachd1)}, which may be involved in regulating voltage-dependent calcium channels, were DE by colony position (two transcripts were up-regulated in bases, one in tips). Transcripts of \textit{Ca}^{2+}-\textit{ATPase} were observed in the dataset, but none was DE by colony position. Another transcription-based study of acidification found no change in the expression of \textit{Ca}^{2+}-\textit{ATPase} in response to CO$_2$-driven acidification (Moya \textit{et al.} 2012). One calcium transport gene was DE for both factors. \textit{Transient receptor potential channel}, a calcium entry channel, was up-regulated in \textit{A. cervicornis} bases. A similar gene was previously found to be down-regulated in \textit{A. millepora} in response to ocean acidification (Moya \textit{et al.} 2012).

Extracellular matrix (ECM) & skeletal organic matrix (SOM)

The aragonite crystals that form at the margins of growing coral skeleton resemble abiotically precipitated calcium carbonate, leading some to suggest that coral skeletal growth is independent of SOM (Barnes 1970; Constantz 1986), yet many studies suggest a role for SOM in some form (Cuif \textit{et al.} 1999; Fukuda \textit{et al.} 2003; Watanabe 2003; Puverel \textit{et al.} 2004; 2005; Helman \textit{et al.} 2008; Ramos-Silva \textit{et al.} 2013). ECM proteins can be incorporated into the skeleton (as skeletal organic matrix; SOM) and/or provide a structured boundary for the growing skeleton. SOM is believed to control skeletal growth by either inducing or inhibiting nucleation of biomineral crystals, but
some SOM proteins may be components of epithelial ECM and cell-adhesion proteins that become incorporated into the skeleton as it grows.

Until recently only one SOM protein, galaxin, had been characterized from a scleractinian coral (Fukuda et al. 2003), yet a number of putative SOM constituents have been proposed, including small cysteine-rich proteins (SCRiPs) (Sunagawa et al. 2009a), unidentified proteins or short peptides with high acidic amino acid (aspartic acid and glycine) content (Puverel et al. 2005), glycosaminoglycans (Puverel et al. 2005), lipids (Puverel et al. 2007), and chitin (Wainwright 1963; Young 1973). Recently, 36 SOM proteins were extracted and identified from A. millepora skeleton, suggesting roles for numerous functional proteins in biomineralization, including ECM-cell adhesion proteins, enzymes, acidic proteins, and a toxin (Ramos-Silva et al. 2013).

ECM: within colony differences

One galaxin, galaxin-2, was up-regulated in tips of both species, while another galaxin was not DE for either factor. Previous studies have noted that some galaxin-related genes are expressed at different stages of development (Reyes-Bermudez et al. 2009b) or respond differently to elevated CO₂ (Moya et al. 2012), supporting distinct roles for various galaxins in calcification or other functions. Two SCRiP-3 transcripts were also DE, but were up-regulated in bases. SCRiPs are coral-specific proteins of unknown function, but their cysteine-rich composition has been suggested as a possible mode of interaction with the SOM protein galaxin (Sunagawa et al. 2009a). SCRiP-3 gene expression has previously shown association with larval development (Hayward et al. 2011; Siboni et al. 2014), as well as localization to developing skeletal septa.
(Hayward et al. 2011); however, up-regulation of SCRIp-3 in bases suggests it is not involved in rapid calcification in branch tips of A. cervicornis. A number of genes DE by colony position resembled those characterized in A. millepora, including a mucin, hemicentin, polycystin-1, protocadherins and collagen type I alpha 1. Additional ECM genes that were DE, including proteoglycan, glycoprotein and endopeptidase transcripts, may regulate cell-cell or cell-matrix interactions that guide coral growth; however, further studies are needed to determine why these genes were DE, since they may have alternate roles in corals.

One mucin, integumentary mucin C.1, was up-regulated in tips and may also serve as a component of the ECM or even play a role in biomineralization, as they do in molluscs (Marin et al. 2000); however, mucins are used by corals for feeding, as a physical barrier against microbes and physical stresses (Brown & Bythell 2005). Four hemicentin (1 and 2) transcripts were DE by colony position, but with up-regulation in tips and bases. In corals, hemicentin is involved in hemidesmosome-mediated attachment of the calicoblastic epithelium to the skeleton (Muscatine et al. 1997). As the skeleton grows, hemicentin may become incorporated with the deposited aragonite (Goldberg 2001). Four cadherins or protocadherins were DE by colony position. One gene, protocadherin Fat 3, was up-regulated in tips, and three genes, protocadherin Fat 4, protocadherin-23, and cadherin EGF LAG seven-pass G-type receptor 3, were up-regulated in bases. Cadherins form adherens junctions, regulate cell adhesion, mobility and communication, but also interact with ß-catenins and are one way in which Wnt proteins may be involved in mediating cell-cell interactions (Akiyama et al. 2004).
Only one collagen (col1a1) has been characterized from A. millepora SOM, but my data indicate that a large number of additional collagens are involved in coral growth. This is not surprising given that collagens comprise about 30% of the total protein content of animals. Twelve collagen transcripts were DE by colony position, with the majority (n=11), including coll1a1, more highly expressed in tips. In addition, four collagen-interacting genes were up-regulated in tips, including loxl2, transmembrane prolyl 4-hydroxylase (P4H-TM), collagenase 3 and fibronectin, and two were up-regulated in bases, procollagen C-endopeptidase enhancer 1 and peroxidase mit-7.

Eleven proteoglycan and proteoglycan synthesis genes were DE by colony position. These included two transcripts of insoluble matrix shell protein 1 (ISMP-1) that were up-regulated in bases and one transcript up-regulated in tips. ISMP-1 was originally identified as a component of the organic matrix in the calcified shell of the Manila clam, Venerupis philippinarum, and may represent a conserved biomineralization protein (Marie et al. 2011). Six glycoproteins were significantly DE by colony position, including uromodulin (Tamm-Horsifall protein), which had two transcripts up-regulated in bases. Uromodulin controls crystal formation in the vertebrate urinary tract and may act as a mineralization inhibitor during skeleton formation, but it has also been implicated in symbiont interactions in non-calcifying cnidarians (Ganot et al. 2011).

ECM: differences between species

Three ECM proteins, similar to those found in coral SOM (Ramos-Silva et al. 2013), were DE for both factors or showed an interaction between factors. hemicentin-1, was up-regulated in A. palmata branch tips. A protocadherin (Fat 2) transcript was most
highly expressed in *A. cervicornis* bases. And a gene annotated as *cephalotoxin* from the squid *Sepia esculenta* showed an interaction between factors, though it was not significantly DE for either; it was up-regulated in branch bases of *A. cervicornis* but branch tips of *A. palmata*.

Since no collagen genes were DE for both factors, direct gene expression of collagens does not appear to determine the morphological differences between *A. cervicornis* and *A. palmata*; however, two collagen-interacting protein genes were up-regulated in branch tips as well as being DE for species. *cthrc1*, a secreted glycoprotein with putative roles in regulating deposition of extracellular collagen matrix and Wnt signaling, was up-regulated in *A. cervicornis* tips, and *lysyl oxidase homolog 2* (*loxl2*), which may have a role in collagen processing, was up-regulated in *A. palmata* tips.

Two C-type lectins were most highly expressed in *A. cervicornis* tips, one of these, Lectin BRA-3, is believed to be involved in biomineralization in barnacles and has been shown to either inhibit or promote crystal growth of calcium carbonate in vitro, depending on the conditions (Muramoto et al. 1994; Kamiya et al. 2002; Matsubara et al. 2008). Lectins have numerous functional roles in animals, including cell adhesion, glycoprotein synthesis, and immunity and may regulate host-symbiont interactions in cnidarians (Kvennefors et al. 2008; Wood-Charlson & Weis 2009; Libro et al. 2013).

**Growth & metabolism**

Increased cellular activity in branch tips was indicated by high expression of genes associated with aerobic respiration and translation relative to branch bases. This is consistent with the observed higher metabolic rate at the tip of *Acropora* branches,
relative to the bases (Gladfelter et al. 1989). However, cytoskeletal construction was up-regulated in branch bases, and genes related to ATP biosynthesis, carbohydrate and lipid metabolic processes were up-regulated both in tips and bases.

Consistent up-regulation of translation in tips of both species was indicated by a large number (n=16) of moderately elevated (~2-fold) ribosomal protein (RP) transcripts, only two RPs were up-regulated in bases. Elevated aerobic respiration in tips was indicated by increased expression of five genes involved in the mitochondrial electron transport chain. One gene, a succinate dehydrogenase, was DE for both factors and was most highly expressed in A. palmata tips. Cytoskeletal construction was up-regulated in branch bases. Almost all cytoskeleton-related genes (including actin, tubulin and dynein) that were DE by colony position (n=31) were up-regulated in bases (n=27). Only four transcripts of cytoskeleton genes were up-regulated in tips (neurofilament medium polypeptide, girdin, tubulin alpha-1D chain, and spectrin).

Sphingolipid metabolism was the most prominent DE lipid biosynthesis pathway. Sphingolipids are lipids with a backbone of sphingoid bases that form a protective layer outside of the cell membrane, and complex glycosphingolipids can be involved in cell recognition, signaling, and immunity. Three genes involved in sphingolipid metabolism were DE by colony position with two up-regulated in tips (alkaline ceramidase and galactosylceramide sulfotransferase) and one up-regulated in bases (ceramide kinase). One transcript, sphingolipid delta(4)-desaturase/C4-hydroxylase DES2 (DEGS2), was DE by species with a significant interaction effect, showing highest expression in A. cervicorns bases and lowest expression in A. palmata bases. In cnidarians, sphingolipids
appear to be involved in stability of the coral-Symbiodinium relationship and may
determine whether heat stress results in coral bleaching (Detournay & Weis 2011).

Genes involved in the biosynthesis of fatty acids showed a pattern of up-
regulation in bases, but lipid catabolic processes appeared to be up-regulated in tips.
Three genes involved in building fatty acid chains (fatty acid synthase, acetyl-CoA
 carboxylase 1, and acyl-CoA desaturase) were up-regulated in bases and at least three
genes involved in fatty acid beta-oxidation (short-chain specific acyl-CoA dehydrogenase,
hydroxyacyl-coenzyme A dehydrogenase and long-chain-fatty-acid-CoA ligase 5) were
up-regulated in tips. Fatty-acid molecule production in bases may serve two functions:
corals may store energy produced by the higher photosynthetic activity of Symbiodinium
as fatty-acids (Bachok et al. 2006), and/or radial polyps increase production of fatty acids
to meet the high lipid demand of egg development (Arai et al. 1993), which is likely to be
higher during the summer spawning season. Another lipid metabolism gene,
phospholipase A2 isozyme PA-12C, which could function in the arachidonic acid pathway,
in repairing oxidized membrane lipids (Yu 1994) or as a toxin in the nematocyst complex
(Grotendorst & Hessinger 2000; Nevalainen et al. 2004), was DE for both factors and
most highly expressed in A. palmata tips.

Response to light & stress

As sessile animals, Acropora corals are unable to change location (unless
disrupted or broken by external forces) and therefore respond to environmental stimuli
and stresses physiologically. Acropora corals use light cues (blue light of 408-508 nm) to
determine their direction of growth and to initiate axial polyp development (Kaniewska et
al. 2009), and like other corals, they may also use light to adapt their polyp behavior (Levy 2003) and coordinate spawning (Brady et al. 2009; Boch et al. 2011). Living in relatively shallow waters, Caribbean Acropora are exposed to high levels of UV and heat stress, as well as oxidative stress resulting from metabolism and photosynthesis. A number of light and stress response genes were DE between tips and bases, and interestingly genes DE for both factors were consistently up-regulated in A. palmata. This is likely due to A. palmata being located in shallower water and exposed to higher levels of UV light compared to A. cervicornis, thus requiring a greater response of chaperone proteins and potentially photoprotective pigment (GFP-like) (Salih et al. 2000; Roth et al. 2010).

Two photoreceptor genes that could be involved in phototropic growth, retinol dehydrogenases (rdhs) 7 and 8, were up-regulated in tips. Rdhs convert retinol to retinal, a polyene chromophore involved in animal vision. In A. millepora, expression of rdh decreased in response to transfer into laboratory conditions, likely in response to lower light conditions (Bay et al. 2013), but increased in response to heat stress (DeSalvo et al. 2010b). Two melatonin receptors were DE by colony position. Melatonin receptor type 1B-B was up-regulated in tips, and melatonin receptor type 1A was up-regulated in bases. Melatonin production is light dependent, with increased production at night being a primary regulator of circadian rhythm in vertebrates. Melatonin has been shown to affect expansion of oral disc in sea anemone polyps (Actinia sp.) (Tsang et al. 1997) and may be involved in polyp behavior. In Caribbean Acropora, polyp behavior during the day differs between radial and axial polyps; symbiont-rich radial polyps are active throughout the day to photosynthesize, but axial polyps are only extended at night to feed. Two
genes involved in photoreception, *rdh8* and *crumbs homolog 1*, were DE for both factors, and both were most highly expressed in *A. palmata* bases,

Heat shock proteins (HSPs) function during times of stress, such as at elevated temperatures, when other proteins may become denatured. One HSP, *HSP70*, was DE by colony position and was up-regulated in bases. Two transcripts of *sacsin*, a DNAJ/HSP40 protein that acts as an HSP70 co-chaperone, were DE for both factors and most highly expressed in *A. palmata* bases. While HSPs protect cells from stress-related damage and may be up-regulated in bases due to higher levels of ROS during the day resulting from photosynthesis, HSP70-related genes have also been shown to interfere with Wnt-related axial development in cnidarians (Duffy *et al.* 2012); therefore, lower expression of these genes in tips may actually prevent interference with proper polyp development.

Another group of proteins showing differential expression were antioxidants that may be involved in redox response. Three putative antioxidants were up-regulated in tips, including *thioredoxin domain-containing proteins* (5 & 12) and *selenoprotein* W. One putative stress response protein, a cyan-emitting GFP-like fluorescent chromoprotein (*amFP486*), was DE for both factors and up-regulated in *A. palmata* tips. GFP-like pigment proteins, which were also found to be up-regulated in branch tips of *A. millepora* (Bay *et al.* 2009a), are thought to provide protection from strong UV radiation (Salih *et al.* 2000; Roth *et al.* 2010).

**Cnidarian-specific gene expression**

Known cnidarian-specific genes that exhibited differential expression were those involved in cnidocyte and nematocyst development. Nematocysts are barb-containing
cnidocysts that fire in response to mechanical stress to capture zooplankton prey or as defense. These organelles are comprised of an outer wall of nematocyst outer wall antigen (NOWA) and an inner wall of minicollagen containing a barb and stored toxins (Meier et al. 2007). A number of transcripts annotating to genes involved in nematocyst development showed increased expression in branch tips, including two minicollagen transcripts, two NOWA transcripts, and a nematoblast-specific protein transcript.

Coral homologs of *N. vectensis* predicted proteins with no additional known function accounted for 92 transcripts DE by colony position, nine that were DE for both factors and one that was DE for species with a significant interaction. These may be cnidarian specific genes deserving of additional investigation.

Division of labor within the coral colony

Within the *Acropora* coral colony there is strong DOL between the actively growing branch tips and the radial polyps of branch bases. Increased expression of transcripts within the data is similarly divided between these regions, indicating that both sections of the coral branch are actively regulating different genetic processes. The high number of DE genes within colonies suggests greater differentiation among polyps in Caribbean *Acropora* than was previously found for *A. millepora* (Bay et al. 2009a). This may be a true difference due to the contrast in colony structure between the much smaller *A. millepora* and the longer branches of Caribbean *Acroporas*, which may affect the extent to which polyps display functional differences, or it may be a function of technique (high-throughput RNA sequencing vs. microarray containing ~8700 UniGene ids). However, the DOL observed in this study between branch tips and bases appears not
to be as distinct as in some hydrozoans, as the proportion of total transcripts $(2,215/22,320 = 10\%)$ DE by colony position is less than that found between functionally different siphonophore zooids $(3,558/19,534$ transcripts $= 18\%)$ (Siebert et al. 2011).

Classical developmental signaling pathways (Wnt, Notch and BMP) were highly DE between branch tips and bases, indicating roles in the growth of mature coral colonies. In particular, up-regulation of a number of Wnt-related genes in branch tips, where new radial polyps are being produced, suggests that this signaling pathway is involved in asexual polyp budding in colonial Acropora corals. In other cnidarians, expression of Wnt signaling genes correlates strongly with the location of oral structures and appears to determine where tentacles and buds are produced (Hobmayer et al. 2000; Broun et al. 2005; Plickert et al. 2006; Lengfeld et al. 2009; Duffy et al. 2010; Trevino et al. 2011). In cnidarians, Notch signaling has been studied in much less depth than Wnt signaling; however, it appears to be important during development for proper cell fate determination, neurogenesis, and for establishing tissue boundaries during the budding of new polyps (Käsbauer et al. 2007; Münder et al. 2010; Marlow et al. 2012). Up-regulation of Notch signaling in branch tips may be related to any of these putative functions.

Studies of BMP signaling in cnidarians are also limited, but BMP genes show localized expression in developing embryos and appear to be involved in axis determination and gastrulation (Hayward et al. 2002; Rentzsch et al. 2006; Saina et al. 2009). In mature cnidarians, $bmp2/4$ is preferentially expressed in the cells that regulate skeleton deposition in corals, the calicoblastic epithelium (Zoccola et al. 2009). Both Wnt and BMP pathways may affect skeleton formation, as related genes are known regulators
of bone morphology and biomineralization in some vertebrate species (e.g. LRP6, Fzd6, Dkk3, BMP) (Williams & Insogna 2009; Mallarino et al. 2012; Cui et al. 2013). Whereas Wnt and Notch signaling were up-regulated in branch tips, BMP genes were primarily up-regulated in branch bases. Interestingly, the primary BMP proteins studied in cnidarians (BMP2/4 and BMP5-8) were not DE, rather genes with putative regulatory roles were up-regulated in bases, including bmp3b, chordin, tolloid, follistatin and scleraxis. Additionally, calcium signaling via calmodulin, a DE calcium signaling gene known to determine morphology in vertebrates (Abzhanov et al. 2006; Mallarino et al. 2012), may influence biomineralization of the coral skeleton. Because crosstalk among signaling pathways is common (Itasaki & Hoppler 2009; Lin & Hankenson 2011; Ann et al. 2012; Collu et al. 2012), it is likely they are not functioning independently.

Skeletal growth by deposition of calcium carbonate occurs more rapidly in the branch tips, where I expect genes involved in calcification to be up-regulated. Though I did not observe increased expression of Ca$^{2+}$-ATPase, a calcium transport protein suspected to be involved in calcification, I did observe differential expression of an L-type calcium channel, which is thought to regulate calcium ion transport into the calicoblastic epithelium (Tambutté et al. 1996); however it was up-regulated in bases. Up-regulation of control of pH and carbonate concentrations in growing tips was indicated by overall increased expression of CA and a bicarbonate transport protein, SLC4A4. Other ion transport genes, including calcium transport, were up-regulated in both branch tips and bases.
Coral skeleton formation is believed to involve ECM, both as SOM and as a boundary region. In these results, *galaxin-2*, a number of proteins with similarity to *A. millepora* SOM (Ramos-Silva *et al.* 2013) (*mucin, hemicentin, polycystin-1, protocadherins and colla1*), and ECM proteins with homologs involved in biomineralization in other species (*ISMP-1, chondroadherin, uromodulin* and collagen types I, II, XI, XXVII) were DE between tips and bases. These may be important candidate genes to investigate further as research continues into the effects of reduced oceanic pH on coral calcification.

In addition to the strong signature of DE developmental signaling genes and the numerous putative skeletal growth-related genes, I observed some differences between branch bases and tips for metabolic functions, response to environmental stimuli and stress and cnidarian-specific genes. Metabolic activity and translation were up-regulated in branch tips, supporting previous findings of increased respiration at distal regions of *Acropora* branches (Gladfelter *et al.* 1989). In this region where new tissue and polyps are being produced and mitotic rate is increased (Gladfelter *et al.* 1989), it is consistent that I observed an increased signature of translation and production of mitochondrial respiratory proteins (ETC). Interestingly, cytoskeletal genes were up-regulated in branch bases, which is unexpected but may be related to the production of gametes in this region. Some carbohydrate and lipid metabolic genes were DE by colony position; in particular, I speculate that DE lipid metabolic genes may be involved with gamete production and sphingolipid metabolism genes may be involved with regulating symbionts (see next section). Up-regulation of light and stress response genes was divided between tips and bases, though *HSP70* was consistently up-regulated in bases. The DE light response
genes, including rdhs and melatonin receptors, may be involved in phototropic growth response, tentacle behavior and/or spawning. Cnidarian-specific genes involved in nematocyst production were consistently up-regulated in growing tips, and a majority of predicted proteins annotating to N. vectensis, which may represent taxonomically-restricted proteins, were also up-regulated in branch tips and may have a role in growth and calcification.

Differences between A. cervicornis & A. palmata

The two coral species investigated, A. cervicornis and A. palmata, are sister species believed to have diverged in the Caribbean Sea approximately 3 million years ago (McNeill et al. 1997). While both species exhibit branching patterns, their morphologies are highly distinct from each other and they occupy different ecological niches. Despite these differences, these species are capable of sexual reproduction to produce F1 hybrids that display an intermediate phenotype known as A. prolifera (Vollmer & Palumbi 2002). RNA-seq results identified many differences in gene expression between these species that may be attributable to physiology, environment, and/or response to symbiont activity. To determine how these two species regulate the processes of growth and reproduction to achieve their distinct growth forms, I evaluated the 69 annotated transcripts that were DE both by colony position and by species, or that showed an interaction between the two factors (Figure 5).

Few of these candidate genes were associated but rather spanned a number of biological functions. Results suggest that differences in growth form between A. cervicornis and A. palmata involve Wnt, Notch, and possibly BMP signaling, regulation
of bicarbonate transport by a sodium-driven chloride bicarbonate exchanger, and ECM proteins. Wnt and Notch genes DE for both factors were consistently up-regulated in tips, with three Wnt-related genes most highly expressed in *A. cervicornis* tips (*cthrc1, apolipophorins, lrp1B*) and two in *A. palmata* tips (*fzd6* and *sox9*). Cthrc, Lrp1 (a protein with similarities to Lrp1B) and Fzd6 are associated with repression of canonical Wnt signaling pathways (Golan *et al.* 2004; Zilberberg *et al.* 2004; Yamamoto *et al.* 2008), while apolipophorins are associated with transport of Wnt and Hedgehog molecules (Panakova *et al.* 2005) and Sox9 is a β-catenin-interacting transcription factor known as a master regulator of cartilage development (Akiyama *et al.* 2004). Due to its critical role in dictating polyp growth form, Wnt regulation has been proposed as a mechanism driving morphological differences among cnidarian species (Duffy 2011). My data suggest that activation/inhibition and transport of Wnt signaling are also important in colonial corals and may play a role in maintaining dominance of a single or few axial polyps in *A. cervicornis* relative to *A. palmata*. Though one Notch receptor gene, *notch1*, was up-regulated in *A. cervicornis* tips, and one tentative BMP signaling gene, *fstl4*, was up-regulated in *A. cervicornis* bases, these pathways are not well characterized in cnidarians, so it is more difficult to speculate on their roles. Notch signaling, which is necessary for cell fate specification, neurogenesis and nematocyte differentiation in cnidarians, may be up-regulated in *A. cervicornis* tips because of the faster linear extension growth rate in this species.

In addition to developmental signaling pathways, some genes that may be more directly related to skeletal growth were DE for both factors. Although *A. cervicornis* has a slightly faster rate of linear branch extension than *A. palmata*, that growth is typically
driven by a single axial polyp, with a number of smaller radial polyps developing along the side of the branch. In contrast, in *A. palmata*, multiple fused axial-type polyps lead branch extension simultaneously. One gene that may be responsible for the broader skeleton of *A. palmata* is the bicarbonate transport protein *SLC4A10*, which was up-regulated in *A. palmata* tips. Interestingly, a calcium transport gene, *calcium entry channel*, was up-regulated in *A. cervicornis* bases, but this gene may be involved in other calcium-signaling related functions such as light-response and spawning (Hilton *et al.* 2012). A number of ECM genes showed species-specific expression, including three genes related to known *A. millepora* SOM proteins, *hemicentin*, *protocadherin Fat 2* and a cephalotoxin (from *S. esculenta*). Other ECM genes with a potential role in morphological differences between species include collagen interacting proteins (*cthrc1* and *loxl2*), and a C-type lectin involved in biomineralization of barnacles, *lectin BRA-3*. It is not understood how these ECM proteins function in corals, but they serve as candidates for further research.

Species-specific expression of the sphingolipid biosynthesis gene *DEGS2*, which was up-regulated in *A. cervicornis* bases and down-regulated in *A. palmata* bases, may be related the regulation of the sphingosine rheostat, a regulatory mechanism that balances signaling sphingolipids involved in cell fate and immunity, and which is suspected to facilitate coral-symbiont interactions (Detournay & Weis 2011). Regulation of sphingolipids may therefore differ between species due to environmental differences that affect symbiont activity, such as temperature and light availability. Light and heat response genes were consistently up-regulated in *A. palmata*, probably due to the greater light intensity of the reef crest environment. Two light-response genes, *rdh8* and *crumbs*
homolog 1, and the HSP70 co-chaperone sacsin were up-regulated in A. palmata bases. Up-regulation of sacsin in A. palmata branch bases may be attributable to increased concentration of reactive oxygen species in symbiont-rich regions of branches. A GFP-like protein up-regulated in A. palmata tips is also possibly involved in photoprotection of coral tissues (Salih et al. 2000; Roth et al. 2010).

These annotated DE genes, however, do not reflect the full extent of gene expression differences between species since many genes were found to be most similar to N. vectensis predicted proteins, which may be cnidarian-specific, and approximately 250 transcripts DE for both factors were not annotated. Further research is needed to identify the precise location and interactions of these candidate genes and to characterize their roles in coral growth. Additionally, a number of transcripts were DE for both factors, but did not meet the criterion of a 2-fold change magnitude of expression for one or both factors. Some of these, such as transcripts of CA2 (up in tips of A. cervicornis), calmodulin (up in tips of A. palmata), chordin (up in bases of A. palmata) were considered as DE for colony position, but may also be involved in differences between species.

The two species investigated are closely related enough to permit hybridization (Vollmer & Palumbi 2002), but display distinct morphological characteristics and occupy different habitats: A. palmata is found in the high-energy reef crest and A. cervicornis inhabits the lower energy fore- and back-reef. As expected, I found that transcriptomes for these species exhibit large differences in gene expression, many of which are certainly due to differences in environmental factors, but some of which likely indicate differences
in genetic regulation of growth form. Though studies on the role of gene expression in species evolution in corals are lacking, more is known in bilaterian systems in which the roles of developmental patterning genes such as Hox, Wnt, BMP and Hedgehog have been more widely explored. Studies in model systems indicate that changes in location, magnitude and timing of expression of functionally conserved genes, particularly during development, are responsible for morphological differences between species (Carroll 2008). While colonial cnidarians differ from these other groups of organisms in that they represent a more ancestral lineage, are comprised of a coordinated group of multiple modular polyps, and display indeterminate growth, my results suggest that similar genetic signaling pathways are associated with their divergent morphologies. Furthermore, the genetic regulation of biomineralization may involve genes similar to those involved in constructing the mineralized structures in higher animals. Vertebrate species with adaptive radiations of bony and cartilaginous features, such as the beaks of finches and jaws of fish, exhibit gene expression patterns and rates of genetic evolution implicating similar genetic pathways (Wnt, BMP, calmodulin) in the development of divergent morphologies that allow adaptation to varied habitats and food sources (Abzhanov et al. 2004; Albertson et al. 2005; Abzhanov et al. 2006; Parsons & Albertson 2009; Mallarino et al. 2012). Additional research into the gene expression of candidate genes in the hybrid A. prolifera and in the highly diverse Pacific Acropora corals can provide additional insights into the role of gene expression the evolution of their growth forms.
Conclusion

These RNA-seq results demonstrate that there are large differences in gene expression representing a strong DOL between polyps in the growing tips of branches compared to branch bases for both Caribbean Acropora. The number of transcripts differentially regulated by position within individual colonies (n=2215) is of the same magnitude as differences between the two species (n=2288). Genes showing differing levels of expression between branch tips and bases point to roles for classical development signaling pathways (Wnt, Notch and BMP) in branch extension and polyp development. Differential expression of CA, ion transport, ECM and putative SOM genes, indicate candidates that may be involved in the active control of skeleton growth by reef-building corals. A small number of genes were identified as DE both by colony position and species, pointing to genes that may play a role in regulating the different growth morphologies between these species.

Availability of supporting data

Additional file 1. Data set of annotated (UniProt/Swiss-Prot e-val < 10⁻⁵) coral transcripts. Includes adjusted P and log₂(fold change) values, and putative functions of genes discussed in the manuscript. This file can be accessed on LabArchives.com (DOI:10.6070/H4JD4TRV).

List of abbreviations

BMP, bone morphogenetic protein; CA, Carbonic anhydrase; DE, differentially expressed; DOL, division of labor; ECM, extra-cellular matrix; GO, Gene Ontology; HSP, heat shock protein; nMDS, non-metric multidimensional scaling; NOWA, nematocyst outer wall antigen; rdh, retinol dehydrogenase; SCRP, small cysteine-rich proteins; SOM, skeletal organic matrix.

Acknowledgements

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### Tables

Table 1. Mean and standard error of RNA-seq library sizes (lib) and mapped reads (mapped) by sample type.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th># Samples</th>
<th>Mean (lib)</th>
<th>Std. Err. (lib)</th>
<th>Mean (mapped)</th>
<th>Std. Err. (mapped)</th>
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</thead>
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<td>5973543</td>
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<td>2064676</td>
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<td>3560176</td>
<td>2055469</td>
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Table 2. Genes from developmental signaling pathways. Number of genes (transcripts in parentheses) found in annotated coral transcriptome (Reference), and in the dataset. Numbers of DE genes for colony position (Pos.) and both factors (Both) indicate transcripts with > 2-fold change in expression.

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>Gene family</th>
<th>Reference</th>
<th>Dataset</th>
<th>Pos.</th>
<th>Both</th>
<th>Int.</th>
<th>Trend</th>
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<td>Wnt</td>
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<td>-</td>
<td>Up tips</td>
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<tr>
<td></td>
<td>Frizzled</td>
<td>8 (16)</td>
<td>8 (12)</td>
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<td>-</td>
<td>Up A. palm tips</td>
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<tr>
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<td>8 (25)</td>
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<td>-</td>
<td>1</td>
<td>Up A. cerv tips</td>
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<tr>
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<td>Kremen</td>
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<td>1</td>
<td>1</td>
<td>-</td>
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</tr>
<tr>
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<td>1 (6)</td>
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<td>Up A. palm tips</td>
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<td>-</td>
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<td>-</td>
</tr>
<tr>
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<tr>
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<td>-</td>
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<td>-</td>
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<td>-</td>
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<tr>
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<td>1 (3)</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>E3 ubiquitin ligase MIB</td>
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<td>2 (14)</td>
<td>1</td>
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<td>-</td>
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<td>Chordin/chordin-like/kielin</td>
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<td>-</td>
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Table 3. Summary of selected DE Wnt pathway genes. Genes include those with roles in cnidarian development and/or cartilage development and biomineralization in other organisms, as well as genes for putative Wnt-interacting proteins. Putative functions and interactions are based on the references. Closest identified cnidarian homolog and GenBank accession ID included for Wnt proteins where applicable.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression higher in:</th>
<th>Functions &amp; Interactions</th>
<th>Study system</th>
<th>Ref.</th>
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</thead>
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<tr>
<td>Wnt2, 2a</td>
<td>Tips</td>
<td>Oral/aboral axis determination</td>
<td>N. v exacerbated</td>
<td>(Kusserow et al. 2005)</td>
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<tr>
<td>NvWnt2 (AAW28132)</td>
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<td>Wnt5a</td>
<td>Tips</td>
<td>Oral/aboral axis determination (NvWnt5)</td>
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<td>(Kusserow et al. 2005)</td>
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<td></td>
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<td>Bud/tentacle formation (hvwnt5)</td>
<td>Hydra</td>
<td>(Philipp et al. 2009)</td>
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<tr>
<td>Wnt7a, 7b</td>
<td>Bases</td>
<td>Oral/aboral axis determination (NvWntA; NvWnt7)</td>
<td>N. v exacerbated</td>
<td>(Kusserow et al. 2005)</td>
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<tr>
<td>NvWntA (AAT02182)</td>
<td></td>
<td>Bud formation/head regeneration (HyWnt7)</td>
<td>Hydra</td>
<td>(Lengfeld et al. 2009)</td>
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<td>A. cerv tips</td>
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Figures

Figure 1. Branch morphology of *A. cervicornis* (A) and *A. palmata* (B), with insets showing branch tips. Regions of sampling for branch tips (red boxes) and bases (white boxes).

Figure 2. nMDS for all samples and transcripts expressed at > 100 total normalized counts (n=23,554). Dashed lines delineate groups of samples.
Figure 3. Number of DE genes for factors (colony position and species) and their interaction considering all coral transcripts (A), annotated transcripts (B), and annotated transcripts with greater than 2-fold difference between treatments (C).
Figure 4. Scatter plots of gene expression for all genes in the dataset (n=22,320). Species (A) and colony position (B) comparisons with DE transcripts in red (P_{adj} < 0.05). Filtering of transcripts expressed at < 100 total normalized counts results in the loss of points around the origin.
**Figure 5. Heat map of transcripts DE for both factors or the interaction between factors (***).** Includes only transcripts with fold change > 2, except for transcripts with a significant interaction effect.

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Figure 6. GO term summary of DE genes for colony position. Values represent the number of transcripts with > 2-fold change in gene expression for selected 'enriched' GO Biological Process terms. N is the total number of transcripts in the dataset annotated with the given GO term.
Figure 7. Heat map of selected transcripts DE by colony position (but not species). Transcripts include those putatively involved in signaling, pH regulation or ion transport, or ECM with > 2-fold DE between branch bases and tips.
Chapter 3

Diurnal and Nocturnal Transcriptomic Variation in the Caribbean Staghorn Coral, *Acropora cervicornis*

Abstract

Reef-building corals experience large diel shifts in their environment, both externally due to changes in light intensity, predator activity and prey availability, and internally as a result of diel fluctuations in photosynthesis by their endosymbiotic algae, *Symbiodinium*. Diel patterns of tentacle behavior, skeletal growth, and gene expression indicate reactions of the coral animal to these regular changes, both in response to light and circadian regulation. Some corals, such as *Acropora*, have strong within-colony division of labor (DOL), including specialized fast-growing axial polyps. Recent work suggests that the within colony DOL in Caribbean *Acropora* is accompanied by large gene expression differences for genes involved in developmental signaling pathways (Wnt, Notch, and BMP), pH regulation, ion transport, and extracellular matrix production. Here I use RNA-seq to evaluate how diel changes in gene expression vary within the branching Caribbean staghorn coral, *Acropora cervicornis*, between the tips and bases of its branches. Multifactor GLM analysis indicates that 6% of transcripts (n=3005) are differentially expressed between branch tips and bases, while 1% of transcripts (n=441) are differentially expressed between day and night. The gene expression patterns of 220 transcripts were affected by both time of day and location within the colony, with 71 transcripts significantly different for both factors and 170 transcripts having a significant interaction effect between factors. Results indicate that photoreceptors, putative circadian
genes, stress response genes and metabolic genes are differentially expressed between
day and night and that some of these, including *Amcry1*, *tef* and *hebp2* have location-
specific regulation within the coral colony.
Introduction

The daily rotation of the earth subjects organisms to predictable oscillations of abiotic factors, such as light, temperature and tides, and biotic factors, such as predation and prey availability. To moderate the effects of these diel changes, organisms often alter their behavior and physiology by responding directly to environmental cues (e.g. light/dark) or through circadian regulation (Reitzel et al. 2013). Circadian rhythms have been observed in animals, plants, fungi and cyanobacteria, and are thought to confer an adaptive advantage by allowing organisms to pre-emptively regulate transcription in anticipation of environmental change (Yerushalmi & Green 2009). Circadian control of gene expression is primarily determined by the circadian oscillator (i.e. circadian clock), which is generally entrained by light and/or temperature, but can function in the absence of environmental cues through the oscillation of transcriptional/translational positive and negative feedback loops over an approximately 24-hour cycle (Roenneberg & Merrow 2005). Some circadian regulation can also occur in the absence of the transcription/translation feedback loops through peroxiredoxin redox cycles (O’Neill & Reddy 2011; O’Neill et al. 2011), but far less is known about this alternate pathway. A significant portion an organism’s transcriptome can exhibit daily rhythms; for example, more than 20% of gene transcripts show daily fluctuations in intertidal mussels (Connor & Gracey 2011) and up to a third of genes are under circadian control in plants (Covington et al. 2008).

Hermatypic (or reef-building) corals inhabit tropical reefs and live in symbiosis with symbiotic dinoflagellate algae (Symbiodinium, aka zooxanthellae). Corals obtain from 40% to 100% of their energy from photosynthetically-fixed carbon, primarily
produced during the day (Levy et al. 2004), that is transferred from the *Symbiodinium* to the coral host (Falkowski et al. 1984; Muscatine et al. 1984). In exchange the coral provides the algae with a sheltered habitat and access to nutrients (Yellowlees et al. 2008). Corals experience large diel changes in their physiology between day and night to deal with the shift from being autotrophic (i.e. photosynthetic) during the day to heterotrophic at night. Daytime stress includes high UV radiation and light exposure and the production of reactive oxygen species (ROS) by *Symbiodinium* during photosynthesis (Lesser 1996; 1997). At night corals switch to heterotrophic feeding to capture demersal zooplankton prey (Heidelberg et al. 2004), at which time the coral can experience intracellular hypoxic conditions and decreased pH due to respiration by both the coral and *Symbiodinium* (Kühl et al. 1995). Of these stresses, the most harmful is oxidative stress from ROS, which causes peroxidation of lipids and oxidation of proteins and nucleic acids and is considered to be largely responsible for the breakdown of the coral-algal symbiosis (i.e. coral bleaching) associated with high temperature and light conditions (Lesser 2006). To neutralize the toxic effects of ROS, both the coral host and its *Symbiodinium* produce a range of enzymatic and non-enzymatic antioxidants (Lesser 1996; Richier et al. 2005; Lesser 2006). Corals may also control *Symbiodinium* growth and activity by restricting the availability of nutrients, particularly nitrogen (Rees 1991), removing symbiont cells by expulsion (Baghdasarian & Muscatine 2000), stimulating photosynthate release via ‘host-release factors’ (HRFs) (Muscatine 1967; Trench 1971; Gates et al. 1995) and inhibiting photosynthesis (Grant et al. 2001). *Symbiodinium* photosynthesis and growth show strong diel variation (Wang et al. 2008), thus there is likely to be a diel component to the coral’s genetic response to shifts in algal activity.
As sessile organisms, coral behavioral changes are limited to polyp and tentacle extension and retraction, which often display diel patterns (Levy et al. 2006). Tentacle behaviors, which may help the animal avoid predation while maximizing photosynthesis, heterotrophic feeding and gas exchange (Sebens & DeRiemer 1977; Levy 2003; Levy et al. 2006), appear to be light-responsive, but may also have a circadian component of regulation (Tsang et al. 1997; Levy 2003). In some corals, such as the Caribbean Acropora corals, tentacle behavior varies between different parts of the colony, suggesting complex regulation beyond uniform light-response. Radial polyps found throughout most of the colony have a high concentration of Symbiodinium, and their tentacles are extended during the day to capture light and remain extended at night. In contrast, axial polyps at the tip of the branch contain few Symbiodinium, are retracted during the day and are only extended at night for feeding on active zooplankton.

Diel gene expression patterns have been observed in anthozoans, including corals, in response to both light and circadian regulation (Levy et al. 2007; Reitzel et al. 2010; Brady et al. 2011; Levy et al. 2011). Transcriptional regulation of gene expression and subsequent protein production at regular intervals based on time of day is probably necessary for the coral to cope with cyclic patterns of stress (UV and ROS), facilitate digestion of molecules obtained from Symbiodinium or heterotrophy, and manage algal densities. Corals and other anthozoans possess a number of insect and vertebrate circadian gene homologs (Vize 2009; Reitzel et al. 2010) as well as photoreceptors, such as cryptochromes (crys) and opsins, that may also interact with circadian genes (Levy et al. 2007; Shoguchi et al. 2013). Coral photoreceptors are suspected to be involved in a number of light-responsive functions, such as phototropic growth (Kaniewska et al.)
2009), polyp expansion or contraction (Levy 2003), and spawning (Gorbunov & Falkowski 2002; Levy et al. 2007; Brady et al. 2009). It is not yet understood what coral functions may be under circadian regulation (Sorek et al. 2014); however, in one study of *A. millepora*, heat shock proteins were expressed with circadian rhythmicity (Levy et al. 2011).

One coral function that is directly related to light availability and *Symbiodinium* photosynthetic activity is calcification of the coral skeleton. In many corals, calcium carbonate deposition increases in light, relative to dark, in a phenomenon referred to as ‘light-enhanced calcification’ (LEC) (Goreau 1959b; Goreau & Goreau 1959; Chalker & Taylor 1975). The Caribbean staghorn coral, *Acropora cervicornis*, has served as a good model for studying LEC and other growth processes (Goreau & Goreau 1959; Pearse & Muscatine 1971; Gladfelter 1982a; 1983b), because of its rapid growth rate. Its branching morphology makes it relatively easy to study linear growth, and there is division of labor (DOL) within the colony. *A. cervicornis* has dimorphic polyps: 1) radial polyps that contain a high concentration of *Symbiodinium* along the branch and are the site of gamete production, and 2) axial polyps at the branch tips that are the location of linear skeletal growth and that contain lower *Symbiodinium* concentrations. Although *A. cervicornis* branch tips contain fewer *Symbiodinium*, it is the region with the highest calcification rates, both in terms of linear extension and CaCO$_3$ accretion (Goreau & Goreau 1959). This is may be due to transfer of *Symbiodinium*-produced photosynthesize among polyps (Pearse & Muscatine 1971), which may be facilitated through a fluid circulating within a connected gastrovascular system (Gladfelter 1983a). Calcification in *A. cervicornis* exhibits a diel pattern in the quality of skeleton deposited at the axial corallite (Gladfelter
and predictable changes in the density of skeleton at different regions along the colony branch (Gladfelter 1982a). While the calcification rate is linked with *Symbiodinium* photosynthetic activity (Moya 2006), we still lack a complete picture of the intermediate proteins or other molecular factors involved. Transfer of fixed carbon may be used to fuel production of proteins suspected to be involved in calcification, such as carbonic anhydrase (CA), skeletal organic matrix (SOM) and ion transport proteins (Tambutté et al. 2011).

The DOL within Caribbean *Acropora* coral colonies is helpful for studying coral growth, but also represents substantial differences in function within these simple colonial organisms. For example, gamete production only occurs in mature radial polyps at least 2 cm from the branch tip (Szmant 1986), and the metabolic rate in branch tips is higher than the rest of the colony (Gladfelter et al. 1989). These corals do not show the kind of functional specialization of zooids observed in some other cnidarians (particularly hydrozoans) (Siebert et al. 2011), but differences in gene expression between branch bases and tips have been found to represent a substantial fraction, approximately 10%, of the transcriptome (chapter 2) and include gene expression differences in developmental signaling, metabolism and calcification (chapter 2). For corals, like *Acropora* species, where the density of *Symbiodinium* varies within the colony, there may also be polyp-specific diel gene expression responses. While studies of Pacific *Acropora* corals have indicated gene expression differences between day and night, or light and dark, with particular interest in circadian regulation or LEC (Levy et al. 2007; Brady et al. 2011; Levy et al. 2011), no study has yet compared full transcriptome differences between day and night in functionally distinct parts of the colony.
To identify diel gene expression differences within the coral colony, I compared transcriptome-wide gene expression patterns of branch tips and bases in the Caribbean staghorn coral, *A. cervicornis*, in its natural reef environment at mid-day versus mid-night using RNA sequencing (RNA-seq). I analyzed full transcriptome data with a multi-factor generalized linear model (GLM) to identify transcripts whose expression differs between day and night (midday vs. midnight) and within the coral colony (branch tips vs. bases), as well as whose expression depends on both factors (significant for both factors or having a significant interaction). I hypothesize that transcripts involved in LEC, phototropism or polyp behavior should show differences in expression between day and night in tips, while transcripts involved in the coral responding to symbiont photosynthetic activity and growth should exhibit differences between day and night in bases. Transcripts with a significant interaction between day and night in bases and tips may also be involved in these processes. Genes expressed differentially between day and night throughout the colony may be involved in stress response, circadian rhythms and other functions that may be shared among all polyps.

**Materials and Methods**

Sample collection & RNA extraction

*Acropora cervicornis* samples were collected from three large coral colonies located at least 10 meters apart at 5-6 meters depth in Crawl Cay, Bocas del Toro, Panama (09 15.517N 082 07.625W). Paired samples were taken from individual branches, with base samples taken at 25-30 cm from the tip of the branch, and the apical sample being the top 1 cm of the branch, including the axial polyp. For each colony, one branch
was sampled at 12:00-13:00 (~6 hrs post-sunrise) on August 2 (sunrise 6:09, sunset 18:39, moonrise 23:46, third quarter), and one branch was sampled at midnight-00:30 (~5.5 hrs past sunset) on August 3, 2010. At the time of the nighttime sampling, corals had been exposed to little, if any, moonlight; however, some exposure to dive lights was unavoidable while collecting. Temperature data was collected from a permanent data logger near Crawl Cay (Cayo Agua; data publicly available at http://biogeodb.stri.si.edu/physical_monitoring/research/bocas). Seawater temperature was 29.7-29.8°C during daytime sampling (12:00-13:00) and 29.6°C during nighttime sampling (24:00-01:00). Coral samples were transported to the boat in covered plastic containers, then wrapped in aluminum foil, immediately preserved in liquid nitrogen, and stored at -80°C.

Total RNA extraction was performed using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH) following the manufacturer’s protocol, with an additional 75% ethanol wash step. For some samples, RNA was extracted up to three times and the best quality extraction was used. To enhance the concentration of RNA extracted, some samples were extracted using 1 ul glycogen during the extraction process, prior to the isopropanol precipitation step. Total RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and RNA Pico Chips, and only extractions with distinct 18S and 28S ribosomal RNA peaks were used (RIN values 4.9-8.0).

Illumina RNA-seq library preparation
mRNA was isolated using Dynabeads® oligo(dT) mRNA isolation beads (Life Technologies, Grand Island, NY) to exclude non-protein coding RNAs and non-eukaryotic mRNAs from the samples. cDNA was produced using random hexamer primers and SuperScript® II reverse transcriptase (Life Technologies) for first strand synthesis and DNA polymerase I (New England BioLabs, Ipswich, MA) for second strand synthesis. RNA-seq libraries were prepared by fragmenting the double stranded cDNA with dsDNA fragmentase (NEB) followed by library preparation using NEBNext® reagents. The cDNA transcripts with barcoded adapters were then size selected at 250 bp by gel extraction and 15 rounds of PCR amplification. RNA-seq libraries were sequenced (6 samples per lane) using single-end 50 bp sequencing on the Illumina Hi Seq 2000 platform (Illumina, Inc., San Diego, CA, USA) at the FAS Center for System Biology at Harvard University.

Bioinformatics

Read quality control and barcode removal were conducted with custom Perl scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). RNA-seq reads were mapped against our published de novo transcriptome for A. cervicornis and A. palmata using CLC Genomics Workbench (CLC bio). Reference transcriptome annotation was conducted using translated nucleotide queries against the curated UniProt/Swiss-Prot database. Reference transcriptome sequences and RNA-seq reads are available on BioProject (accession number PRJNA222758). Coral genes were identified by aligning the de novo reference transcriptome contigs to the genomes of congeners A. digitifera (Shinzato et al. 2011) and A. millepora (Miller, unpublished). Transcripts with
a significant BLAST hit (E-value < 10^{-10}) were identified as coral and retained for the analyses. Putative non-coral transcripts were removed.

Statistical analysis of differential expression

Using the DESeq package v. 1.14.0 (Anders & Huber 2010) in R v. 3.0.2 (R Development Core Team, 2012), the RNA-seq read count data were normalized by sample library size. Non-metric multidimensional scaling (nMDS; isoMDS) and PERMANOVA (Adonis) were conducted on the normalized data using the MASS v. 7.3.29 and vegan v. 2.0.10 packages (Venables & Ripley 2002; Oksanen et al.) in R to examine transcriptome-wide differences in gene expression. Differentially expressed (DE) gene transcripts were then identified using a three-factor negative binomial Wald test (design = ~ genet + position * time) in DESeq2 v 1.2.10 (Anders & Huber 2010; Love et al. 2014a) with colony position (base vs. tip), time (day vs. night) and genet (genetically distinct colony) as the main factors. I also tested for an interaction between the two main factors, position and time. Genet was included as a factor only to control for inter-colony variation. A significant interaction indicates that the level of expression for one factor is dependent on the level of the other factor. An adjusted p-value < 0.05 was used to evaluate significance (Benjamini & Hochberg 1995). For the colony position factor, I assigned gene expression values in bases as the baseline for comparison, so DE transcripts are considered ‘up-regulated’ or ‘down-regulated’ in tips, relative to bases. For the time of day factor, I assigned gene expression values in night samples as the baseline, so DE transcripts are considered ‘up-regulated’ or ‘down-regulated’ during the day. Log2-fold change estimates presented in table 2 represent an a posteriori calculation
of fold change calculated in DESeq2 based on data-driven prior distributions (Love et al. 2014b).

Functional genetic pathway analysis

Using UniProt accession IDs, contigs were annotated with Gene Ontology (GO) terms (www.geneontology.org). Transcripts of interest were determined based on GO terms and a review of the literature for transcripts that were DE between day and night, DE for both factors or that had a significant interaction between the two primary factors. Heat maps of gene expression data were created using log-transformed normalized count data in GENE-E (Gould). For the cryptochromes, an additional annotation step was performed using nucleotide BLAST (blastn) through NCBIs reference databases to more precisely discuss their known roles as putative circadian and light-response genes in cnidarians.

Results

RNA-seq libraries ranged in size from 8.4 to 22.5 million reads (16.6 million ± 1.2 million, mean ± SE) (Table 1), with 4.1 to 15.8 million reads (9.2 million ± 0.9 million) mapping to coral transcripts. PERMANOVA and nMDS analyses revealed significant differences in transcriptome-wide gene expression patterns between branch bases and tips (PERMANOVA, d.f. = 1, Pseudo-F = 5.9631, P = 0.001), but not between day and night (d.f. = 1, Pseudo-F = 1.9308, P = 0.072) or among genets (d.f. = 1, Pseudo-F = 1.9992, P = 0.08). The interaction between colony position and time of day was also not significant (d.f. = 1, Pseudo-F = 1.1943, P = 0.261). The nMDS plot (Figure 1)
demonstrates the difference in gene expression profiles between tips and bases, which separate primarily along axis 1.

Of the 47,688 coral transcripts in the analysis, 3005 transcripts were significantly differentially expressed (DE) between tips and bases, while only 441 transcripts were DE between day and night (Figure 2A). Similar numbers of transcripts were up-regulated (n=1396) and down-regulated (n=1609) in tips and up-regulated (n=204) versus down-regulated (n=237) during the day. Seventy-one transcripts were DE for both factors (colony position and time of day), and 171 transcripts had a significant interaction. In order to characterize the genetic response of the coral, I focused on the 1902 DE coral transcripts with strong protein annotations to known genes (UniProt/Swiss-Prot BLASTx e-value < 10^{-5}). This includes 1686 transcripts that differed by colony position and 234 transcripts that differed by time of day (Additional file 1, Figure 2B). Of these, 47 transcripts were DE for both factors. Ninety-one transcripts had a significant interaction. I focused on DE transcripts falling into functional categories of interest, including circadian regulation and photoreception, stress response and immunity, nutrient, lipid and carbohydrate metabolism, calcification, and transport (Figure 3).

DE by colony position

Out of the 3005 transcripts that were significantly DE between tips and bases, 940 or 31% also differed significantly in my previous analyses of within-colony gene expression patterns between branch bases and tips in both A. cervicornis and its congener, the elkhorn coral A. palmata (chapter 2). Another 1273 (42%) of these transcripts had been excluded from the previous analysis due to filtering based on low abundance or high
variance prior to DE analysis. The observed within-colony differences in this study include many of the same annotated genes and patterns that were previously found and discussed at length in chapter 2. These include 1) the up-regulation of putative Wnt pathway genes \textit{dkk3}, \textit{wnt proteins (5a, 5b, 8a, 8b)}, \textit{fzd6}, \textit{lrps (1, 1b, 6)} and \textit{cthrc1} in tips, 2) down-regulation of BMP pathway genes \textit{(bmp3b, toloid, kielin/chordin)} in tips, 3) up-regulation of cytoskeletal transcripts (actin, tubulin, and dynein) in bases and 4) the comparable patterns of up- and down-regulation of possible calcification-related genes: carbonic anhydrases (2, 3, 7), ECM proteins (e.g. \textit{galaxin-2}, \textit{coll1a1}, \textit{ISMP-1}, \textit{uromodulin}) and bicarbonate transport proteins (\textit{SLC4A4, SLC4A10}).

In addition to these previously discussed genes, transcripts that were DE by colony position but not by time of day included proteins involved in circadian or photoreceptor development, lipid and carbohydrate metabolism, and UV and oxidative stress response (Table 2). Some photoreception and putative circadian genes show within-colony differences, without being DE between day and night. For example, two genes involved in entrainment of the circadian clock in vertebrates, a melatonin receptor and a melanopsin-type opsin, were up-regulated in tips, whereas three other genes that may be involved photoreceptor development, \textit{retinol dehydrogenase 8}, \textit{crumbs} and \textit{sine oculis}, were down-regulated in tips. Two patterns of expression were observed for lipid metabolism genes. Lipid biosynthesis, including seven transcripts of \textit{fatty acid synthase}, was down-regulated in tips; however, the arachidonic acid/allene oxide pathway was elevated in tips, indicated by up-regulation of \textit{arachidonate 5-lipoxygenase} and seven transcripts of \textit{allene oxide synthase}. Elevated mitochondrial metabolism in tips was indicated by the up-regulation of four transcripts of ATP synthase. Stress response genes
that were DE by colony position included chaperone protein genes (hsp5, DnaJ, and sacsin), redox and oxidative stress response genes (thioredoxin, superoxide dismutase, and glutathione pathway genes), and known and possible UV stress response genes (DNA photolyase and GFP-like chromoproteins).

Day versus night

Of the 176 annotated transcripts DE only by time of day, 86 were up-regulated and 90 were down-regulated during the day. Up-regulated genes include two with putative roles in circadian clock regulation, cryptochrome 2 (Amcry2 and a mammalian cry2) and neuronal PAS domain-containing protein 2 (NPAS2), as well as two genes I hypothesize may be involved in circadian regulation, a retinoic acid nuclear receptor (nr2c1), and mapk6. Cryptochromes (Crys) are blue light photoreceptors that also regulate animal circadian clocks. NPAS2 is a bHLH–PAS transcription factor that can act as one of the primary circadian regulatory transcription factors in mammals. The nuclear receptor NR2C1 is a retinoic acid responsive nuclear receptor whose transcription may be regulated by the circadian-related orphan nuclear receptor Rev-erba/Nr1d1. Mitogen Activated Protein Kinase (MAPK) proteins, including MAPK6, may be involved in circadian regulation through phosphorylation of Cry or other proteins. Other genes up-regulated during the day include three carbohydrate transport genes, sodium/glucose transporters 2 & 5 (slc5a2, slc5a10) and sodium/myo-inositol cotransporter 2 (slc5a11), a protein phosphatase involved in glycogen metabolism (ppp1r3b), two iron homeostasis genes, soma ferritin and mitoferrin-2, and two glutamine metabolism genes, carbamoyl-phosphate synthase I (cpsI) and putative glutamine amidotransferase YLR126C (GATase).
Genes down-regulated during the day include a rhodopsin-like opsin photoreceptor gene and two putative circadian genes, *clock* and *D site-binding protein* (*dbp*). Rhodopsins are low-light photoreceptors made up of a transmembrane opsin protein bound to retinal, a photoreactive chromophore. Clock is a paralog of NPAS2 and is one of the primary transcription factors involved in the circadian oscillator, whereas DBP is a PAR bZip transcriptional factor that modulates circadian output genes. In addition, the nitrogen metabolism gene *glutamate synthase* (*gogat*), and a potential SOM protein, *polycystic kidney disease protein 1-like 1* (*pkd1l1*) were down-regulated.

Day *versus* night, and base *versus* tip

Forty-two annotated transcripts were DE for both factors only. Of these, 26 were up-regulated in tips and during the day, nine were up-regulated in tips and down-regulated during the day, five were down-regulated in tips and up-regulated during the day and two were down-regulated in tips and during the day. DE transcripts that were up-regulated in tips during the day include genes involved in the mitochondrial electron transport chain (ETC), lipid metabolism, light response, UV and oxidative stress response and calcification. ETC components include *electron transfer flavoprotein subunit alpha*, *cytochrome c1 heme protein*, *NADH* and *succinate dehydrogenases*. The lipid metabolism gene *delta(5) fatty acid desaturase* is involved in biosynthesis of unsaturated fatty acids. *Retinol dehydrogenase 7* (*rdh7*), which may be involved in photoreceptor development, and the light-induced DNA repair protein *deoxyribodipyrimidine photolyase* (*phr*), were also up-regulated in tips during the day. Two transcripts that may be involved in calcification, the enzyme *carbonic anhydrase 2* (*CA2*), and a putative SOM
protein, *mucin-4*, were up-regulated in tips during the day. Two redox-related proteins, *thioredoxin* and the glutathione metabolism gene *glutathione-S transferase 1 (GST)*, and a retinoic acid nuclear receptor (*nr2f5*) were also up-regulated in tips during the day. One transcript of an immune-related gene, *hemagglutinin/amebocyte aggregation factor (HAAF)*, was down-regulated in tips and up-regulated during the day, and one transcript of the lipid biosynthesis gene *fatty acid synthase* was down-regulated in tips and during the day.

**Interactions**

Forty-six annotated transcripts were DE for colony position and had a significant interaction effect. Six of these were up-regulated and forty were down-regulated in tips. The antioxidant enzyme *extracellular superoxide dismutase (sod)* was overall down-regulated in tips, but while its expression was elevated during the day in bases, it was elevated at night in tips. Three genes were highly down-regulated in tips, and had higher expression at night only in bases. These include three transcripts of *fatty acid synthase*, the egg yolk protein gene *vitellogenin-6*, the lipid metabolism gene *pancreatic lipase-related protein 2*, and the signaling gene *casein kinase I*. One nitrogen metabolism gene, *putative L-amino-acid oxidase YobN*, was down-regulated in tips with the highest expression during the day. Three other genes were down-regulated in tips but lacked a clear pattern of expression between day and night. These were a possible SOM protein, *hemicentin-1*, and two collagen-related genes, *collagen triple helix repeat-containing protein 1 (cthrc1)* and *collagen alpha-1(II) chain (col2a1)*.
Eleven DE transcripts differed by time of day and had a significant interaction effect. Eight of these were up-regulated and three were down-regulated during the day. All transcripts of interest were up-regulated during the day had a greater fold change in tips than bases. These transcripts include two putative circadian or light-responsive genes, two transcripts of the *A. millepora* cryptochrome homolog *Amcry1* and *thyrotroph embryonic factor* (*tef*). TEF is a non-essential circadian PAR bZip transcription factor, similar to DBP, that activates circadian target genes. In addition, an iron homeostasis protein that may function in oxidative stress response, *heme-binding protein 2* (*hebp2*), and a lipid metabolism enzyme, *Acyl-CoA desaturase*, were up-regulated during the day with an interaction effect.

Five annotated transcripts were DE for both factors and had a significant interaction effect. These were *fatty acid synthase*, *GFP-like non-fluorescent chromoprotein*, *cytosolic phospholipase A2*, *VWFA and cache domain-containing protein 1* (*cachd1*) and an uncharacterized protein. The lipid biosynthesis gene *fatty acid synthase* was down-regulated in tips and during the day and had almost no expression in tips at either time point. *Phospholipase A2*, a lipid metabolism gene that may be involved in the arachidonic acid pathway, repairing oxidatively damaged lipids or as a toxin in the nematocyst complex was also down-regulated during the day, but showed much higher expression in tips than bases. The GFP-like chromoprotein was up-regulated during the day, and also showed higher up-regulation in tips than bases. And *cachd1*, which may be involved in regulating voltage-dependent calcium channels, was expressed at a much higher level in tips overall, but showed up-regulation during the day only in bases.
Twenty-nine annotated transcripts were significant for the interaction between factors without being DE for either factor. Three transcripts of interest include the nitrogen metabolism gene glutamine synthetase (gs) and a carnitine transport protein (slc25a20), which were most highly expressed during the day in bases, but at night in tips, and retinol dehydrogenase 3 (rdh3), which was most highly expressed in tips at night. Glutamine synthetase is an enzyme in the glutamate metabolic pathway, which catalyzes the condensation of glutamate and ammonia to form glutamine. Carnitine is a quaternary ammonium compound that is necessary for lipid metabolism in eukaryotic cells to facilitate the transport of fatty acids into the mitochondria.

**Discussion**

Greater differential expression was observed between the tips and bases of the *A. cervicornis* colony (3005 transcripts) than between the day and night (441 transcripts). This indicates strong within-colony division of labor (DOL) between branch tips and bases in addition to moderate diel variation. The 231 transcripts that differed by colony position and time of day (or had an interaction) demonstrate that gene expression patterns underlying the within-colony DOL include a diel component. While corals have previously been shown to exhibit clear diel patterns of calcification (Barnes & Crossland 1980; Gladfelter 1983b; Moya 2006; Schneider *et al.* 2009) and tentacle behavior (Levy 2003; Levy *et al.* 2006), as well as light-responsive and circadian patterns of gene expression (Levy *et al.* 2007; Brady *et al.* 2011; Levy *et al.* 2011; Sorek *et al.* 2014), these results suggest that diel shifts in gene expression occur at only 1% of *A. cervicornis*’s transcriptome. The diel effects on transcriptome-wide gene expression
patterns in staghorn corals are relatively modest compared to those observed in other marine organisms, like mussels, where over 20% of the transcriptome shows diel oscillations (Connor & Gracey 2011). In A. cervicornis, genes that differed significantly between day and night were involved in a variety of processes including regulating circadian rhythm and photoreception, mediating stress responses and immunity, as well as nitrogen, lipid, and carbohydrate metabolism and carbohydrate transport.

Circadian rhythms & Photoreception

Eight transcripts representing regulatory and response components of the animal circadian clock (*clock*, *npas2*, cryptochromes, *dbp*, *tef*) were DE by time of day (Table 2; Figure 4). Two of these transcripts, *Amcry1* and *thyrotroph embryonic factor* (*tef*), had a significant interaction effect in which each exhibited a much higher (doubled) fold change up-regulation in day in tips compared to bases. In animals, the core circadian oscillation of genes occurs through positive and negative transcriptional feedback loops. In mammals, positive regulation by a heterodimer comprised of two bHLH-PAS transcription factors, Clock and BMAL1, activates transcription of E-box regulated genes, including Cry and Period (Gekakis 1998). Together Cry and Period repress their own transcriptional activation by binding to the Clock:BMAL1 complex, making up the negative component of the oscillator (Duong et al. 2011). A related but distinct circadian oscillation is found in insects (e.g. *Drosophila*), Clock and Cycle (a BMAL1 ortholog) activate transcription of Period and Timeless, which repress the activity of Clock (Bae et al. 1998; Stanewsky 2002), while Crys act as photoreceptors that entrain the oscillator to light (Stanewsky et al. 1998).
Down-regulation of *clock* during the day in corals was unexpected, as this gene has previously been shown to be up-regulated during the day in the coral *A. millepora* (Brady *et al.* 2011). Interestingly, *npas2*, a *clock* paralog that acts as a distinct circadian regulator in some mammalian tissues (Reick 2001), and which has not previously been studied in cnidarians, was up-regulated during the day, indicating that Clock and NPAS2 may function independently of one another in cnidarians. The other circadian gene down-regulated during the day, *dbp*, is a circadian output PAR bZip transcription factor that may enhance production of enzymes involved in detoxification and metabolism important for processing food, as it appears to do in mammals (Gachon *et al.* 2006). Corals would have a greater requirement for such enzymes at night when a greater abundance of demersal planktonic prey are available for consumption (Heidelberg *et al.* 2004).

An important distinction between the two well-described animal circadian pathways is the different functionality of Crys. These blue light sensitive photolyase-like proteins act as photoreceptors to entrain the circadian clock in insects, but not in mammals, where they act as a light-independent part of the circadian oscillator feedback loop (Lin & Todo 2005). Cnidarian *crys* are more closely related to their mammalian counterparts, yet cnidarian *cry* gene expression appears to be light-induced (Levy *et al.* 2007; Reitzel *et al.* 2010) with only *Amcry2* showing some circadian periodicity (Brady *et al.* 2011). Correlation of *Amcry2* with lunar light exposure may be involved in regulation of coral spawning behavior (Levy *et al.* 2007). In this dataset, *Amcry2* and a transcript annotating to a mammalian-type *cry2*, were up-regulated during the day, whereas two *Amcry1* transcripts were up-regulated during the day and had a significant interaction effect due to greater fold change in tips, in a similar pattern to that expressed
for the circadian output transcription factor tef. This within-colony variation observed in the expression of Amcry1 and tef genes might reflect an effect of Symbiodinium on coral circadian gene expression, possibly through photosynthetic activity which strongly alters the oxygen content of the polyp tissue (Kühl et al. 1995). In a previous study of symbiont-containing adult versus aposymbiotic A. millepora larvae, a higher fold change of Amcry1 between light and dark was found for larva than adults (Brady et al. 2011). It is possible that Amcry1 expression change may be dampened in cells containing or located near a high concentration of Symbiodinium through the effect of redox state on the circadian clock (Stangherlin & Reddy 2013). For example, in light-responsive cells of zebrafish, H$_2$O$_2$ is produced by light and induces expression of circadian genes, including crys, and may act as a messenger between photoreceptors and the circadian clock (Hirayama et al. 2007). In A. cervicornis during the day, polyps located in the branch bases having a high density of Symbiodinium are likely to experience higher levels of ROS, including H$_2$O$_2$, compared to those in branch tips with lower density of algal cells. H$_2$O$_2$ is able to diffuse through cell membranes (Lesser 2006), and could therefore move freely between the endoderm where Symbiodinium are located and ectoderm where cryptochrome proteins have been found (Levy et al. 2007). A link between tef expression and ROS may be the potential circadian regulation of DNA repair and ROS stress response proteins by tef, which has been observed in zebrafish (Gavriouchkina et al. 2010). The possible connection between ROS stress and regulation of circadian genes in corals warrants further investigation.

An alternate hypothesis for a greater magnitude change in expression of Amcry1 in tips is a potential role outside of circadian regulation, specifically in the blue-light-
activated phototropic growth observed in branching *Acropora*. In *Acropora*, the production and direction of growth of new axial corallites respond to blue light (Kaniewska *et al.* 2009), but the mechanism for this is not known. Crys, which are known to modulate phototropic growth in plants (Goyal *et al.* 2013) and may play a phototaxic role in sponges (Rivera *et al.* 2012), should also be considered as a potential candidate for phototropism in corals.

Although the transcriptional/translational feedback loops involving Clock:BMAL or Cycle and Cry:Period or Timeless are the core of the animal circadian clock, additional mechanisms of regulation have been described and are important in supporting the core clock function. These include a feedback loop regulated by nuclear receptors REV-Erbα (NR1D1), REV-Erbβ (NR1D2) and the retinoid nuclear receptor RORα (NR1F1) that regulate transcription of BMAL1 and other genes with ROR response elements (Preitner *et al.* 2002; Cho *et al.* 2012), as well as post-translational regulation through degradation of Cry and Period proteins involving kinases (Lee *et al.* 2000) and F-box/LRR proteins (Skaar *et al.* 2013). Both retinoic acid signaling and MAPK activity appear to have a diel component in *A. cervicornis* as two nuclear hormone receptors involved in retinoic acid signaling and eye development, *nr2f5* and *nr2c1* (a gene regulated by REV-Erbα) (Mollem *et al.* 2011), and *mapk6* were up-regulated during the day. MAPK6, which has been found to phosphorylate Cry1 (Wang *et al.* 2009), may have a role in determining the activity or degradation of Crys as part of the circadian cycle.

Genes involved in production of components of rhodopsin-like photoreceptors were found to be DE both by time of day and colony position. Rhodopsin, comprised of a
transmembrane opsin apoprotein with photosensitive retinal chromophore, is a low-light photoreceptor that allows nighttime vision in animals. The production of retinal from retinol (vitamin A) is catalyzed by the enzyme Retinol dehydrogenase (Rdh). One rhodopsin-like opsin was down-regulated during the day and three transcripts of rdh were observed with differing patterns of expression. rdh3 was most highly expressed in tips at night, rdh7 was most highly expressed in tips during the day, and rdh8 was down-regulated in tips. In addition to its role in producing retinal, Rdh can also produce retinaldehyde, which can be further oxidized to retinoic acid, an important signaling molecule used in embryonic patterning as well as circadian gene regulation (Shirai et al. 2006). The multiple gene expression patterns found for rdh genes suggest that Rdhs are involved in multiple functional pathways. The up-regulation of the rhodopsin-like opsin and rdh3 at night indicates that a rhodopsin-like photoreceptor may be involved with spawning coordination (Sweeney et al. 2011); however because moonlight was not a factor at the time of sampling, the night-time elevation of rhodopsin-like opsin and rdh3 cannot be attributed to a direct response to moonlight. Up-regulation of rdh7 in tips during the day suggests it may be involved in phototropic growth.

Some transcripts of putative circadian or light-response related genes were DE only by colony position. These DE genes include two putative circadian-related genes, a melanopsin-like opsin and a melatonin receptor, which were up-regulated in tips, and two genes that are involved in eye development in Drosophila, crumbs and sine oculis, which were down-regulated in tips. Melanopsin is a blue-light sensitive photoreceptor comprised of an opsin and retinal that facilitates photoentrainment of the circadian clock in mammals (Hankins et al. 2008). In mammals, light reception by melanopsin is one of
the mechanisms for suppressing the synthesis of melatonin (Panda 2003). Evidence from the anemone *Nematostella* suggests the hormone melatonin can regulate the circadian clock in cnidarians; however, tissue specific expression indicates that it may also function in gametogenesis, larval development, and nervous system development (Roopin & Levy 2012; Peres *et al.* 2014). There is no clear explanation for the patterns observed within the coral colony for these groups of putative circadian and photoreceptor related genes; however, genes may confer a different degree of sensitivity to light and other stimuli to distinct regions within the coral colony.

### Stress response & immunity

Predictably, UV and ROS stress response genes were mostly up-regulated during the day, but interestingly the degree of up-regulation often varied within the colony. The light-induced DNA-repair enzyme gene *deoxyribodipyrimidine photo-lyase (phr)* was up-regulated both during the day and also in branch tips. Photoreactivation, the light-induced repair of UV-damaged DNA through correction of pyrimidine dimers, is the primary DNA-repair mechanism in coral larvae (Reef *et al.* 2009), and it appears to be important for mature colonies as well, particularly in the growing tips. Growing tips may be more vulnerable to UV-induced DNA damage due to the production of new polyps through asexual reproduction, the high rate of mitosis, and the lower concentration of *Symbiodinium*, which absorb and block UV light through shading and mycosporine-like amino acids (Dunlap & Shick 1998). Additionally, while three transcripts of GFP-like fluorescent chromoproteins (FP486 and FP506) were up-regulated in tips only, one *GFP-like non-fluorescent chromoprotein (gtCP)*, was up-regulated both in tips and during the
day. Elevated expression of GFP-like proteins in growing regions of corals, has
previously been observed in Acropora and other species (Bay et al. 2009a; D’Angelo et al. 2012), and up-regulation during the day is consistent with the observed increase in transcription and production of GFP-like proteins in response to blue light (D’Angelo et al. 2008). My results indicate that different chromoproteins may be functioning differently within the colony. Numerous roles have been suggested for GFP-like proteins, including photoprotection, stress/redox response, symbiont interactions and a potential role in growth (D’Angelo et al. 2008). Higher expression of both known and putative UV stress genes in branch tips suggests that this region of the colony must cope with elevated UV stress compared to branch bases.

Excessive oxidative stress resulting from ROS produced by Symbiodinium and coral under high light and temperature conditions is believed to be the primary cause of mass coral bleaching episodes (Weis 2008). Multiple oxidative stress response genes have been proposed to be important in maintaining the symbiotic partnership (Downs et al. 2002; DeSalvo et al. 2008; Sunagawa et al. 2009b). Three of these genes showed both diel and within-colony differential expression. These include thioredoxin and glutathione S-transferase (gst), and extracellular superoxide dismutase (sod). Thioredoxin is a small redox protein found in all organisms that can participate in redox signaling or antioxidant activity by reducing other proteins by cysteine thiol-disulfide exchange. GST is an enzyme that inactivates the oxidizing activity of substrates by conjugation with the antioxidant glutathione. Both thioredoxin and gst were up-regulated during the day, particularly in branch tips. While higher ROS during the day is expected to increase oxidative pressure on the host and trigger elevated expression of these proteins, it is not
clear why these redox proteins were also up-regulated in tips, which due to a lower concentration of photosymbionts are expected to have lower ROS stress. Interestingly, although counterintuitive based on the expectation of ROS generation by algal symbionts (Lesser 1997; 2006), an inverse relationship between Symbiodinium and ROS response genes, including gst and sod, has been observed in multiple studies comparing symbiotic and non-symbiotic cnidarians (Rodriguez-Lanetty et al. 2006; Ganot et al. 2011; Lehnert et al. 2014). This may be due to alleviation of ROS stress by production of antioxidants by the symbiont itself (Rodriguez-Lanetty et al. 2006). One transcript of sod had a significant interaction effect with the lowest expression during the day in tips and highest at night in tips. SOD is an antioxidant enzyme that catalyzes the dismutation of the free radical superoxide (·O₂) into hydrogen peroxide (H₂O₂). The pattern of expression observed for sod suggests that it has a function other than (or in addition to) coping with Symbiodinium-produced ROS, possibly a role related ROS produced during metabolism of heterotrophically obtained food. As mentioned earlier, transcription of stress response genes may also be directly or indirectly linked to circadian rhythm. For example, in mammals gst transcription may be stimulated by tef and/or dbp transcription factors (Gachon et al. 2006).

Three iron homeostasis genes with putative roles in oxidative stress response, heme-binding protein 2 (hebp2), soma ferritin (sot), and mitoferrin-2, were up-regulated during the day, indicating differential use of iron between day and night. The reaction of ferrous iron (Fe²⁺) with H₂O₂ produces the highly reactive hydroxyl radical (·OH); therefore, proteins that chelate iron or bind heme can act as antioxidants (Yu 1994). In
this dataset, *hebp2* had a similar expression pattern with *Amcry1* and *tef*, being up-regulated during the day with a 2-fold greater change in tips than bases. *hebp2* was previously observed to follow a synexpression pattern with *α-carbonic anhydrase*, *cry1* and *cry2* in *A. millepora*, indicating a potential link between redox state, *hebp2* and circadian expression (Levy et al. 2011). *sof*, which codes an iron storage protein, is considered a putative stress indicator in corals due to its potential to minimize iron availability for ROS production (Bay et al. 2013). Up-regulation during the day of mitoferrin, which transports iron into the mitochondria, is more likely to be due to increased demand by ETC proteins requiring iron or heme as a co-factor, such as cytochromes and NADH and succinate dehydrogenases, which were also up-regulated during the day.

Regulation of *Symbiodinium* by the coral host

The breakdown of the coral-algal symbiosis during coral bleaching under thermal and UV stress is considered one of the most serious threats to the future of coral reefs (Hoegh-Guldberg 1999). For this reason, determining the cellular and molecular basis for maintenance of successful symbiosis has become the objective of numerous studies (reviewed in Davy et al. 2012). The healthy symbiosis depends on the coral regulating the population biomass of algae through three proposed mechanisms: expulsion or degradation of excess symbionts or inhibition of symbiont growth and division (Davy et al. 2012). I identified two candidate genes that may be involved in such regulation: a nitrogen metabolism gene, *glutamine synthetase* (*gs*), and an immunity-related gene, *hemagglutinin/amebocyte aggregation factor* (*haaf*). In addition to *gs*, other
glutamine/glutamate pathway genes (Figure 5) had diel and within-colony differences, indicating that the coral may use transcriptional regulation of this nitrogen metabolic pathway to regulate its symbiont population.

Nitrogen metabolism may play an important role in regulating *Symbiodinium* population growth by restricting the available nitrogen (Rees 1986). Glutamine synthetase (GS), which is required for the assimilation of nitrogen from ammonium into glutamine (Figure 5), has been suggested as the mechanism of symbiont control (Rees 1986). The pattern of *gs* expression, which was up-regulated during the day in branch bases and down-regulated during the day in branch tips, with a significant interaction effect, supports the hypothesis of nitrogen control in symbiont-rich base polyps during the day when *Symbiodinium* growth would be at its height (Wang *et al.* 2008). Interestingly, the highest expression of *gs* was in symbiont-poor tips at night, which may be related to processing nitrogen waste derived from heterotrophy. *Glutamate synthase [NADH] (gogat)*, which breaks down glutamine to glutamate, was elevated at night, particularly in branch bases. GOGAT produces two molecules of glutamate from glutamine and 2-oxoglutarate, without producing ammonia. Glutamate is an excitatory amino acid that may be used in signaling and to synthesize other amino acids. Genes for carbamoyl-phosphate synthase I (CPS1), which catalyzes the synthesis of carbamoyl phosphate from glutamine-derived ammonia and bicarbonate, and a putative glutamine amidotransferase (GATase), which transfers the ammonia group from glutamine to other substrates, were up-regulated during the day and had the lowest expression in bases at night. These genes suggest that nitrogen cycling is elevated in bases during the day, where there is a higher concentration of symbionts, and that nitrogen availability for
*Symbiodinium* may be controlled by production of glutamine followed by transfer of the ammonia group to carbamoyl phosphate and ultimately to urea or to other molecules.

The daytime up-regulation of the immune response gene *haaf* in bases, suggests enhanced amebocyte activity in branch bases to promote phagocytic clearance of dysfunctional or damaged *Symbiodinium*. Mobile amebocytes are part of the coral’s innate immune response and remove infecting pathogens or necrotic tissue via phagocytosis (Libro *et al*. 2013), but *haaf* and amebocytes have also been found to respond to elevated heat stress in soft corals (octocorallia) (Woo *et al*. 2006; Mydlarz *et al*. 2008). It has also been suggested that amebocytes may be activated under heat stressed conditions to dispose of damaged *Symbiodinium* cells (Strychar *et al*. 2004). Phagocytosis is one potential mechanism of coral bleaching, but may also be a way for corals to manage symbiont populations when subjected to stressful (e.g. high light and ROS) conditions, even when bleaching is not the ultimate outcome (Stat *et al*. 2006). Because no sign of disease, bleaching or wounding was observed on these corals prior to collection and there was no difference in the way the corals were collected, the location and timing of elevated expression of an amebocyte-related gene suggests a role for these cells in regulating *Symbiodinium* populations under high light conditions.

Light-enhanced calcification

In *Acropora* quantity and quality of CaCO₃ deposited into the skeleton differs between day and night. At night the linear branch extension rate is higher and CaCO₃ crystals accumulate in random orientation atop axial spines (Barnes & Crossland 1977; Gladfelter 1983b; Gladfelter *et al*. 1989). During the day, more CaCO₃ is incorporated
into the skeleton as ‘reinforcement’ of the spines and lateral bars occurs (Barnes & Crossland 1977; Gladfelter 1983b). Functionally, this suggests that nighttime conditions promote abiotic precipitation of CaCO₃, while in daytime, coral-regulated activity requiring energetic inputs encourages directed skeleton formation. Because the accumulation of CaCO₃ appears to be randomly precipitated at night, if SOM is involved in skeletal construction, it would likely be deposited during the day. I have identified two genes with possible roles in LEC, *carbonic anhydrase 2* (*CA2*) and *mucin-4*, and multiple genes that may provide the energy for LEC through metabolic functions or transport. Additionally, genes indirectly related to LEC include mitochondrial respiration genes (elevated in tips during the day) and carbohydrate transport genes (elevated in bases and tips during the day) involved in providing energy or molecules used in growth and LEC.

*Carbonic anhydrase*, including one transcript of CA2, was previously identified as highly up-regulated in the growing tips of Acropora corals (chapter 2) and is known to be involved in coral skeleton growth (Goreau 1959b; Tambutté et al. 2006). However, expression of CA genes tends to vary among studies. α-CA is suspected to be involved in LEC due to increased expression during the day in colonies of *A. millepora* (Levy et al. 2011), yet a previous study in *Stylophora pistillata* found up-regulation of a CA (STPCA) at night suggesting that this enzyme functions to drive the conversion of carbon dioxide to bicarbonate for calcification under lower pH conditions (Moya et al. 2008a). CA, which catalyzes the interconversion of carbon dioxide and water to bicarbonate and protons, provides a source of bicarbonate for CaCO₃ production but may also be involved in providing inorganic carbon for symbiont photosynthesis (Yellowlees et al. 2008). In these results, two transcripts of CA2, an α-CA, were up-regulated in tips and also up-
regulated during the day, but another transcript of CA2 was up-regulated in bases but did not show differential expression between day and night. Within the transcriptome multiple additional CAs (1, 3, 4, 5b & 7) are present and some of these (1, 3 & 7) were also DE within the coral colony, indicating that different CAs serve different functions.

A potential SOM protein, *mucin-4*, that was up-regulated during the day in tips is similar to a protein extracted from *A. millepora* SOM (Ramos-Silva *et al.* 2013), and mucins are known matrix proteins in mollusc shell formation (Marin *et al.* 2000). Other potential SOM proteins DE in this dataset, including *PKD1L1* and *hemicentin-1*, were down-regulated in tips, and are therefore not likely to be involved in LEC.

Carbohydrate and lipid metabolism and transport

Carbon metabolic processes, particularly mitochondrial respiration, were largely up-regulated during the day, consistent with a previous study in *A. millepora* (Brady *et al.* 2011) and corresponding to the increased production of photosynthetically-fixed carbon by *Symbiodinium*. Elevated respiration in tips indicates a high amount of energy consumption involved with growth during the day, which may permit the increase in mitotic activity at night previously observed in *A. cervicornis* tips (Gladfelter 1983c).

The elevated expression of lipid synthesis genes in branch bases (e.g. *fatty acid synthase*) observed in this study confirms a similar finding previously observed for *Acropora* corals (chapter 2). Lipid synthesis also appears to be elevated during the day (*acyl-coA desaturase*), which may be a result of increased effort to store energy produced by symbiont photosynthesis. Up-regulation of lipid synthesis, storage, and catabolism genes has previously been associated with the symbiotic state (Ganot *et al.* 2011; Lehnert
et al. 2014). However, lipid synthesis may also be increased in branch bases to support sexual reproduction. Elevated expression of vitellogenin in bases indicates active production of gametes (eggs), which have a high lipid content (Arai et al. 1993).

Lipid metabolism genes that were up-regulated in tips, including delta(5) fatty acid desaturase, which was also up-regulated during the day, and phospholipase A2, which was also down-regulated during the day, have putative roles in arachidonic acid metabolism and eicosanoid production. Eicosanoids are thought to function as part of the coral’s innate immune system (Libro et al. 2013). Interestingly, arachidonate 5-lipoxygenase, involved in production of leukotriene eicosanoids, and seven transcripts of allene oxide synthase-lipoxygenase, which are involved in biosynthesis of allene oxide from arachidonic acid, were also up-regulated in tips. The highest expression of phospholipase A2 at night in tips, however, suggests that this protein may act as a toxin in the nematocyst complex (Nevalainen et al. 2004).

Transport of carbohydrates is critical for coral growth, both among cells within individual polyps and among the polyps of the colony. Symbiodinium are isolated within vacuoles called symbiosomes comprised of both coral-derived and symbiont-derived membranes (Wakefield et al. 2000); therefore, carbohydrates produced during symbiont photosynthesis and transferred to the host must be transported across the symbiosome membranes and from the endoderm to other cells of the polyp. In Acroporas, they must also be transported from radial polyps to the growing tip of the branch (Pearse & Muscatine 1971), which may be facilitated by the inter-polyp gastrovascular canals (Gladfelter 1983a). The three carbohydrate transport genes that were up-regulated during
the day (slc5a2, slc5a10, slc5a11) are sodium/glucose and sodium/myo-inositol transporters, which have previously been observed to be upregulated in symbiotic compared to aposymbiotic anemones (Lehnert et al. 2014). These are strong candidates for involvement in transfer of fixed carbon involved in LEC.

**Conclusion**

Though conditions within the coral animal have previously been shown to fluctuate dramatically between day and nighttime conditions, the coral response via gene expression is relatively modest. Transcriptomic differences between day and night reflect putative circadian clock functions, response to light and oxidative stress, lipid and nitrogen metabolic functions and carbohydrate transport. By exploring the effect of location within the colony (branch bases versus branch tips) on diurnal versus nocturnal gene expression, I have identified genes that show within-colony temporal differences and provide candidates for further exploration into LEC, phototaxis and the regulation of *Symbiodinium* by the host coral. My results support roles for carbonate ion regulation (CA2) and a mucin SOM protein in LEC, and two photoreceptor-related genes, a retinol dehydrogenase and *Amcry1*, in blue-light phototaxis. Furthermore, my results support the hypothesis that corals control *Symbiodinium* population densities by limiting nitrogen availability through uptake of ammonia by Glutamine synthetase activity, and additionally suggest removal of dysfunctional symbionts by phagocytotic amebocytes. These findings add to a growing body of knowledge about coral growth and coral host-symbiont interactions.
Availability of supporting data

Additional file 1. Data set of annotated (UniProt/Swiss-Prot e-val < 10^-5) coral transcripts. Includes adjusted P and log2(fold change) values. This file can be accessed on LabArchives.com (DOI:10.6070/H4DN432C)


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Tables

Table 1. Mean and standard error of RNA-seq library sizes and number of reads mapped to coral transcripts for each treatment group of samples.

<table>
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<th></th>
<th># samples</th>
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<th>std. err. (lib)</th>
<th>mean (mapped)</th>
<th>std. err. (mapped)</th>
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Table 2. Number of differentially expressed transcripts for functional categories of interest. For transcripts with a significant interaction, *indicates also DE by colony position, †indicates also DE by time of day, §indicates DE for both factors.

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<th>Function</th>
<th>Total DE</th>
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<th>Time of Day only</th>
<th>Both Factors</th>
<th>Interaction</th>
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<tr>
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<td></td>
<td></td>
<td></td>
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<tr>
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<td>2</td>
<td></td>
<td></td>
<td>†2</td>
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<tr>
<td>D site-binding protein</td>
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<td>Melatonin receptor</td>
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<td></td>
<td></td>
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<td>Melanopsin</td>
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<td></td>
<td></td>
<td></td>
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<td>Fatty acid desaturase</td>
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<td>Fatty acid synthase</td>
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<td></td>
<td>*1, §1</td>
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<tr>
<td>Mitochondrial metabolism</td>
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<td>ATP synthase</td>
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<td>Electron Transfer Chain</td>
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<td>341</td>
<td>60</td>
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Figures

Figure 1. **nMDS plot of transcription profiles** for tips (triangles) and bases (squares), day (red) and night (blue). Samples collected from the same colony (genet) are indicated by the number within the data point.
Figure 2. Number of DE genes for model factors (colony position and time) and their interaction considering all coral transcripts (A) and annotated transcripts (B).
Figure 3. Heat map of selected annotated DE transcripts. Sample identity indicated above heat map: day base (BD), night base (BN), day tip (TD) and night tip (TN). Significance of factors indicated by bands to the left of expression data: time of day (black), colony position (light gray), interaction (dark gray). Annotation for cryptochromes based on blastn results. Expression data is log-transformed.
Figure 4. Possible circadian and light-responsive gene interactions and roles in diel processes in corals. Genes DE by time of day or showing an interaction between factors were up-regulated in day (left) or night (right). § indicates a significant interaction effect. Dashed lines indicate hypothetical relationships. Rdh enzymes (purple) may either produce retinal or retinaldehyde; retinal interacts with the opsin apoprotein to form rhodopsin, while retinaldehyde may be further reduced to retinoic acid. Retinoic acid receptors (RARs, blue) may be involved in light reception and/or circadian regulation. Photo-lyase family proteins (gray), Amcrys and phr, may be light-responsive, and may be phosphorylated by MAPK. Cryptochrome expression may regulate the circadian clock, Clock and NPAS2 activity in a light-dependent manner. Clock and NPAS2 proteins (red) are two of the primary bHLH-PAS transcription factors that, when bound to BMAL1 as a heterodimer, direct the circadian rhythm of animals and activate expression of Crys. TEF and DBP are PAR bZip transcription factors (green) activate transcription of circadian-regulated genes.
Figure 5. Glutamate and glutamine biosynthesis pathways including enzymes (boxes) with genes DE by time of day or with an interaction (black text) and present but non-DE enzymes (gray text). §Indicates a significant interaction effect. Enzymes are GLDH, Glutamate dehydrogenase; GOGAT, Glutamate synthase; GLN, Glutaminase; GS, Glutamine synthetase; GATase, Glutamine amidotransferase; and CPSI, Carbamoyl phosphate synthetase I.
Chapter 4
Diel Variation in the Transcriptomic Response of the Coral Algal Symbionts *Symbiodinium*

Abstract

The photosynthetic dinoflagellate algae (*Symbiodinium*) living in symbiosis with reef-building corals are the energetic foundation of tropical coral reefs, supplying up to 100% of a host coral’s energy budget. The breakdown of the coral-algal symbiosis due to factors including heat stress, known as coral bleaching, is a critical concern given rising sea surface temperatures associated with global warming. Understanding how the coral and algal symbiont function under both normal and stressed conditions is a key component for predicting how corals will cope with future environmental conditions. Recent studies have focused on the genetic response of the coral host during the onset of symbiosis and during heat stress. Relatively little is known about the natural variation in *Symbiodinium* gene expression over diel cycles, as well as within the coral host. Recent RNA-seq analyses indicate that gene expression in the Caribbean staghorn coral, *Acropora cervicornis*, shows strong diel (day vs. night) and within-colony differences (branch tips vs. branch bases). Here I use matching RNA-seq data from *Symbiodinium*, collected at mid-day and mid-night from two regions of the coral colony, to examine the *in hospite* differences in algal gene expression. I identify over 50 algal transcripts that are differentially expressed (DE) between branch tips and bases, and over 400 transcripts that differ between day and night. DE genes between branch tips and bases include the up-regulation of photosynthesis light reactions genes in branch tips. DE genes between day
and night are involved in translation, photosynthesis, redox, proteolysis and apoptosis.

These results suggest that diel variation in gene expression varies within the coral colony and that there is diel aspect of transcription-driven photoacclimation in *Symbiodinium*. The photosystem II D1 reaction center protein was DE between regions of the colony, but not between day and night, suggesting that both transcriptional and post-transcriptional regulation may be important for determining protein levels under some conditions.
Introduction

All tropical reef-building corals live in association with photosynthetic endosymbiotic dinoflagellate algae of the genus *Symbiodinium* (Freudenthal 1962), commonly called zooxanthellae. In what is generally considered a mutualistic relationship (but see Lesser *et al.* 2013), the coral host provides the endosymbiont with a relatively protected and stable environment and nutrients, while the *Symbiodinium* supply 40 to 100 percent of the coral’s energy via transferred photosynthetic products (Muscatine *et al.* 1984; Yellowlees *et al.* 2008). Environmental stress, particularly thermal stress combined with high UV or PAR exposure, can cause this symbiotic relationship to break down, resulting in a loss of *Symbiodinium* or algal pigments from the host, which is known as ‘coral bleaching’ (Hoegh-Guldberg 1999). Because bleaching deprives the coral of its primary source of energy, severe bleaching can lead to reduced growth rates (Goreau & Macfarlane 1990), reproduction (Szmant & Gassman 1990) and mortality (Brown & Suharsono 1990; Glynn & D'Croz 1990). Increasing thermal stress events linked to global climate change over the past three decades have resulted in more numerous and extreme bleaching events, making bleaching one of the most imminent threats to the future of coral reef ecosystems (Hoegh-Guldberg 1999).

Large-scale mass bleaching events have correlated with thermal spikes in sea surface temperature (Goreau 1989; Glynn & D'Croz 1990; Hoegh-Guldberg 1999), and bleaching often preferentially affects light-exposed regions of coral colonies (Goenaga *et al.* 1989; Brown *et al.* 1994), indicating an effect of either UV or photosynthetically active radiation (PAR). UV and PAR do not cause mass expulsion of *Symbiodinium* on
their own, but act as a trigger for bleaching in thermally or otherwise stressed corals (Hoegh-Guldberg & Smith 1989; Lesser 1996). At the cellular level, the primary cause of bleaching is thought to be damage to photosynthetic elements of the *Symbiodinium* chloroplasts, leading to excessive production of reactive oxygen species (ROS), which can damage lipids, proteins and DNA (Lesser 2006). Three photosynthetic components have been implicated in susceptibility to bleaching, including the D1 reaction center protein of photosystem II (PSII D1) (Warner *et al.* 1999), Rubisco (Jones *et al.* 1998) and thylakoid membrane lipids (Tchernov *et al.* 2004). Damage to any of these photosynthetic components causes a build up of the electrons that would normally be channeled through the photosystems and electron transport chain to NADPH. These electrons can reduce O$_2$ to the highly reactive superoxide radical (・O$_2^-$), which can be converted into additional ROS species (Weis 2008). Build up of ROS and reactive nitrogen species that reach the host tissue may be a signal used by the host anthozoan to initiate a bleaching response (Perez & Weis 2006).

Bleaching is an extreme and sudden alteration of the host-algal relationship when environmental conditions exceed the tolerance limits. However, under normal daily and seasonal cycles, the coral and *Symbiodinium* respond physiologically to cope with variation in light and oxidative stress. Corals and *Symbiodinium* both produce antioxidant enzymes (Weis 2008), heat shock proteins, and suspected UV-protective proteins or metabolites (e.g. GFP-like proteins in corals (Salih *et al.* 2000) and mycosporine-like amino acids in *Symbiodinium* (Dunlap & Shick 1998)). *Symbiodinium* also respond to changes in light availability by adjusting the concentration of chloroplast pigments,
indicating photoacclimation. *Symbiodinium* in shade-adapted corals have a substantially higher chlorophyll a content than those in light-adapted corals (Falkowski 1981; Porter et al. 1984), and both *in hospite* (in symbiosis with the host coral) and cultured *Symbiodinium* respond to decreased light with corresponding increase in photosynthetic capacity by increasing chlorophyll-protein complexes (Falkowski 1981; Iglesias-Prieto & Trench 1997; Torres-Pérez et al. 2007). The coral may also actively regulate *Symbiodinium* populations through expulsion, degradation, or inhibition of algal growth and reproduction (division) (Davy et al. 2012). In this dynamic symbiosis, the density of *Symbiodinium* has been seen to change seasonally and in response to environmental factors such as the concentration of bio-available nitrogen (Marubini & Davies 1996; Fagoonee 1999).

The health of the coral-algal symbiosis requires a balancing act on the part of the host animal to maintain a population of algae that can supply energy to meet the metabolic requirements of both partners, while not allowing the symbiont population to overwhelm the host tissues or the host’s capacity to cope with oxidative stress. *Symbiodinium* photosynthesis provides the coral with energy, but also affects the internal chemistry and functioning of the animal host (Kühl et al. 1995; Gordon & Leggat 2010). Photosynthesis activity peaks during mid-day and afternoon (Levy et al. 2004; Schneider et al. 2009) and tissues during the day experience hyperoxic conditions (>250% of air O₂ saturation) and relatively high pH (7.4-8.5), while in the absence of light, aerobic metabolism quickly consumes the available oxygen, producing a hypoxic (< 2% of air saturation) and a lower pH (7.0-7.3) intracellular environment (Kühl et al. 1995; Venn et al. 2009; Laurent et al. 2013).
In efforts to better understand the molecular basis of the coral-<em>Symbiodinium</em> interaction and the cellular basis for the bleaching response, a number of gene expression studies have examined the genetic response of coral (or other symbiotic anthozoans) to the symbiotic state (Rodriguez-Lanetty <em>et al.</em> 2006; Voolstra <em>et al.</em> 2009; DeSalvo <em>et al.</em> 2010a; Lehnert <em>et al.</em> 2014), to light stress (DeSalvo <em>et al.</em> 2011; Bay <em>et al.</em> 2013) and/or under thermal stress (DeSalvo <em>et al.</em> 2008; Richier <em>et al.</em> 2008; Leggat <em>et al.</em> 2011a; Bellantuono <em>et al.</em> 2012; Kenkel <em>et al.</em> 2014; Maor-Landaw <em>et al.</em> 2014); however, relatively little is known about the natural variation in <em>Symbiodinium</em> gene expression. Most gene expression analyses of <em>Symbiodinium</em> have used algal cultures, rather than in hospite samples, and have almost always evaluated a limited number of genes (Rosic <em>et al.</em> 2010; Leggat <em>et al.</em> 2011a; McGinley <em>et al.</em> 2012; 2013). Two recent studies have evaluated <em>Symbiodinium</em> gene expression at a broader scale using RNA-seq (Baumgarten <em>et al.</em> 2013; Barshis <em>et al.</em> 2014). One of these studies, Barshis <em>et al.</em> (2014), which was conducted on in hospite <em>Symbiodinium</em> in association with the Indo-Pacific coral <em>Acropora hyacinthus</em>, found a very low transcriptional response of the symbiont to thermal stress, compared with the coral transcriptional response. Like some other dinoflagellates (Van Dolah <em>et al.</em> 2007; Morey <em>et al.</em> 2011; Roy & Morse 2013), <em>Symbiodinium</em> seem to have few transcription factors (Bayer <em>et al.</em> 2012) and may depend highly on post-transcriptional and post-translational mechanisms to regulate their protein content in response to certain conditions (Baumgarten <em>et al.</em> 2013).

I recently evaluated gene expression activity of the host coral using the Caribbean staghorn coral, <em>Acropora cervicornis</em>, in which the abundance of <em>Symbiodinium</em> naturally differs between regions of the colony (Pearse & Muscatine 1971). The branch tips of A.
*cervicornis* colonies are sites of rapid growth (Goreau & Goreau 1959) and contain a much lower concentration of *Symbiodinium* compared to the rest of the colony. I compared within-colony gene expression differences between branch bases and tips and diel gene expression differences between day and night (chapter 3). Diel variation in coral gene expression included circadian and light-response genes, as well as lipid, carbohydrate and nitrogen metabolism, some of which showed within-colony differences in expression as well. Within-colony difference in expression of some genes, including the circadian-related genes *Amcry1* and *tef*, the iron homeostasis and oxidative stress response gene *hebp2*, and the nitrogen metabolism gene *glutamine synthetase* may be due to a differing effect of *Symbiodinium* activity within the colony. Gene expression data from the coral host suggests that photosynthetic activity by the symbiotic algae may affect coral gene expression associated with oxidative stress or redox state of the cell. Differentially expressed genes in the glutamine/glutamate pathway also suggest that the coral may alter its nitrogen metabolism to control *Symbiodinium* population growth (chapter 3).

Transcriptome-wide gene expression comparison between day and night in *in hospite* *Symbiodinium* may help to clarify how the algal symbiont responds to normal diel variation, and in turn how algal activity may influence the coral host. Yet, no such study has yet been conducted. Because *A. cervicornis* host corals exhibit within-colony differences in gene expression (chapters 2 & 3), *Symbiodinium* gene expression may also be affected by their location in branch bases or tips. Here I evaluate differences in gene expression between day and night for *in hospite Symbiodinium* living in association with *A. cervicornis*, using RNA-seq. I compare gene expression at noon and midnight for
Symbiodinium in two parts of the coral colony, the branch bases and branch tips. Using multi-factor GLM analyses, I identify algal transcripts that differ due to time of day (mid-day vs. mid-night) and within the coral colony (branch tips vs. branch bases).

Materials and methods

Acropora cervicornis samples were collected from three large staghorn coral colonies located at least 10 meters apart at 5-6 m. depth in Crawl Cay, Bocas del Toro, Panama (09 15.517 N, 082 07.625 W). Paired branch base and tip samples were taken from individual branches, with base samples taken at 25-30 cm from the tip of the branch, and tip samples being the top 1 cm of the branch, including the axial polyp. For each of the three colonies, base and tip samples were collected from one branch at 12:00-13:00 (~6 hrs after sunrise) on August 2 (sunrise 6:09, sunset 18:39, moonrise 23:46, moonset 11:46, third quarter), and a second branch at midnight-00:30 (~5.5 hrs after sunset) on August 3, 2010. Temperature data was collected from a permanent data logger located at 3 m. depth near Crawl Cay (Cayo Agua; data publicly available at http://biogeodbo.stri.si.edu/physical_monitoring/research/bocas). Seawater temperature was 29.7-29.8°C during daytime sampling (12:00-13:00) and 29.6°C during nighttime sampling (00:00-01:00). Coral fragments were transported to the boat in a plastic box and flash frozen in liquid nitrogen.

Total RNA extraction was performed using TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH) and the manufacturer’s protocol with an additional 75% ethanol wash step. For some samples, RNA was extracted up to three times and
extraction with the best quality was used. To enhance the concentration of the extracted RNA, 1 ul glycogen was added to some samples during the extraction process, prior to the isopropanol precipitation step. Total RNA quality was assessed using Agilent Bioanalyzer 2100 RNA Pico Chips, and only extractions with distinct 18S and 28S ribosomal RNA peaks were used (RIN 4.9-8.0).

Illumina RNA-seq library preparation

mRNA was isolated using Dynabeads® oligo(dT) mRNA isolation beads (Life Technologies, Grand Island, NY) to exclude non-protein coding RNAs and non-eukaryotic mRNAs from the samples. cDNA was produced using random hexamer primers and SuperScript® II reverse transcriptase (Life Technologies) for first strand synthesis and DNA polymerase I (New England BioLabs, Ipswich, MA) for second strand synthesis. RNA-seq libraries were prepared by fragmenting the double stranded cDNA with dsDNA fragmentase (NEB) followed by library preparation using NEBNext® reagents. The cDNA transcripts with barcoded (4 bp) adapters were size selected at 250 bp by gel extraction and amplified with 15 rounds of PCR. RNA-seq libraries were sequenced (6 samples per lane) using single-end 50 bp sequencing on the Illumina Hi Seq 2000 platform (Illumina, Inc., San Diego, CA, USA) at the FAS Center for System Biology at Harvard University.

Bioinformatics

Read quality control and barcode removal were conducted with custom Perl scripts in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), and RNA-seq quantification of gene expression against a de novo combined A. cervicornis and A.
*palmata* reference transcriptome was performed in CLC Genomics Workbench (CLC bio). Reference transcriptome sequences and RNA-seq read data are available on BioProject (accession number PRJNA222758). *Symbiodinium* sequences were identified by nucleotide BLAST alignment to the transcriptomes of *Symbiodinium* clades A (KB8), B (Mf104b), C and D (Bayer et al. 2012; Ladner et al. 2012), and coral sequences were identified by alignment to genomes for *A. millepora* (Miller, unpublished) and *A. digitifera* (Shinzato et al. 2011), both using a threshold E-value \( \leq 10^{-10} \). Only transcripts identified exclusively as *Symbiodinium* (i.e. matching *Symbiodinium* transcriptomes and not matching coral genomes) were included in the analyses.

Sequences of COI and cytochrome b in our reference transcriptome indicate that the *Symbiodinium* in *A. cervicornis* at the collection site are most likely to be clade A. The *Symbiodinium* clade present in these samples is most likely A3, which is commonly associated with *A. cervicornis* at the sampled site and depth (Vollmer, unpublished data) and elsewhere in the Caribbean (LaJeunesse 2002; Thornhill et al. 2006).

Statistical analyses: ‘Two-factor’ analysis

I used a ‘two-factor’ design to test the effects of time of day (day vs. night), colony position (base vs. tip) and the interaction between time of day and colony position. I added a third factor, genet (genetically distinct coral colony), to the model to control for inter-colony variation in gene expression; however, results for this factor are not discussed in detail. I conducted non-metric multidimensional scaling (nMDS; isoMDS) and PERMANOVA (Adonis) on the full gene expression profiles of the samples using normalized count data and the vegan package (Oksanen et al.) in R (R Development Core
Team, 2012). The transcript count data were normalized by sample library size (size factor) in DESeq2 (Anders & Huber 2010) in R. The same program was used to test for gene expression differences by colony position, time of day and genet, as well as the interaction between colony position and time of day, using a three-factor negative binomial Wald test (design = ~ genet + position * time). An adjusted p-value < 0.05 was used to evaluate significance (Benjamini & Hochberg 1995). For the colony position factor, gene expression values in bases were assigned as the baseline for comparison, so DE transcripts are considered ‘up-regulated’ or ‘down-regulated’ in tips, relative to bases. For the time of day factor, gene expression values in night samples were assigned as the baseline, so DE transcripts are considered ‘up-regulated’ or ‘down-regulated’ during the day.

Statistical analyses: ‘One-factor’ analyses

Based on the results of the ‘two-factor’ analyses, which suggested that the size of the RNA-seq libraries was limiting the ability to detect genes differentially expressed between day and night, I used two ‘one-factor’ analyses to look more closely at the influence of day versus night on gene expression. Again for these analyses, the genet factor was included only to control for inter-colony variation. The ‘one factor: position’ design was used to test the effect of colony position (base vs. tip) on gene expression by testing differences between bases and tips for day and night samples separately. Differential expression was evaluated using a two-factor negative binomial Wald test (design = ~ genet + colony position) in DESeq2. The ‘two factor: time’ design was used to test the effect of time of day (day vs. night) on gene expression by testing differences
between day and night in base and tip samples separately. nMDS, PERMANOVA and differential gene expression were evaluated for both models. Differential expression was evaluated using a two-factor negative binomial Wald test (design = ~ genet + time of day) in DESeq2. Heat maps of selected photosynthesis genes were created using log-transformed normalized count data in GENE-E (Gould).

Results and discussion

Of the 95,389 transcripts in the reference coral transcriptome (including algal transcripts), 36,909 (39%) were identified bioinformatically as Symbiodinium transcripts by BLAST against Symbiodinium-only reference transcriptomes. Of these, 267 transcripts also aligned to symbiont-free larval Acropora genomes and may represent horizontal gene transfer; they were therefore excluded from subsequent analyses. Symbiodinium-only RNA-seq libraries varied from 2.7 ± 0.4 million (mean ± SE) reads in base samples to 0.3 ± 0.1 million reads in tip samples. These correspond to approximately 20-30% of the total base and 6% of the total tip RNA-seq reads from the mixed coral/algal samples. Symbiodinium library sizes were significantly different between bases and tips (paired t(5)=5.17, P=0.0036), but not between day and night (paired t(5)=0.40, P= 0.7044).

Transcriptome profiles and differentially expressed genes

For the analyses considering the ‘two-factor’ model (colony position and time of day, with genet to control for inter-colony variation), transcription-wide gene expression profiles were significantly different between branch bases and tips (PERMANOVA, df = 1, Pseudo-F=2.69676, P = 0.012), but not between day and night (df = 1, Pseudo-
F=0.73130, P = 0.656), among genets (df = 1, Pseudo-F=0.87545, P = 0.491) or for an interaction between colony position and time of day (df = 1, Pseudo-F=0.65438, P = 0.786). nMDS analysis for all samples indicated a clustering of base samples, but no clear clustering of samples by time of day or genet (Figure 1). ‘Two-factor’ differential expression analysis identified 52 transcripts that were differentially expressed (DE) by colony position (tip vs. base; Figure 2A, Table 1) but no DE transcripts by time of day (day vs. night; Figure 2B) or with significant interactions. Of the 52 transcripts DE by colony position 47 were up-regulated and five were down-regulated in tips.

Given the expectation of finding DE genes between day and night in this photosynthetic alga, the result of the ‘two-factor’ analysis suggests that the power to detect DE genes was constrained by the small sizes of the branch tip RNA-seq libraries (averaging 0.3 million reads) (Liu et al. 2014), likely related to much lower symbiont densities. To see if I could get better resolution of the gene expression differences, I performed independent ‘one-factor’ GLM analyses. ‘One-factor’ analyses run independently for the day and night data identified 39 transcripts DE by colony position during the day (37 up-regulated and 2 down-regulated in tips; Table 1, Figure 3A) and 18 transcripts DE by colony position at night (all up-regulated in tips; Figure 3B). ‘One-factor’ analyses run independently for the base and tip data identified 443 transcripts DE by time of day in branch bases, and no transcripts DE in tips (Table 1, Figure 4). Of the transcripts DE between day and night in bases, 336 were up-regulated and 107 were down-regulated during the day.

The effect of colony position: branch base vs. branch tip
In the ‘two-factor’ analysis, the 18 annotated genes that were up-regulated in tips included eight photosynthesis-related genes \((psbA, psbC, psbD, psbE, petd, cyb6, atpA\) and \(atpB\); Table 2, Figure 5), a mitochondrial respiration gene \((cytochrome b)\), two transcripts involved in transcription \((high mobility group B protein 6\) and \(pentatricopeptide repeat-containing protein At1g74850\), two transcripts involved in translation or protein regulation \((a ubiquitin-60S ribosomal protein (RL40)\) and \(methionine aminopeptidase 2\)\) and a vacuolar amino acid transporter \((avt1)\). The photosynthesis proteins coded by \(psbA\) (PSII D1 reaction center protein, aka photosystem Q(B)), \(psbC\) (PSII CP43 chlorophyll apoprotein), \(psbD\) (D2 reaction center protein) and \(psbE\) (cytochrome b559 subunit alpha) are components of photosystem II. Cytochrome b6 \((petB)\) is part of the cytochrome b6-f complex in the electron transport chain between PSII and PSI, while ATP synthase subunits alpha \((atpA)\) and beta \((atpB)\) are components of the ATP-synthase complex. The four annotated transcripts that were down-regulated in tips included two transcripts of the photosynthesis-related gene \(ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)\), in addition to \(fatty acid desaturase 2\) and \(sodium:pantothenate symporter\).

In the ‘one factor:position’ analyses, of the seventeen annotated transcripts up-regulated in tips in the daytime samples, nine were also DE in the ‘2-factor’ analysis, including a \(vacuolar amino acid transporter (avt)\) and a number of photosynthesis light reactions genes (Table 2). The two annotated transcripts down-regulated in tips during the day were ‘uncharacterized proteins’. Of the nine annotated transcripts up-regulated in tips during the night, seven were DE in both the ‘two-factor’ and ‘one factor’ daytime analyses, including \(avt\), and five photosynthesis light reactions proteins (Table 2). One
transcript, *cytochrome b*, was also DE only in the ‘two factor’ but not in the ‘one-factor’
daytime analysis, and a predicted protein was only DE in the nighttime samples.

Nine photosynthesis transcripts were DE between regions of the colony in the
‘two-factor’ analysis, and twelve were DE in at least one of the analyses (Table 2). The
eleven photosynthesis genes that were up-regulated in tips are related to the light
reactions of photosynthesis in which light energy is used to generate ATP and NADPH,
while the two photosynthesis transcripts that were down-regulated in tips (only in the
‘two-factor’ analysis) are Rubisco, the protein involved in the first step of the Calvin
cycle, in which ATP and NADPH are expended to produce glyceraldehyde-3-phosphate
from inorganic CO$_2$.

Up-regulation of light reaction genes in coral tips may be due to a higher rate of
*Symbiodinium* asexual reproduction in the tips of *A. cervicornis* (Wilkerson *et al.* 1988;
Gladfelter *et al.* 1989), requiring a higher initial production of photosynthesis proteins in
the growing algal cells. In the event that the coral host restricts nitrogen availability to
*Symbiodinium* in branch bases to control algal population growth, the corresponding
higher population growth rate in tips, itself, or enrichment of photosynthetic proteins in
each algal cell could be related to increased availability of nutrients. Corals grown in
higher nitrogen (ammonium or nitrate) conditions experience increased *Symbiodinium*
growth and higher chlorophyll concentrations (Muscatine *et al.* 1989; Dubinsky *et al.*
1990; Marubini & Davies 1996). Another explanation may be the availability or relative
concentrations of CO$_2$ and O$_2$ in tips compared to bases. *Symbiodinium* concentrations
are lower in the tips of *Acropora* branches, and therefore there may be a lower amount of
O$_2$ produced and CO$_2$ consumed per unit area during the day in this part of the colony. Previous direct evaluation of respiration (Gladfelter et al. 1989) as well as gene expression analysis (chapters 2 & 3) for Acropora corals suggest that there is a higher rate of mitochondrial respiration in branch tips, which would simultaneously consume O$_2$ and produce CO$_2$. Although total photosynthetic productivity of Symbiodinium is lower in branch tips compared to branch bases (Gladfelter et al. 1989), less competition for CO$_2$ among the lower density of Symbiodinium, and/or a lower concentration of O$_2$, could result in a higher per unit efficiency in tips. Stylophora colonies in which ammonium enrichment leads to increased algal densities experience decreased photosynthetic efficiency of individual algal cells, possibly due to CO$_2$ competition (Dubinsky et al. 1990). Additional analysis of O$_2$ and CO$_2$ concentrations within the tissue of the coral polyps at these different regions of the colony, as well as analysis of photosynthetic efficiency per algal cell would be necessary to confirm this hypothesis.

This same scenario may explain the higher expression of Rubisco in branch bases. Symbiodinium Rubiscos are a multi-gene family of nuclear encoded form II Rubisco, which are primarily found in prokaryotes and have a much lower specificity for CO$_2$ over O$_2$, compared to the eukaryotic form I Rubisco. As a result, Symbiodinium likely depend upon a CO$_2$ concentrating mechanism within the chloroplast (Leggat et al. 1999), which may employ carbonic anhydrase to convert bicarbonate to CO$_2$ (Weis et al. 1988). In an environment with a low CO$_2$:O$_2$ ratio, Rubisco may engage in a higher level of photorespiration (the addition of oxygen, instead of CO$_2$, to ribulose-1,5-bisphosphate), resulting in a loss of photosynthetic efficiency. In this case, the Symbiodinium may require a higher Rubisco concentration to produce an equivalent amount of fixed carbon.
Higher expression of *fatty acid desaturase* by *Symbiodinium* in branch bases is consistent with earlier observations in the coral gene expression data, in which fatty acid synthesis was higher in branch bases (chapter 3). Higher fatty acid production in corals and *Symbiodinium* may be due to the higher overall photosynthetic productivity and lower mitotic index of the algae in this region of the colony. The reproductive (mitotic) rate of *Symbiodinium* is higher in branch tips of *A. cervicornis* (Wilkerson et al. 1988, Gladfelter 1983c), which may be due to regulation of *Symbiodinium* population growth by the coral in the parts of the colony with high algal abundance (Davy et al. 2012). As a result, energy diverted from algal growth may be stored or incorporated into lipids. This may be directly or indirectly related to restriction of nitrogen availability to *Symbiodinium* as a means of population growth control. In cultured *Symbiodinium*, nitrogen deprivation results in a higher accumulation of lipid droplets (Jiang et al. 2014).

Up-regulation of a vacuolar amino acid transporter in tips may indicate increased availability of amino acids and nitrogen-containing molecules within *Symbiodinium* in branch tips. Coral regulation of *Symbiodinium* population growth may occur through restriction of nitrogen through coral uptake of nitrogen by the glutamate/glutamine metabolic pathway (Rees 1986). I previously found up-regulation of *glutamine synthetase* in during the day in branch bases, indicating increased assimilation of nitrogen by the host in the symbiont-rich region of the colony (chapter 3). If corals actively restrict nitrogen availability to *Symbiodinium* in branch bases, algal nitrogen compound metabolism and transport would likely be depressed. Finally, one transcript, *methionine aminopeptidase* 2 was 10.5-fold up-regulated by *Symbiodinium* in branch tips. Methionine aminopeptidase proteins remove N-terminal methionine residues from newly
translated peptides and are important for post-translational processing. This may be a way in which *Symbiodinium* regulate protein abundances downstream of transcription.

The effect of time of day: day vs. night

Gene expression of *Symbiodinium* in association with *A. cervicornis* varied between day and night in branch bases (n=443 transcripts; Table 1) and exhibited a similar number of DE transcripts between day and night as the coral host (n=441, chapter 3). However, few *Symbiodinium* genes showed large fold changes in expression (only seven transcripts had > 2-fold change), compared to the host coral, in which more than half of the 441 DE transcripts had > 2-fold change between day and night (chapter 3). *Symbiodinium* have repeatedly demonstrated relatively low magnitude gene expression changes in response to different conditions, suggesting that regulation of protein expression in these organisms may largely be post-transcriptional or post-translational (Leggat et al. 2011a; Barshis et al. 2014). Bayer et al. (2012) suggest that transcriptional regulation in *Symbiodinium* may differ from that in other eukaryotes due to a low number of transcription factors (TFs) present in their transcriptome. Indeed, in comparison to the coral host, which contains numerous DE TFs, such as circadian regulators that are DE between day and night (chapter 3), few TFs were DE in *Symbiodinium*.

The majority of annotated transcripts were up-regulated (n=228) rather than down-regulated (n=73) during the day (Table 1). Cellular processes showing the greatest diel shifts in gene expression for *Symbiodinium* were up-regulation of translation-related genes during the day (n=67; table 3), primarily 40S and 60S ribosomal proteins, indicating increased cellular activity, and differential expression of transcripts involved in
photosynthesis (n=22; Figure 6). Moderately up-regulated transcripts involved in cell redox homeostasis and proteolysis (Table 3) suggest a response to oxidative stress and/or redox signaling involved in photosynthesis, as well as removal of damaged proteins. The results for the ‘one-factor:position’ analyses indicated that transcripts DE by colony position vary between day and night, and suggest that diel variation in Symbiodinium may also be affected by location within the colony; however, additional analyses with larger RNA-seq library sizes may be needed to detect diel variation of transcription in branch tips.

Photosynthesis

For the branch base samples, a greater number of photosynthesis-related genes were up-regulated (15 transcripts representing 14 genes) than down-regulated (8 transcripts representing 6 genes) during the day (Table 2, Figures 6 & 7). The fourteen photosynthesis-related genes that were up-regulated during the day showed only a moderate magnitude change in expression (log_2(fold change)=0.3-0.7) and included a gene involved in chlorophyll biosynthesis, tetrapyrrole-binding protein (gun4), and genes involved in many steps of the light reactions: two oxygen evolving complex (OEC) subunits (psbU & psbV), two fucoxanthin-chlorophyll binding proteins (fcp & fcpf), PSI RC subunits III (psaf), IV (psae, psae1), and iron-sulfur center protein (psac), chlorophyll a-b binding protein L1818, a PSII RC subunit (psbN), cytochrome c6 (petJ), and two ATP synthase subunits, delta (atpD) and b’ (atpG). Of the six photosynthesis genes that were down-regulated during the day in bases, three genes showed greater than 2-fold change in expression, all of which were genes of reaction center chlorophyll binding proteins of PSI (psaA & psaB) and PSII (psbB). Three other genes showed
moderate (<2-fold) down-regulation, including a component of the light-harvesting complex (LHC) of both PSI and PSII (caroteno-chlorophyll a-c-binding protein; ccac), a fatty acid biosynthesis gene (palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase; FAD5), and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco).

These DE genes indicate that Symbiodinium within the coral are actively regulating the transcription of some photosynthetic proteins between day and night, even though the magnitude of transcriptional regulation is small. Many photosynthesis-related genes in Symbiodinium are coded for by chloroplastic DNA found in plasmid-like minicircles (Leggat et al. 2011b), which appear to be targets of transcriptional regulation (Baumgarten et al. 2013). However, the DE genes identified here also include Rubisco, which in Symbiodinium is a nuclear-encoded gene (Morse et al. 1995; Rowan et al. 1996). The genes DE between day and night are involved in three aspects of photosynthesis thought to be important in bleaching: photoacclimation, Rubisco, and the thylakoid membrane (FAD5). Interestingly, the psbA gene that codes for the PSII D1 reaction center protein, which was DE by colony position, was not DE by time of day.

Photoacclimation is the process by which photosynthetic organisms adjust to changing levels of PAR and involves increasing the concentrations of light-harvesting pigments (e.g. chlorophylls) under low light to capture the maximum available energy, and reducing them under high light to prevent excessive energy absorption (Dubinsky & Stambler 2009). In daylight, excitation energy absorbed by the pigments is dissipated either by photochemistry (passing energy from the light-harvesting complex to a reaction center protein), by fluorescence (release of a photon) or by heat (non photochemical
quenching). When the energy absorbed exceeds the capacity of the reaction center proteins or primary electron acceptor (plastoquinone) and cannot be sufficiently dissipated by accessory pigments or the xanthophyll cycle, ROS (singlet oxygen, superoxide, and hydroxyl radicals) can be produced, leading to cellular damage (Demmig-Adams 1990; Lesser 2006) and a reduction in photosynthetic efficiency known as photoinhibition (Long et al. 1994). In addition to reducing the light-absorbing pigments, photoacclimation often leads to an increase in the concentrations of photo-protective pigments (e.g. xanthophylls) in high light, which helps to dissipate excess energy and reduce the production of ROS (Demmig-Adams & Adams 1996; Lesser 2006). For *Symbiodinium*, photoacclimation through decreased chlorophyll concentration (Falkowski 1981; Porter et al. 1984; Torres-Pèrez et al. 2007) and increased xanthophyll (Iglesias-Prieto & Trench 1997) concentration have been observed in response to increased light availability.

Down-regulation of reaction center chlorophyll binding protein genes (*psaA, psaB, psbB*) during the day indicates that high light may inhibit transcription of these genes and helps to explain the lower chlorophyll levels observed for corals grown under high light conditions (Falkowski 1981; Porter et al. 1984; Torres-Pèrez et al. 2007; Lesser et al. 2010). However, for these genes and others, location within the coral colony also appears to influence gene expression. For the same three genes (*psaA, psaB, psbB*), expression was up-regulated in tips, but only in daytime samples, not samples taken at night. This appears to be due to a greater down-regulation of these genes during the day in bases, compared to tips (Figure 5). This suggests the transcription of these genes does not depend on light-availability alone, but may also be influenced by redox state or other
factor differing between bases and tips. Unfortunately, due to small library size, it was not possible to determine whether these genes are also DE between day and night in branch tips.

One photosystem reaction center gene that was DE by colony position but not by time of day was \textit{psbA}, the gene for the PSII D1 reaction center protein. The PSII D1 reaction center protein is a key structural component of PSII and provides the plastoquinone binding pocket, which is essential for transfer of electrons through the electron transport chain (McGinley \textit{et al.} 2012). PSII D1 is particularly sensitive to damage from light and heat, undergoing a high rate of synthesis, repair and degradation under normal conditions (Andersson & Aro 2001). The sensitivity of PSII D1 and its repair mechanisms (Takahashi \textit{et al.} 2004) are considered to be a major factor in the breakdown of the coral-\textit{Symbiodinium} relationship that leads to coral bleaching (Warner \textit{et al.} 1999). The ability for \textit{Symbiodinium} to maintain high levels of \textit{psbA} transcription may be associated with thermal tolerance of some clades (e.g. D1, A20 and F2) (McGinley \textit{et al.} 2012). Though the \textit{psbA} gene was not DE between day and night, it was between base and tip and was the most highly expressed annotated transcript with an order of magnitude higher gene expression compared with the photosynthesis-related genes. This suggests that a high rate of regeneration for PSII D1 protein is maintained both during the day and at night.

Gene expression analyses of \textit{psbA} in \textit{Symbiodinium} in other corals have given varied and contrasting results. In \textit{Stylophora pistillata}, expression of \textit{psbA} was found to increase in the light, relative to dark (Moya \textit{et al.} 2008b); however, in cultured
*Symbiodinium, psbA* showed little response to changes in light intensity (McGinley *et al.* 2013). In *Symbiodinium, psbA* shows a clade-specific transcriptional response to heat stress (McGinley *et al.* 2012), and therefore, transcriptional response of *psbA* to light may also be clade-specific or related to the symbiotic condition. My results for *psbA* suggest two different modes of regulation for this gene. The similarity of *psbA* levels between day and night suggests that the level of PSII D1 protein content under conditions of normal diel variation may be determined by post-transcriptional processes, whereas, the up-regulation of *psbA* in tips suggests that *Symbiodinium* in differing environments may regulate *psbA* transcriptionally.

Two of the genes that were up-regulated during the day, PSI RC subunit IV (*psae*) and a fucoxanthin-chlorophyll binding protein (*fcpf*), have been found to have elevated rates of non-synonymous nucleotide evolution in clade D *Symbiodinium* and may also be involved in thermal tolerance (Ladner *et al.* 2012). The protein coded by *psae*, which is located on the reducing side of PSI and is responsible for the connection between PSI and the electron acceptor ferridoxin, appears to be important for preventing production of light-induced ROS (Jeanjean *et al.* 2008). Another gene involved in the xanthophyll cycle for non-photochemical quenching, *zeaxanthin epoxidase*, was also up-regulated during the day. Zeaxanthin epoxidase catalyzes the epoxidation of zeaxanthin to violaxanthin and facilitates the dissipation of excess energy as heat from PSII (Eskling *et al.* 1997).

*Rubisco*, which was up-regulated at night in bases and down-regulated in tips (‘2-factor’ analysis), was the only DE transcript involved in the Calvin cycle reactions of photosynthesis. Rubisco is another *Symbiodinium* protein suspected to play a role in
susceptibility to bleaching (Jones et al. 1998). Under this hypothesis, heat damage to Rubisco resulting in a loss of function and carboxylation capacity is the primary cause of bleaching, followed by a secondary incapacitation of an overwhelmed PSII under light stress (Jones et al. 1998). Like psbA, Rubisco gene expression studies in Symbiodinium have found inconsistent transcriptional and translational activity. Rubisco gene expression was previously found to be up-regulated in light by Symbiodinium within the coral S. pistillata (Moya et al. 2008b) and in culture (Mayfield et al. 2014); however, in symbiosis with Aiptasia, Rubisco gene expression was elevated in the dark (Mayfield et al. 2014). In both symbiotic and cultured Symbiodinium Rubisco gene expression appears to have little correlation with the protein content of the cells (Mayfield et al. 2014). Therefore, Symbiodinium Rubisco protein content may be affected both by symbiotic state, light cycles and post-transcriptional regulation. In the current samples, both time of day and location appear to influence Rubisco transcription, and as mentioned earlier may be related to factors such as CO$_2$ and O$_2$ concentrations.

The lipid content of the thylakoid membrane, particularly the degree of saturation of the membrane lipids, is correlated with thermal tolerance of Symbiodinium clades (Tchernov et al. 2004). For thermally sensitive Symbiodinium, temperature increases result in thylakoid membrane degradation (Tchernov et al. 2004). In general, the saturation of membrane lipids may be adjusted with temperature to maintain a balance of stability and fluidity under changing thermal conditions: increased saturation of lipids during warmer conditions provides membrane stability, while desaturation of lipids in cooler conditions allows fluidity of the membrane. In thylakoid membranes, unsaturation of membrane fatty acids can speed recovery of PSII after photoinactivation.
(Allakhverdiev et al. 2001) and confers higher light and thermal (cold) stress tolerance (Wada et al. 1994), possibly by facilitating membrane protein replacement (Gombos et al. 1997). However, polyunsaturated fatty acids may be more easily damaged by oxidative stress (Allakhverdiev et al. 2009). The degree of fatty acid/lipid saturation is controlled by fatty acid desaturases. Upregulation of FAD5, a palmitoyl-monogalactosyldiacylglycerol delta-7 desaturase, at night is an indication that the symbiont is either repairing the thylakoid membrane or making the membrane less rigid by increasing the unsaturated lipid content. In Arabidopsis, FAD5 appears to determine the lipid content of the thylakoid membrane and is related to chlorophyll content and recovery of PSII after photoinhibition by high light stress (Heilmann et al. 2004). The FAD5 gene has previously been suggested as a candidate contributing to thermal tolerance because of an elevated ratio of non-synonymous mutations in clade D Symbiodinium (a clade with high bleaching tolerance) (Ladner et al. 2012).

Interestingly there was little overlap in the genes DE between base and tip in and those DE between day and night (Table 2). Only Rubisco was both down-regulated in tips and down-regulated during the day in bases (‘2-factor’ analysis), and three reaction center chlorophyll apoprotein genes (psaA, psaB and psbB) were up-regulated in tips during the day and down-regulated during the day in bases. As discussed earlier, these genes indicate that location within the coral colony can have an effect on transcriptional regulation, though additional analyses will be needed to understand whether these transcriptional differences have a corresponding effect on protein content.
Post-transcriptional or post-translational regulation of proteins may be the dominant mode of protein regulation in *Symbiodinium* as suggested previously by the low degree of transcriptional change observed in response to varied conditions including heat stress (Leggat *et al.* 2011a; Barshis *et al.* 2014). Post-transcriptional regulation may occur through microRNA and RNAi-mediated degradation (Baumgarten *et al.* 2013), induction or inhibition of translation due to factors such as redox state (Trebitsh *et al.* 2000; Trebitsh & Danon 2001; Kim & Mayfield 2002) and protein degradation.

**Cell redox and UV stress response**

The up-regulation of redox proteins during the day is likely driven by two major functions within the *Symbiodinium*: redox signaling and protection from oxidative stress. The light reactions of photosynthesis are essentially a series of redox reactions in which an electron, derived from the light-induced splitting of water by PSII, is passed through a chain of membrane-bound proteins and ultimately delivered to NADP+, reducing it to NADPH (Figure 9). In photosynthetic organisms, redox signaling based on the redox demand of the system is used to modulate expression, processing and translation of genes encoded within the chloroplast and nucleus (Durnford & Falkowski 1997; Pfannschmidt 2003). The light-responsiveness of photosynthesis-related genes, including light-harvesting proteins and chlorophyll biosynthesis proteins, may be related to the redox states of plastoquinone, the cytochrome b6f complex, ferredoxin and soluble compounds such as thioredoxin, glutathione and ROS (Pfannschmidt 2003). In addition to their roles as signaling molecules, thioredoxin and glutathione are important antioxidants and may ameliorate the damaging effects of ROS (Carmel-Harel & Storz 1999).
Three thioredoxin-containing redox proteins, *thioredoxin*, *thioredoxin domain containing protein 9* and *protein disulfide-isomerase*, were up-regulated in daytime samples. In the dinoflagellate *Chlamydomonas reinhardtii*, thioredoxin domain-containing proteins facilitate photo-regulation of *psbA* translation (Trebitsh et al. 2000; Trebitsh & Danon 2001; Kim & Mayfield 2002), which may be a form of post-transcriptional regulation also employed in *Symbiodinium*. One gene involved in the metabolic pathway of the redox molecule glutathione, *glutamate-cysteine ligase*, was down-regulated during the day. Glutathione synthesis requires glutamate as a substrate in the initial step, and one enzyme involved in one glutamate biosynthesis pathway, *glutamate synthase*, was also down-regulated during the day, both in *Symbiodinium* and in the coral host (chapter 3). In addition to its role as a precursor of glutathione, glutamate has numerous roles in the cell, including signaling and synthesis of other amino acids; therefore, it is difficult to predict why *glutamate synthase* may be down-regulated during the day.

*Symbiodinium* are known to produce UV-absorbing mycosporine-like amino acids, which are hypothesized to protect both coral and symbiont from the high levels of UV exposure on tropical reefs (Dunlap & Shick 1998). The complete biosynthetic pathway for producing these compounds has not yet been elucidated (Singh et al. 2007), but it is believed to be derived from the shikimate pathway, which is used to synthesize aromatic amino acids (phenylalanine, tyrosine, and tryptophan). Two transcripts in the shikimate pathway were elevated during the day, *shikimate dehydrogenase* and *chorismate synthase*. It should be noted that the enzymes coded by these genes are not specifically believed to
be involved in MAA biosynthesis, but are farther downstream in the shikimate biosynthetic pathway (Singh et al. 2007).

Proteolysis & apoptosis

A number of proteases were moderately upregulated during the day, including plasminogen, pepsin A (an aspartyl protease), and three papain-like cysteine proteases (cathepsin K, thiol proteinase SEN102, and digestive cysteine proteinase 2). Increased proteolysis is likely to be a response to increase of oxidized proteins during the day. Papain-like cysteine proteases, which have been shown to trigger apoptosis in the freshwater dinoflagellate Peridinium gatunense (Vardi et al. 2007), may also activate apoptosis in Symbiodinium. Another type of cysteine protease known to activate apoptosis, metacaspase, was DE; however, one transcript was upregulated and one transcript was down-regulated during the day.

Conclusions

Using RNA-seq whole-transcriptome sequencing, I quantified in hospite gene expression of Symbiodinium associated with the Caribbean staghorn coral, A. cervicornis. By comparing transcription profiles of Symbiodinium between branch tips and branch bases, I found over 50 DE genes, the majority of which were up-regulated in tips. Among these, the most prominent biological function was photosynthesis. All photosynthesis transcripts up-regulated in tips were related to the light reactions, while Rubisco was down-regulated in tips. By comparing gene expression between day and night in the branch bases, I identified over 400 DE transcripts; however, the magnitude of gene expression change was much smaller than that previously observed between day and
night in the host coral. The most prominent patterns of genes DE between day and night were related to photosynthesis and translation, while some smaller differences were observed for genes involved in redox homeostasis and protein degradation.

The results for colony position suggest that *Symbiodinium* located in branch tips may have a greater individual photosynthetic capacity, compared to those in branch bases, while Rubisco may be more highly expressed in branch bases to compensate for higher rates of photorespiration in a more oxygenated environment. The results for time of day support diel transcriptomic regulation of some photosynthesis genes involved in the light reactions, Rubisco and thylakoid membrane integrity. Down-regulation of multiple reaction center chlorophyll binding protein genes during the day suggests a transcriptional mode of photoacclimation to prevent excessive absorption of light that may lead to ROS production and photoinhibition. The *psbA* gene, coding for the PSII D1 reaction center protein, was the most highly expressed transcript in the dataset, but was only DE by colony position. This suggests that transcription of this gene can be regulated under varying environmental conditions, yet other modes of post-transcriptional regulation are employed to regulate abundances of this protein under the normal variation between day and nighttime conditions. Transcripts for the redox response protein thioredoxin were up-regulated during the day and may be involved in ROS stress response and redox signaling pathways, including light-responsive regulation of transcription and translation of photosynthesis-related genes.
Availability of supporting data


Acknowledgements

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Table 1. DE transcripts for three comparisons.

<table>
<thead>
<tr>
<th>Model</th>
<th>Factor</th>
<th>Samples used</th>
<th>Highest exp.</th>
<th>Total</th>
<th>Annot.</th>
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<td>Colony position</td>
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<td>Tip</td>
<td>47</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Base</td>
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<td>4</td>
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<td></td>
<td>Time of day</td>
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<td>Day</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Night</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Both factors</td>
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</tr>
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<td></td>
<td>Interaction</td>
<td>All</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-factor</td>
<td>Colony position</td>
<td>Day samples</td>
<td>Tip</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Base</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Night samples</td>
<td>Tip</td>
<td>18</td>
<td>9</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td>Base</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Time of day</td>
<td>Tip samples</td>
<td>Day</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Night</td>
<td>0</td>
<td>0</td>
</tr>
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<td></td>
<td>Base samples</td>
<td>Day</td>
<td>336</td>
<td>228</td>
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<td></td>
<td></td>
<td>Night</td>
<td>107</td>
<td>73</td>
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</table>
Table 2. Photosynthesis genes DE in at least one comparison. The treatment with highest expression is indicated. Abbreviation column includes location of genes known to be located in the nucleus (n) or on minicircles in the chloroplast (m) (Leggat et al. 2011b).

<table>
<thead>
<tr>
<th>Function</th>
<th>Gene</th>
<th>Abbr.</th>
<th>'2-factor'</th>
<th>'1-factor'</th>
<th>Colony Position</th>
<th>Daytime samples</th>
<th>Nighttime samples</th>
<th>Base samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calvin cycle</td>
<td>Rubisco</td>
<td>Rbl (n)</td>
<td>base</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>night</td>
<td></td>
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<tr>
<td>PSI</td>
<td>P700 chlorophyll a apoprotein A1</td>
<td>psaA (m)</td>
<td>-</td>
<td>tip</td>
<td>-</td>
<td>-</td>
<td>night</td>
<td></td>
</tr>
<tr>
<td>PSI</td>
<td>P700 chlorophyll a apoprotein A2</td>
<td>psaB (m)</td>
<td>-</td>
<td>tip</td>
<td>-</td>
<td>-</td>
<td>night</td>
<td></td>
</tr>
<tr>
<td>PSI</td>
<td>iron-sulfur center</td>
<td>psaC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>day</td>
<td></td>
</tr>
<tr>
<td>PSI</td>
<td>reaction center subunit IV / IVA</td>
<td>psae/</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>day</td>
<td></td>
</tr>
<tr>
<td>PSI</td>
<td>reaction center subunit III</td>
<td>psaf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>day</td>
<td></td>
</tr>
<tr>
<td>PSI</td>
<td>D1 reaction center/ Q(B)</td>
<td>psbA (m)</td>
<td>tip</td>
<td>tip</td>
<td>tip</td>
<td>-</td>
<td>-</td>
<td></td>
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<tr>
<td>PSI</td>
<td>CP47 chlorophyll apoprotein</td>
<td>psbB (m)</td>
<td>-</td>
<td>tip</td>
<td>-</td>
<td>-</td>
<td>night</td>
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<tr>
<td>PSI</td>
<td>Cp43 chlorophyll apoprotein</td>
<td>psbC (m)</td>
<td>tip</td>
<td>tip</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>D2 reaction center</td>
<td>psbD (m)</td>
<td>tip</td>
<td>tip</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PSI</td>
<td>cytochrome b559 subunit alpha</td>
<td>psbE (m)</td>
<td>tip</td>
<td>tip</td>
<td>tip</td>
<td>-</td>
<td>-</td>
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<tr>
<td>PSI</td>
<td>psbN</td>
<td>psbN</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>day</td>
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<td>PSI</td>
<td>12 kDa extrinsic protein</td>
<td>psbU</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>PSI</td>
<td>cytochrome c-550</td>
<td>psbV</td>
<td>-</td>
<td>-</td>
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<td>Fucoxanthin-chlorophyll a-c binding protein / F</td>
<td>fcp/fcpf</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>day</td>
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<td>-</td>
<td>-</td>
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<td>carotenochlorophyll a-c binding protein</td>
<td>ccac</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>night</td>
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<td>ATP synthase</td>
<td>subunit alpha</td>
<td>atpA (m)</td>
<td>tip</td>
<td>tip</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>ATP synthase</td>
<td>subunit beta</td>
<td>atpB (m)</td>
<td>tip</td>
<td>tip</td>
<td>tip</td>
<td>-</td>
<td>-</td>
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<td>subunit delta</td>
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<td>-</td>
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<td>Cyt. b6-f</td>
<td>cytochrome b6</td>
<td>petB (m)</td>
<td>tip</td>
<td>tip</td>
<td>tip</td>
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<td>-</td>
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<td>petD (m)</td>
<td>tip</td>
<td>tip</td>
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<td>cytochrome c6</td>
<td>petU</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>day</td>
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<td>thylakoid memb.</td>
<td>palmitoyl-monogalactosyldiac glycerol delta-7</td>
<td>FAD5</td>
<td>-</td>
<td>-</td>
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<td>-</td>
<td>night</td>
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<td>desaturase</td>
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<td>gun4</td>
<td>-</td>
<td>-</td>
<td>day</td>
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Table 3. GO biological process categories with multiple DE transcripts between day and night in branch bases.

<table>
<thead>
<tr>
<th>GO Term</th>
<th>GO code</th>
<th>Gene/Functional group</th>
<th>UniProt ID</th>
<th># DE transcripts</th>
<th>Log$_2$ (fold change)</th>
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<td>Translation</td>
<td>GO:0006412</td>
<td>40S ribosomal proteins (various)</td>
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<td>60S ribosomal proteins (various)</td>
<td>Multiple</td>
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<td>0.37 - 0.95</td>
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<tr>
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<td></td>
<td>Ubiquitin-40S ribosomal protein S27a</td>
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<td>Ubiquitin-60S ribosomal L40</td>
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<td>Cell redox homeostasis</td>
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<td>Protein disulfide-isomerase</td>
<td>Q9LJU2</td>
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<td>Thioredoxin domain-cont. prot. 9</td>
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<td>Glutamate-cysteine ligase cat. subunit</td>
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<td>Proteolysis</td>
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<td>0.7</td>
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<td>Multiple</td>
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<td>2</td>
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<td>Q949X0</td>
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<td>Shikimate pathway</td>
<td>Multiple</td>
<td>2</td>
<td>0.6 - 0.7</td>
</tr>
</tbody>
</table>
Figures

Figure 1. nMDS plot for sample transcription profiles for bases (squares) and tips (triangles), day (red) and night (blue). Stress = 0.06.
Figure 2. Scatter plots of whole transcriptome gene expression for ‘2-factor’ analysis. (A) bases vs. tips, positive is up in tips; and (B) day vs. night comparisons, positive is up during the day. DE transcripts are indicated in red ($P_{adj} < 0.05$).
Figure 3. Scatter plots of whole transcriptome gene expression for 1-factor:colony position analysis. (A) bases vs. tips in daytime samples; and (B) bases vs. tips in nighttime samples. DE transcripts are indicated in red ($P_{adj} < 0.05$).
Figure 4. Scatter plot of whole transcriptome gene expression for day vs. night in bases. DE transcripts are indicated in red ($P_{adj} < 0.05$).

Figure 5. Heat map of photosynthesis related genes DE between base and tip.
*indicates only significant in ‘2-factor’ analysis. †indicates DE for ‘2-factor’ and both ‘1-factor’ analyses. §indicates only DE for daytime samples in ‘1-factor’ analysis.
Figure 6. Heat maps for base (left) and tip (right) samples showing expression of *Symbiodinium* photosynthesis-related genes that were DE by time of day in the branch base comparison. * Indicates DE > 2-fold change in bases. Heat maps are based on log-transformed normalized counts. Normalization of expression counts and row min. and max. values were calculated for base and tip samples separately (i.e. base and tip heat maps are not directly comparable).
Figure 7. Photosynthesis diagram showing DE genes in branch base samples upregulated during the day (red) and at night (blue), and the D1 reaction center gene psbA (green), which was not DE, but is believed to be important in maintaining the coral/Symbiodinium symbiosis. Dotted lines indicate the movement of electrons (e-) and protons (H+) through the oxygen evolving complex (OEC), Photosystems (PSI & II), plastoquinone (PQ)/plastoquinol (PQH2), the cytochrome b6f complex, plastocyanin (PC), ferredoxin (Fd), ferredoxin-NADP reductase (FNR), and ATP synthase.
Chapter 5

Genetic Diversity and Connectivity in the Threatened Staghorn Coral (Acropora cervicornis) in Florida

Abstract

Over the past three decades, populations of the dominant shallow water Caribbean corals, Acropora cervicornis and A. palmata, have been devastated by white-band disease (WBD), resulting in the listing of both species as threatened under the U.S. Endangered Species Act. A key to conserving these threatened corals is understanding how their populations are genetically interconnected throughout the greater Caribbean. Genetic research has demonstrated that gene flow is regionally restricted across the Caribbean in both species. Yet, despite being an important site of coral reef research, little genetic data has been available for the Florida Acropora, especially for the staghorn coral, A. cervicornis. In this study, I present new mitochondrial DNA sequence data from 52 A. cervicornis individuals from 22 sites spread across the upper and lower Florida Keys, which suggest that Florida’s A. cervicornis populations are highly genetically interconnected (F_{ST} = -0.081). Comparison between Florida and existing mtDNA data from six regional Caribbean populations indicates that Florida possesses high levels of standing genetic diversity (h = 0.824) relative to the rest of the greater Caribbean (h = 0.701 ± 0.043). I find that the contemporary level of gene flow across the greater Caribbean, including Florida, is restricted (Φ_{CT} = 0.117), but evidence from shared

haplotypes suggests the Western Caribbean has historically been a source of genetic variation for Florida. Despite the current patchiness of *A. cervicornis* in Florida, the relatively high genetic diversity and connectivity within Florida suggests that this population may have sufficient genetic variation to be viable and resilient to environmental perturbation and disease. Limited genetic exchange across regional populations of the greater Caribbean, including Florida, indicates that conservation efforts for *A. cervicornis* should focus on maintaining and managing populations locally rather than rely on larval inputs from elsewhere.
Introduction

Coral reefs have declined rapidly over the past three decades, due in large part to the loss of dominant reef-building corals (Gardner et al. 2003; Mumby & Steneck 2008). A major factor contributing to the decline of coral reefs has been the rise in coral diseases, particularly in the Caribbean, which is now regarded as a “disease hot spot” (Weil 2004; Harvell et al. 2007). White band disease (WBD), in particular, has transformed Caribbean reefs by causing an unprecedented die-off of the two dominant shallow-water Caribbean corals, the staghorn coral (*Acropora cervicornis*) and the elkhorn coral (*A. palmata*). Since WBD was first observed in the late 1970s, record losses of up to 95% of live *Acropora* cover have been observed throughout the Caribbean (Green & Bruckner 2000; Miller et al. 2002), and recovery has been slow to non-existent at most locations over the past two decades (Aronson & Precht 2001) (but see Grober-Dunsmore et al. 2006; Zubillaga et al. 2008). As a result, both species have been listed as threatened under the U.S. Endangered Species Act (Oliver 2005; Hogarth 2006) and as critically endangered under the International Union for the Conservation of Nature (IUCN) Red List criteria (Carpenter et al. 2008).

One key to designing appropriate management strategies and conserving the remaining Caribbean *Acropora* is knowledge about the extent to which populations of each species are interconnected via larval dispersal. Genetic exchange over large spatial scales (i.e. hundreds of kilometers) might allow distant healthy populations to rescue damaged reefs; whereas, restricted gene flow would indicate that populations rely on local recruitment and require local management. Information about the genetic make-up of Caribbean *Acropora* populations is also important since both species rely heavily on
asexual fragmentation to propagate locally (Tunnicliffe 1981; Highsmith 1982) but must reproduce sexually during yearly mass spawning events to produce dispersing larvae (Szmant 1986; Vargas-Angel & Thomas 2002). Because both species are largely self-incompatible (Baums et al. 2005a; Fogarty N, Vollmer SV, unpublished data), successful sexual reproduction requires that multiple genets are present and spawn on a reef. While genetic surveys indicate that multiple genets are often present in stands of both species (Baums et al. 2006a; Vollmer & Kline 2008), it is unknown if small remnant Acropora populations have too few individuals to spawn consistently and successfully. The genetic make-up of Acropora populations may also affect their resiliency. For example, recent research indicates that 6% of A. cervicornis individuals are resistant to WBD (Vollmer & Kline 2008), suggesting that populations with higher frequencies of resistant individuals may be more sustainable.

Recent genetic research on both Caribbean Acropora species indicates that gene flow is geographically restricted among populations separated by 500 km or more (Baums et al. 2005b; Vollmer & Palumbi 2007). Microsatellite data further indicate that A. palmata can be subdivided into distinct Western and Eastern Caribbean subpopulations (Baums et al. 2005b; 2006b), and mitochondrial and nuclear sequence data from A. cervicornis detected fine-scale genetic differences among populations separated by as little as 2 km (Vollmer & Palumbi 2007). Regionally restricted gene flow in the Caribbean Acropora argues for regionally-based management (Baums et al. 2005b; Vollmer & Palumbi 2007), but evidence for additional fine-scale differentiation in A. cervicornis suggests that the scale of dispersal and thus management at some locations may need to be much smaller (i.e. on the order of individual reefs) in this species.
(Vollmer & Palumbi 2007). Genetic studies of Indo-Pacific Acroporids have found evidence for population structure as well, but at a much larger geographic scale and generally of a smaller magnitude (Ayre & Hughes 2000; Mackenzie et al. 2004; but see Benzie et al. 1995; Ayre & Hughes 2004).

One area where population genetic information from the Caribbean Acropora is lacking is the Florida Keys reef tract, which is the largest continuous barrier reef in the U.S. and a focal point for U.S. coral research in the Caribbean. The Florida Keys reef tract sits downstream of most Caribbean reefs, which makes it a possible sink for immigrant larvae from upstream source populations (Roberts 1997). The predominant currents influencing larval transport into Florida’s reefs are the Florida Current and the Loop Current, which is derived from the Caribbean Current after its passage between the Yucatan peninsula and Cuba. Oceanographic models indicate that northern Central American and Cuban reefs are the most likely sources for larval immigration into Florida, although larval exchange between the Bahamas and Florida is also possible (Roberts 1997; Cowen 2006). Microsatellite data from A. palmata support the genetic relationship between Florida and Western Caribbean, clustering Florida in the Western Caribbean subpopulation with Panama and Mexico, as well as with the Bahamas (Baums et al. 2005b). DNA sequence data for A. cervicornis also suggest a genetic connection between the Western Caribbean and Florida, but too few samples (n = 5) were available to estimate the extent of population genetic structure between Florida and the greater Caribbean.
To date, the five genets from Florida used for the study by Vollmer and Palumbi (2007) represented the entirety of our knowledge about the genetic state of this *A. cervicornis* population. Florida is at the northernmost limit of this species in the Caribbean (Precht & Aronson 2004), and Florida’s *A. cervicornis* have a sparse and patchy distribution and have been heavily impacted by WBD (Miller *et al.* 2002; Williams & Miller 2005). Due to Florida’s location upstream of many other Caribbean reefs, genetic diversity of these corals might be influenced, and possibly increased, by receipt of immigrant larvae from upstream spawning populations (Roberts 1997). However, within the Florida Keys, exposure to differing environmental factors may contribute to isolation of its populations. For example, the middle Keys reefs are exposed to higher inputs of water from Florida Bay, while the upper Keys, closer to the mainland, are subjected to more intensive terrestrial and anthropogenic influences (Murdoch & Aronson 1999). If local recruitment is the primary source of *A. cervicornis* throughout the Keys, which is possible given the observation of genetic isolation over distances as short as 2 km in other *A. cervicornis* populations (Vollmer & Palumbi 2007), one would expect to see genetic differentiation within the 200 km long Florida Keys reef tract.

Here I use mitochondrial DNA sequence data from 52 *Acropora cervicornis* individuals from 22 sites across the Florida Keys to evaluate the population genetic structure within the Florida Keys and compare these data to published sequence data from across the greater Caribbean. Genetic comparisons between Florida and the rest of the Caribbean allow us to estimate genetic connectivity within Florida and between Florida and the greater Caribbean and to evaluate the genetic diversity within Florida relative to other Caribbean populations. Based on oceanographic models and genetic data
from *A. palmata*, I hypothesize that the Florida Keys reef tract is a sink for *A. cervicornis* larvae and genetic diversity from upstream sources, predominantly the Western Caribbean. I also evaluate the possibility of local recruitment and genetic structure within the Florida Keys reef tract, given previous observations of genetic structure in *A. cervicornis* over distances as small as 2 km (Vollmer & Palumbi 2007).

**Materials and Methods**

**Sampling and Data Collection**

For this study, 52 mtDNA sequences were produced from staghorn corals sampled from 22 populations spread across the Florida Keys. *A. cervicornis* specimens were collected July-September 2008 from 22 sites spanning the Florida Keys from southwest of Key West to Key Largo (Table 1, Figure 1), a distance approximately 200 km long. Each site represents a patch of *A. cervicornis* individuals. One to six corals were sampled per site (i.e. staghorn coral patch), which reflects the biological reality of the typically small patch sizes within Florida. Tissue samples were collected by sampling a small (1 cm) branch tip from staghorn coral colonies spaced at least 10 meters apart to minimize collection of clones produced by asexual fragmentation within each patch (Vollmer, in prep). Tissue samples were preserved in Chaos DNA extraction buffer and stored at room temperature. Extraction of DNA from the samples was conducted using a modified phenol-chloroform procedure (Fukami *et al*. 2004). The dataset used here also includes published data from Vollmer and Palumbi’s (2007) Caribbean-wide population genetic survey of *A. cervicornis*, consisting of mtDNA sequence data from 148 individuals from six geographic regions plus Florida (Figure 1): the Bahamas (n = 32 individuals), Turks
and Caicos (n = 32), Puerto Rico (n = 26), Curacao (n = 19), Belize (n = 12), Panama (n = 25), and Florida (n = 2). Eight additional sequences from Belize were also added to improve the previous sample size (from 12 to 20).

Population genetic analyses were conducted using the putative mitochondrial control region (van Oppen et al. 1999), a 941-bp fragment that has been shown to have high haplotype diversity and the ability to resolve population genetic structure in A. cervicornis across the Caribbean (Vollmer & Palumbi 2007). Three nuclear genes previously evaluated for A. cervicornis have few native alleles (2 in MiniCollagen and 1 in both Calmodulin and PaxC), and thus signatures of population structure are dominated by introgression (Vollmer & Palumbi 2007). Multiple microsatellite loci designed from A. palmata (Baums et al. 2005a) are also available for A. cervicornis; however, preliminary analyses of these loci indicate that they are confounded by introgression and homoplasy (S. Vollmer, pers. obs.). In order to avoid the confounding effects of introgressed alleles, I used mtDNA sequence data, which allowed for identification of introgressed and native haplotypes. Polymerase chain reaction (PCR) amplifications and DNA sequencing of the putative mtDNA control region were carried out according to (Vollmer & Palumbi 2002), and sequencing was performed on ABI sequencers (Applied Biosystems, Foster City, CA). Sequences were edited and aligned manually using Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI). Ends of the sequences were trimmed to a total sequence length of 814 base pairs. The region contained two informative insertion/deletion (indel) regions, which were coded as single base changes for population genetic analyses.

Due to the small sample sizes within Florida (n = 1-6 samples per site), it was not possible to examine population structure between each site, rather the sites were more
broadly classified into two regions, upper Keys (n = 22) and lower Keys (n = 30), for analyses of structure within Florida (Table 1). Samples were assigned to these regions based on the natural break in the dataset due to a distance of 80 km between the most southwestern upper Keys site (62) and most northeastern of the clustered lower Keys sites (53) (Figure 1); the one sample from site 54 was grouped with the lower Keys due to proximity. Upper and lower regions of the Keys are subjected to different environmental conditions, including currents and proximity to terrestrial and anthropogenic influences. While lower Keys reefs run west to east and may be influenced by the Pourtales Gyre (Lee et al. 1992), the upper Keys have a more north-south orientation and primarily experience the northeastward flow of the Florida Current. In addition, Florida Bay water delivered to the reef tract through channels in the middle Keys may influence coral and larval survival and reef connectivity between upper and lower Keys as well. However, the population structure analysis detected no population structure within the Florida Keys reefs (see results), and hence all Florida samples were treated as a single population for the Caribbean-wide population genetic comparisons.

It has been shown that Acropora cervicornis hybridizes and exchanges genes with its congener A. palmata (van Oppen et al. 2000; Vollmer & Palumbi 2002), and that the pattern of this introgressive gene flow is one-way from A. palmata into A. cervicornis (Vollmer & Palumbi 2002; 2007). This one-way gene flow allows for the identification of mtDNA haplotypes that are either introgressed (i.e. from A. palmata) or native to A. cervicornis (Vollmer & Palumbi 2002; 2007). Vollmer and Palumbi (2007) have shown that including introgressed genes in population genetic analyses of A. cervicornis obscures native population structure across the Caribbean, but adds to the genetic
structure between local populations (i.e. reefs) due to strong differences in introgression frequencies among local staghorn coral populations. To account for the differences between native mtDNA haplotype variation (i.e. reflecting intra-species gene flow only) and variation in the complete dataset including introgressed genes (i.e. reflecting intra- and inter-specific gene flow), I split the mtDNA data into two datasets for analyses: one complete dataset including all haplotypes (i.e. native and introgressed haplotypes) and one dataset including only native (non-introgressed) haplotypes. Native and introgressed haplotypes in the sampling were identified after Vollmer and Palumbi (2002, 2007).

Significance of differences in introgression frequencies between populations was compared using a G-test of independence (Sokal & Rohlf 1995).

Population Genetic Statistics

DNA sequence polymorphism for each population was characterized using DnaSP 4.0 (Rozas et al. 2003). A Statistical Parsimony Network was constructed in TCS version 1.21 (Clement et al. 2000). Haplotypes were identified as shared between two or more populations or as unique to a single population (private haplotypes), and introgression frequencies were calculated for each population as the percentage of haplotypes sampled identified as originating in the A. palmata lineage. Using the native haplotypes I tested for deviations from neutral expectations using standard tests: Tajima’s D (Tajima 1989b), Fu and Li’s D (Fu & Li 1993), and Fu and Li’s F (Fu & Li 1993). In addition, I ran mismatch analyses to detect signatures of population expansion or contraction against the null hypothesis of a constant-sized population and used coalescent simulations to test the significance of population size changes using the R² statistic (Ramos-Onsins & Rozas...
Population Genetic Structure

Hierarchical Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992) was conducted using Arlequin 2.0 (Schneider et al. 2000) to test for population genetic structure among four regions across the seven sampled populations. The regions were defined as follows: Western Caribbean (Belize and Panama), Eastern Caribbean (Curacao and Puerto Rico), Greater Bahamas (Bahamas and Turks and Caicos), and Florida. To estimate genetic structure between populations, pairwise $F_{ST}$ ($\Phi_{ST}$) values were calculated between each population. Significance was determined by 10,100 permutations and $P$-values were adjusted using sequential Bonferroni (Rice 1989). Genetic structure between regions within the Florida Keys (as stated above) was evaluated by calculating $F_{ST}$.

Results

Fifty-two control region sequences were produced for the Florida Keys, including 22 representing the upper Keys region, and 30 representing the lower Keys region (Table 2). In addition, two sequences from Florida and 146 sequences from six regional populations throughout the Caribbean from Vollmer and Palumbi (2007) were used in the population genetic analyses. Thirty unique mtDNA control region haplotypes were observed in the Caribbean-wide sample (Figure 2, Table 2). Seventeen haplotypes are native to $A. cervicornis$, whereas 13 haplotypes represent introgressed haplotypes from $A. palmata$ (resulting from interspecific hybridization) (Vollmer & Palumbi 2002; 2007). Seven native and four introgressed haplotypes were observed in the Florida Keys. The four native and two introgressed haplotypes found in the upper Keys were also found in
the lower Keys, and an additional three native and two introgressed haplotypes were present in the lower Keys sample.

Table 3 presents genetic diversity statistics for the native mtDNA haplotypes and includes the percentage of introgressed mtDNA haplotypes in each population sample. Florida had the highest proportion of introgressed haplotypes (61%) and no significant difference in introgression frequencies was detected between the lower and upper Keys (G = 0.26, df = 1, P > 0.5). The high frequency of introgressed haplotypes in Florida was matched only by Panama, with an introgression frequency of 60%. Other Caribbean populations have introgressed haplotype frequencies between 19% - 47%. Belize was an exception with no introgressed haplotypes (n = 20). Introgression frequencies of haplotypes varied significantly across the seven Caribbean samplings (G = 14.02, df = 6, P < 0.05).

Nucleotide and haplotype diversity values for the native mtDNA haplotypes were similar among populations across the greater Caribbean (Table 3). Bahamas had the lowest haplotype and nucleotide diversity (h = 0.574 and π = 0.00094), whereas Florida (h = 0.824 and π = 0.00242) and the two Central American populations (Panama, h = 0.800 and π = 0.00197; Belize, h = 0.847 and π = 0.00284) had the highest values. Within Florida, although the upper Keys had fewer haplotypes (n = 4) than the lower Keys (n = 7), when adjusted for sample size, both had similar levels of haplotype and nucleotide diversity (upper Keys h = 0.810, π = 0.00211; lower Keys h = 0.879, π = 0.00236). Theta values were comparable across Caribbean populations, and neutrality tests (Tajima’s D, Fu and Li’s F and D, mismatch analysis and R2) do not suggest significant deviations from neutral expectations including population bottlenecks or expansions, except in the
Bahamas and Turks and Caicos Islands where $R_2$ was significant.

Phylogeographic Distribution of mtDNA Haplotypes

The haplotype network shows the relatively high levels of mtDNA diversity of native (n) and introgressed (i) haplotypes and their distributions across the four Caribbean regions (Figure 2). For the native mtDNA haplotypes, a single haplotype (n11) was distributed across all four regions; this is the most likely ancestral native haplotype. Three native haplotypes were distributed across three regions; n1 was present in the Western Caribbean, Florida and Greater Bahamas, whereas n2 and n13 were present in Florida, the Greater Bahamas and Eastern Caribbean. The remaining 13 native haplotypes had geographically restricted distributions and were observed either in two geographic regions (five haplotypes: n4, n5, n8, n9, n14) or in a single region or population (eight haplotypes). Geographically restricted haplotypes tended to be more derived and should reflect contemporary genetic connections among regions. Of the five geographically restricted haplotypes shared across two of the four regions, one haplotype was shared between the Greater Bahamas and Eastern Caribbean (n4), two haplotypes were shared between the Western Caribbean and Greater Bahamas (n9 and n14), and two haplotypes were shared between the Western Caribbean and Florida (n5 and n8); the Western Caribbean and Eastern Caribbean lacked shared haplotypes. The remaining eight native haplotypes were observed only in a single region or population (i.e. private haplotypes). Four of these haplotypes were observed in Belize (n6, n7, n16 and n17), one in the Bahamas (n3), one in the Turks and Caicos (n19), and one in Florida (n18).

With respect to the phylogeographic distribution of the seven native haplotypes
observed in Florida, four haplotypes had broad geographic distributions (n1, n2, n11, n13), whereas the remaining three haplotypes had geographically restricted distributions. Of the geographically restricted haplotypes found in Florida, which are likely to reflect recent gene flow, two were shared between Florida and the Western Caribbean (n5 and n8), providing evidence of a link between these two regions. The other haplotype was exclusive to Florida (n18).

Thirteen introgressed mtDNA haplotypes were detected in _A. cervicornis_ across the Caribbean. Haplotype i2 was the most common introgressed haplotype and was found in all four regions; it is also the most common and ancestral haplotype in _A. palmata_ (Vollmer & Palumbi 2002). Haplotype i2 was the only introgressed haplotype observed in the Western Caribbean. Two introgressed haplotypes were found in two (i10) or three (i11) regions, whereas the remaining 10 introgressed haplotypes were found in only one region and often only in one population, including one introgressed haplotype found only in Florida (i13). The relatively high proportion of private introgressed haplotypes most likely reflects both the rarity of local introgression events (Vollmer & Palumbi 2002) and possibly the restricted geographic distribution of these haplotypes in _A. palmata_.

Regarding the four introgressed haplotypes found in the Florida sample, two haplotypes had broad geographic distributions (i2 and i11), and two were restricted to one (i13) or two (i10) regions. Haplotype i10 is a restricted haplotype shared between the Eastern Caribbean and Florida; however, this could reflect gene flow in either _A. cervicornis_ or _A. palmata_. Because of the high variance in introgression frequency across populations, the distribution of introgressed haplotypes may not accurately reflect gene flow among populations of _A. cervicornis_; rather, it may represent differential gene flow between _A.
palmata and A. cervicornis occurring in different regions.

AMOVA and Population Genetic Structure

Within Florida, due to the small sample sizes within sites, AMOVA and analysis of population structure at the level of reefs were not possible. To evaluate gene flow among the Florida Keys, collection sites were classified as upper Keys or lower Keys according to geography (Figure 1). No evidence for significant population structure between the upper Keys and lower Keys in either the complete dataset ($F_{ST} = -0.024, P = 0.66$) or the native only dataset ($F_{ST} = -0.081, P = 0.79$) was observed, and both upper and lower Keys had similar levels of introgressed haplotypes. As a result, the upper Keys and lower Keys samples were combined into a single Florida population for the Caribbean-wide population genetic analyses.

Hierarchical AMOVA was used to compare the levels of population genetic differentiation among regions ($\Phi_{CT}$), among populations ($\Phi_{ST}$), and among populations within regions ($\Phi_{SC}$). Both the complete dataset and native only dataset reveal strong and significant levels of genetic differences among populations ($\Phi_{ST}$, Table 4). For the native haplotypes, the AMOVA indicates that strong population genetic differences exist among the four regions, Western Caribbean, Eastern Caribbean, Greater Bahamas and Florida ($\Phi_{CT} = 0.117, P = 0.02$), but not among populations within regions ($\Phi_{SC} = 0.030, P = 0.11$). In contrast, AMOVA for the complete dataset indicates structure among populations within regions ($\Phi_{SC} = 0.176, P < 0.001$) but not among regions ($\Phi_{CT} = -0.048, P = 0.66$). This demonstrates that the inclusion of introgressed haplotypes in the analysis increases the variation among populations, but obscures the regional structure revealed in
the native haplotypes.

Pairwise $F_{ST}$ values show varying degrees of population genetic structure across the greater Caribbean in both the native and complete datasets. For native haplotypes, pairwise $F_{ST}$ across all comparisons ranged from 0 to 0.477 (mean ± SE = 0.141 ± 0.028). Curacao was the most distinct population with all pairwise comparisons being significant and four of six comparisons remaining significant after sequential Bonferroni correction. For the native dataset, significant population structure was observed between Florida and all other Caribbean populations ($F_{ST} = 0.117 ± 0.011$), except Panama ($F_{ST} = 0.042$); however, after sequential Bonferroni correction, none of the comparisons remained significant (Table 5). For the complete dataset, pairwise $F_{ST}$ across all comparisons ranged from 0 to 0.424 (mean ± SE = 0.133 ± 0.027). For this dataset, Belize was the most distinct population with four of six pairwise comparisons being significant after Bonferroni correction, largely due to the absence of introgressed haplotypes in the Belize sample. In the complete dataset, significant population structure was observed between Florida and three of the four Caribbean populations, but after sequential Bonferroni correction only two comparisons are significant (Belize and Turks and Caicos).

**Discussion**

The results demonstrate that significant genetic structure exists among *Acropora cervicornis* populations across the greater Caribbean (native Φ_{CT}), indicating that gene flow is restricted over regional scales (500 km or more). High levels of genetic differentiation detected in the native mtDNA haplotypes among the four Caribbean regions (native Φ_{CT} = 0.117) corresponds to a rate of inter-regional gene flow on the
order of 3.8 migrants per generation across the greater Caribbean (after Wright 1949).

The relatively large proportion of native mtDNA haplotypes with restricted geographic distributions [i.e. in only one or two regions (47% and 29%, respectively)] provides additional support for the regional genetic structure detected with F-statistics and AMOVA and reflects the limits on gene flow over large spatial scales.

The data indicate that Florida’s A. cervicornis population is not genetically depauperate and has similar levels of genetic diversity to regions elsewhere in the Caribbean. I detected no evidence for population structure within Florida (between the upper and lower Keys) in this dataset. However, I detected relatively high levels of genetic structure between Florida and other populations in the Caribbean (average native pairwise $F_{ST} = 0.105 \pm 0.016$). Panama was genetically most similar to Florida (native $F_{ST} = 0.042$), whereas Curacao was most different (native $F_{ST} = 0.157$). Native $F_{ST}$ values for all other populations with Florida were between 0.097 – 0.126. Florida and the Western Caribbean share geographically-restricted haplotypes, suggesting that the Western Caribbean has historically been an upstream source of genetic diversity for Florida.

Genetic Connections Between Florida and the Rest of the Caribbean

Evidence presented here for restricted gene flow in Acropora cervicornis across large swaths of the Caribbean support the previous findings of Vollmer and Palumbi’s (2007) multi-locus Caribbean-wide survey. In addition to the genetic structure among Caribbean populations observed in the previous study ($\Phi_{CT} = 0.249$) and supported here ($\Phi_{ST} = 0.143$), the data demonstrate significant structure among regional groupings
Despite this population structure, analysis of shared haplotypes indicates a historical and possibly on-going connection between *A. cervicornis* populations in the Western Caribbean and Florida, which Vollmer and Palumbi (2007) tentatively suggested with a small sample of Florida *A. cervicornis* (a total of five genets). While Florida, the Greater Bahamas and the Eastern Caribbean also share some haplotypes, strong genetic connections are not evident between these regions. Although the Bahamas is much closer to the Florida Keys (200 km) than the other populations, the strong Gulf Stream current appears to act as a barrier to gene flow. Shared haplotypes between Florida and the greater Caribbean are likely to be the result of gene flow to Florida, rather than from Florida into the other Caribbean regions due to prevailing currents (Hare & Walsh 2007). Yet, a protracted pelagic larval stage and favorable currents may allow for gene flow from the Florida Keys to the Bahamas (Cowen 2006).

Ocean currents support a link between the Western Caribbean and Florida (Cowen 2006), but coral reefs in Panama should be less interconnected with Florida than those of Belize based on dispersal routes and distances and the retaining influence of the Colombia-Panama Gyre (contrary to the data presented here). In addition, the transfer of genetic variation between Central America and Florida (1000 km from Belize and 1900 km from Panama) would likely require multiple generations of dispersal in a stepping stone fashion via intermediate populations. With a relatively short larval stage (~4 days, Vollmer SV, Fogarty N, unpublished data), the dispersal potential of *A. cervicornis* larvae should be on the order of tens of kilometers at most. Sharing of haplotypes between Western Caribbean and Bahamas (1200 km apart) may also be accomplished via a similar
route of connectivity along the reefs of Cuba (Cowen 2006). Larvae with such a short competency period still have a very low probability of surviving each leg of this journey. Thus, the phylogeographic connections in these data may reflect historical patterns of gene flow that occurred decades, centuries or longer ago in this species, which is both long-lived and able to propagate indefinitely through asexual reproduction (Botsford et al. 2009).

Significant differences in the frequencies of introgressed haplotypes among populations provides another strong indication that gene flow among these populations is geographically restricted. With free exchange of larvae among populations, introgression frequencies would homogenize, but the data demonstrate that this is not the case. The extremely high introgression frequencies in Florida and Panama distinguish these populations from elsewhere in the Caribbean, including the Greater Bahamas. While the similarity in introgression frequencies between Florida and Panama may be due to ongoing gene flow, I consider this unlikely given the distance and genetic structure of both populations with Belize, an intermediate population. It is possible that A. cervicornis in Panama and Florida share similar characteristics that favor introgressive hybridization, although it is not clear what those characteristics might be. The rarity of A. cervicornis in Florida could increase the likelihood of inter-specific fertilization, but this cannot explain the high introgression frequency in Panama, which has dense thickets of A. cervicornis.

Given the relatively short dispersal potentials of the Caribbean Acropora coral (3-5 days) and reef corals in general (Nishikawa et al. 2003; Wellington & Fitt 2003; Baums et al. 2006b), it is perhaps not a surprise that population genetic studies of a diversity of Indo-Pacific and Caribbean reef coral species indicate that gene flow tends to be
restricted over hundreds of kilometers (summarized in Vollmer & Palumbi 2007). Regionally restricted gene flow has been detected in both of the major reef-building Caribbean coral groups, *Acropora* (Baums et al. 2005b; Vollmer & Palumbi 2007) and *Montastrea* corals (Severance & Karl 2006). Caribbean-wide population genetic analyses for *A. palmata* using microsatellites have shown strong genetic structure across the greater Caribbean with a genetic break between the Western Caribbean and the Eastern Caribbean occurring at the Mona Passage between Puerto Rico and the Dominican Republic (Baums et al. 2005b; 2006b). Genetic data from *A. palmata* support a connection between Florida and Western Caribbean reefs (Baums et al. 2005b; 2006b) similar to *A. cervicornis* (Vollmer & Palumbi 2007). But unlike for *A. cervicornis*, in *A. palmata* there appears to be a strong genetic connection between Florida and the Bahamas (Baums et al. 2005b; 2006b). Microsatellites and RFLP analysis of two *Montastrea* species within the same genus revealed that one species, *M. annularis*, exhibits high population differentiation while the other, *M. faveolata*, appears panmictic between the Western Caribbean (Yucatan), Eastern Caribbean (Puerto Rico) and Florida (Severance & Karl 2006). Thus, in both the Caribbean *Acropora* and *Montastrea*, genetic data suggest that related coral species with similar life-histories and dispersal potentials can have contrasting population structures. Future research is needed to explain these differences.

Differing degrees of population genetic structure have also been detected in a variety of other Caribbean reef taxa and ascribed to a variety of causes. Within the Florida Keys, structure was observed in damselfish over a few meters and attributed to either a local genetic bottleneck (Lacson & Morizot 1991) or recruitment from
genetically divergent source populations, such as the Bahamas and Western Caribbean (Roberts 1997). Across the Caribbean, the significant population genetic structure detected in fishes including gobies (*Elacatinus* spp) (Taylor & Hellberg 2006), damselfish (*Stegastes partitus*) (Purcell *et al.* 2009) and wrasses (Rocha *et al.* 2005) has been explained by isolation by distance (Purcell *et al.* 2009) as well as differences in environmental factors (Rocha *et al.* 2005). Invertebrates have shown varying levels of genetic connectivity, from high genetic structure in an octocoral (*Pseudopterogorgia elisabethae*) (Gutiérrez-Rodríguez & Lasker 2004; Gutiérrez-Rodríguez *et al.* 2009) to almost no genetic structure in the economically important species queen conch (*Strombus gigas*) (Mitton *et al.* 1989) and spiny lobster (*Panulirus argus*) (Silberman *et al.* 1994).

Genetic Diversity and Connectivity Within Florida’s Staghorn Corals

The data revealed no significant population structure among *A. cervicornis* within the Florida Keys. This is surprising, given that the samples were collected over a distance of 200 kilometers and a range of environmental conditions. Previous research on *A. cervicornis* by Vollmer and Palumbi (2007) detected multiple instances in which *A. cervicornis* populations separated by 2 – 15 kilometers were genetically distinct. In Florida, however, no population genetic differences were detected between the upper Keys and lower Keys in either native or introgressed haplotypes. The absence of population genetic structure within Florida may indicate that gene flow is high across the Florida Keys reef tract, but alternate explanations are possible. In particular, barriers to gene flow between reefs may exist at a small geographic scale, but due to the limited sampling available from this diminished *A. cervicornis* population such fine-scale
analyses could not be applied. Similar haplotype frequencies between upper and lower Keys may also result from recent mortality due to WBD that may be exhibiting positive selective pressure on resistant genotypes (Vollmer & Kline 2008) and reducing local diversity; however, my analyses indicate relatively high diversity along the Florida Keys as a whole. While it is possible that highly polymorphic genetic markers, such as microsatellites, might reveal additional population genetic structure within the Florida Keys, preliminary microsatellite data for *A. cervicornis* indicate that no such structure exists (I. Baums, personal communication). Thus, it may well be the case that Florida is characterized by having high gene flow within the region.

It is not entirely clear why Vollmer and Palumbi (2007) detected such fine-scale differences among reefs elsewhere. Much of the fine-scale genetic differences in their dataset were driven by highly localized introgression signatures at one or more of the mtDNA or nuclear intron loci surveyed, including the putative mtDNA control surveyed here. Florida is distinctive for having the highest frequencies of introgressed mtDNA haplotypes detected to date across the greater Caribbean, but interestingly these high frequencies do not differ between the upper and lower Keys \( (G = 0.26, \text{df} = 1, P > 0.5) \). The absence of localized differences in introgression frequencies provides additional support for high gene flow across Florida Keys reef tract; however, the low abundance of *A. cervicornis* may have resulted in a higher proportion of hybrid recruits. Additional investigation of the geographic patterns of hybridization and introgression may shed light on this matter.

High gene flow across the Florida Keys is a possible indication that the genetic diversity present in this population is sufficient to allow sexual reproduction via
outcrossing. Sufficient genetic diversity and larval recruitment are essential for recovery of at risk populations of corals, and the results indicate that Florida’s genetic diversity of native haplotypes ($h = 0.824$) is comparable to, and even higher than, the rest of Caribbean ($h = 0.701 \pm 0.043$). In addition, while a number of haplotypes are regionally restricted, Florida’s $A. cervicornis$ population contains haplotypes found in all other regions. Historical recruitment from the Western Caribbean and other regions is one possible explanation for the relatively high diversity in Florida.

The relatively high genetic diversity and the results of tests for population size fluctuations (Tajima’s D, Fu & Li’s D, Fu & Li’s F, and $R_2$) do not indicate that there has been a significant loss of gene diversity (i.e. a genetic bottleneck) associated with the recent declines of $A. cervicornis$ in Florida due to WBD. This may not be a surprise given that it should take multiple generations of random genetic drift for population size reductions to be reflected in genetic diversity estimates (Tajima 1989a), especially in large populations. This could take many years in a clonal species with an indefinite life span. Even in species with short life spans, genetic diversity may not immediately reflect dramatic population size reductions. For example, Caribbean populations of the long-spined black sea urchin ($Diadema antillarum$), which suffered an analogous decline of up to 97% throughout the Caribbean as a result of disease in the early 1980s, also retained high genetic diversity in an mtDNA marker surveyed for individuals collected between 1987 and 1999 (Lessios et al. 2001). In both cases, it may take time before a genetic bottleneck is evident in genetic diversity and effective population size estimates.

The effective population size of $A. cervicornis$ in Florida can be estimated using the estimated theta value from the native mtDNA diversity and the equation $\Theta = 2N_e(\theta)\mu$ for
mitochondrial DNA [where $N_{e(f)}$ is the effective number of females, which is equivalent to $N_e$ (the effective population size) because *A. cervicornis* is hermaphroditic, and $u$ is the mutation rate per generation]. To determine the neutral mutation rate of the putative mitochondrial control region ($u$), I used the current estimated divergence time between *A. palmata* and *A. cervicornis* of 3.6-2.6 mya, which corresponds to 350,000 to one million generations ago using a generation time of 3-8 years (van Oppen *et al.* 2000). Based upon the presence of six diagnostic mutations between *A. palmata* and *A. cervicornis* sequences of 814 bp, I estimated a neutral mutation rate between $1.053 \times 10^{-8}$ and $3.686 \times 10^{-9}$ mutations per basepair per generation\(^2\). Given the estimated theta (per site) for Florida (0.00171), this corresponds to an estimated range of effective population size of 81,200-232,000 individuals within the Florida Keys.

This estimate is far less than the 2007 abundance estimates of Miller *et al.* (2008) (Miller *et al.* 2008), which indicate 13.8 ± 12.0 million colonies of *A. cervicornis* in the Florida Keys. Clearly, more precise estimates of current census population sizes are needed. One reason for the discrepancy between census and effective population size estimates is that the effective population size reflects effective number of genets rather than ramets, and there are likely to be many ramets per genet in this asexually reproducing species. In addition, effective population size is often much smaller than the census size due to gender imbalance (not a factor in hermaphroditic species), variance in reproductive success (Hedrick 2005), fluctuating population size, population subdivision

\(^2\) In the published version of the manuscript, the mutation rate was incorrectly calculated as $2.106 \times 10^{-8}$ and $7.371 \times 10^{-9}$ mutations per basepair per generation. This was based on all six mutations occurring in one lineage, rather than three mutations per lineage. This resulted in an estimated effective population size of 40,600-116,000.
with frequent extinction and recolonization (Maruyama & Kimura 1980) or a combination of these factors (Avise 1994). However, due to the widespread evidence of recent population decline of *A. cervicornis* and the tendency of effective population size estimates to reflect the long-term average (harmonic mean) population size (Hartl & Clark 1997), this effective population size estimate may not significantly underestimate the true census size of genetically distinct colonies of this species in the Florida Keys.

The high standing genetic diversity in the Florida Keys is a hopeful sign for future resilience of *A. cervicornis* along these reefs, but over time the effect of genetic drift in a small population may result in a future genetic bottleneck. Even with relatively high genetic diversity, successful reproduction will occur at the level of individual reefs and requires that multiple genotypes are present. Future research should address the extent of localized genotypic diversity on Florida Keys reefs with additional sampling and long-term monitoring.

**Conclusions**

The significant levels of population structure detected between Florida and other regions in the Caribbean reveal that ongoing rates of recruitment to the Keys from reefs elsewhere are low. Restricted gene flow between Florida and other Caribbean populations indicates high dependence on local larval retention within the Florida Keys on the whole. Overall, the data suggest that the *A. cervicornis* in the Florida Keys comprise a unique population within the Caribbean and should be treated as a distinct management unit for conservation. Monitoring of genetic diversity should continue for the Florida Keys region as the effect of a genetic bottleneck may be lagging behind the observed decrease in
population size resulting from WBD. Furthermore, the genetic make-up may shift if
WBD takes a greater toll on disease susceptible genotypes (Vollmer & Kline 2008) or if
high recruitment of larvae occurs from elsewhere. Continued genetic analysis of
additional samples, as they become available, will help to reveal the extent of local
genotypic diversity and clarify whether barriers to gene flow exist between individual
reefs in the Florida Keys. Current data showing limited genetic inputs from the greater
Caribbean and gene flow within the Florida Keys suggest that the persistence of
populations of this important reef-building species in Florida in the immediate future will
depend on self-recruitment, and thus must be managed as a local resource.

Acknowledgements

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2008-006 and FKNMS-2007-061. I would like to thank Mark Chiappone and Steven
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Delaney). Thank you to Rasit Bilgin, Steven Miller, Sean Kent and two anonymous
reviewers for their comments on the manuscript.
Tables

Table 1. Florida sampling locations listed roughly southwest to northeast. Sites 53-65 are technically middle Keys, but have been grouped by proximity into either upper Keys or lower Keys.

<table>
<thead>
<tr>
<th>Region</th>
<th>Site #</th>
<th>Site location</th>
<th>Lat. (N)</th>
<th>Long. (W)</th>
<th>Acropora cervicornis samples</th>
</tr>
</thead>
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<td>Lower Keys</td>
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<td>Middle Ground</td>
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<td></td>
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<td>Middle Ground</td>
<td>24° 28.801’</td>
<td>81° 52.949’</td>
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<td>115</td>
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<td>81° 50.217’</td>
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<td>99</td>
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<td>81° 43.729’</td>
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<td>South of Duck Key</td>
<td>24° 42.993</td>
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Table 2. Native (n1-n19) and introgressed (i1-i14) haplotypes observed in each population in the Caribbean and in the upper (Up) and lower (Low) regions of the Florida Keys. * Indicates a haplotype observed in Vollmer and Palumbi (2007), but absent in this study.

<table>
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<th>Haplotype</th>
<th>Panama</th>
<th>Belize</th>
<th>Bahamas &amp; Caicos</th>
<th>Puerto Rico</th>
<th>Curacao</th>
<th>Florida</th>
<th>Total</th>
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<td>i14</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>0</td>
<td>15</td>
<td>6</td>
<td>7</td>
<td>8</td>
<td>33</td>
<td>84</td>
<td>18</td>
</tr>
</tbody>
</table>
Table 3. Diversity values for native haplotypes of an 814 basepair fragment of *A. cervicornis* putative Control Region. Total number of sequences analyzed per population ($N_t$), percent of total sequences found to be introgressed *A. palmata* haplotypes (%I), number of native haplotypes in the population used to calculate polymorphism statistics ($N_n$), Length of sequences (L), number of polymorphic sites (S), Number of haplotypes, Haplotype diversity ($h$), Nucleotide diversity ($\pi$), Theta per sequence ($\theta_{seq}$) and Theta per site ($\theta_{site}$) calculated from S, Tajima D, Fu & Li D, Fu & Li F and R$_2$.

<table>
<thead>
<tr>
<th>Population</th>
<th>$N_t$</th>
<th>%I</th>
<th>$N_n$</th>
<th>S</th>
<th>#hap</th>
<th>h</th>
<th>$\pi$</th>
<th>$\theta_{seq}$</th>
<th>$\theta_{site}$</th>
<th>Tajima D</th>
<th>Fu &amp; Li D</th>
<th>Fu &amp; Li F</th>
<th>R$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerto Rico</td>
<td>26</td>
<td>27</td>
<td>19</td>
<td>3</td>
<td>4</td>
<td>0.678</td>
<td>0.00128</td>
<td>0.85834</td>
<td>0.00105</td>
<td>0.57845</td>
<td>1.01467</td>
<td>1.02929</td>
<td>0.1735</td>
</tr>
<tr>
<td>Curacao</td>
<td>19</td>
<td>42</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>0.618</td>
<td>0.00107</td>
<td>1.02425</td>
<td>0.00126</td>
<td>-0.50634</td>
<td>-0.87363</td>
<td>-0.88004</td>
<td>0.2096</td>
</tr>
<tr>
<td>Belize</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>10</td>
<td>8</td>
<td>0.847</td>
<td>0.00284</td>
<td>2.8187</td>
<td>0.00346</td>
<td>-0.63045</td>
<td>-0.96607</td>
<td>-1.00748</td>
<td>0.1099</td>
</tr>
<tr>
<td>Panama</td>
<td>25</td>
<td>60</td>
<td>10</td>
<td>4</td>
<td>4</td>
<td>0.800</td>
<td>0.00197</td>
<td>1.41394</td>
<td>0.00174</td>
<td>0.50521</td>
<td>1.23914</td>
<td>1.1866</td>
<td>0.2000</td>
</tr>
<tr>
<td>Bahamas</td>
<td>32</td>
<td>47</td>
<td>17</td>
<td>4</td>
<td>5</td>
<td>0.574</td>
<td>0.00094</td>
<td>1.18318</td>
<td>0.00145</td>
<td>-1.08236</td>
<td>-0.66882</td>
<td>-0.8952</td>
<td>0.1042*</td>
</tr>
<tr>
<td>Turks &amp; Caicos</td>
<td>32</td>
<td>19</td>
<td>26</td>
<td>7</td>
<td>8</td>
<td>0.686</td>
<td>0.00135</td>
<td>1.83440</td>
<td>0.00225</td>
<td>-1.23319</td>
<td>-1.40617</td>
<td>-1.57613</td>
<td>0.0763*</td>
</tr>
<tr>
<td>Florida</td>
<td>54</td>
<td>61</td>
<td>21</td>
<td>5</td>
<td>7</td>
<td>0.824</td>
<td>0.00242</td>
<td>1.38976</td>
<td>0.00171</td>
<td>1.26153</td>
<td>0.37162</td>
<td>0.71852</td>
<td>0.1924</td>
</tr>
<tr>
<td>Lower Keys</td>
<td>30</td>
<td>56</td>
<td>12</td>
<td>5</td>
<td>7</td>
<td>0.879</td>
<td>0.00236</td>
<td>1.6557</td>
<td>0.00193</td>
<td>0.83404</td>
<td>0.56268</td>
<td>0.71557</td>
<td>0.1944</td>
</tr>
<tr>
<td>Upper Keys</td>
<td>22</td>
<td>68</td>
<td>7</td>
<td>4</td>
<td>4</td>
<td>0.810</td>
<td>0.00211</td>
<td>1.63265</td>
<td>0.00201</td>
<td>0.23902</td>
<td>-0.06863</td>
<td>0</td>
<td>0.2259</td>
</tr>
</tbody>
</table>

*P < 0.05
Table 4. AMOVA results showing levels of genetic structure among regions ($\Phi_{CT}$), among populations within regions ($\Phi_{SC}$), and among populations ($\Phi_{ST}$). Data were analyzed separately for combined native and introgressed haplotypes and for native haplotypes only. Significance tests are based on 10,100 permutations.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>Variance components</th>
<th>Variation (%)</th>
<th>Fixation Index</th>
<th>Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All haplotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Regions</td>
<td>3</td>
<td>-0.11</td>
<td>-4.85</td>
<td>$F_{CT} = -0.048$</td>
<td>0.638</td>
</tr>
<tr>
<td>Among Populations within Regions</td>
<td>3</td>
<td>0.42</td>
<td>18.43</td>
<td>$F_{SC} = 0.176$</td>
<td>0.000</td>
</tr>
<tr>
<td>Within Populations</td>
<td>201</td>
<td>1.98</td>
<td>86.42</td>
<td>$F_{ST} = 0.136$</td>
<td>0.000</td>
</tr>
<tr>
<td>Total</td>
<td>207</td>
<td>2.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native haplotypes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Among Regions</td>
<td>3</td>
<td>0.10</td>
<td>11.65</td>
<td>$F_{CT} = 0.117$</td>
<td>0.020</td>
</tr>
<tr>
<td>Among Populations within Regions</td>
<td>3</td>
<td>0.02</td>
<td>2.62</td>
<td>$F_{SC} = 0.030$</td>
<td>0.113</td>
</tr>
<tr>
<td>Within Populations</td>
<td>117</td>
<td>0.70</td>
<td>85.73</td>
<td>$F_{ST} = 0.143$</td>
<td>0.000</td>
</tr>
<tr>
<td>Total</td>
<td>123</td>
<td>0.82</td>
<td></td>
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</tr>
</tbody>
</table>
Table 5. Pairwise $F_{ST}$ between populations. Upper right calculated for the combined native and introgressed haplotype dataset, and lower left calculated with native *A. cervicornis* haplotypes. Significance tests are based on 10,100 permutations.

<table>
<thead>
<tr>
<th></th>
<th>Puerto Rico</th>
<th>Curacao</th>
<th>Panama</th>
<th>Belize</th>
<th>Bahamas</th>
<th>Turks &amp; Caicos</th>
<th>Florida</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puerto Rico</td>
<td>0.007</td>
<td>0.186**</td>
<td>0.114**</td>
<td>0.060</td>
<td>-0.003</td>
<td>0.177**</td>
<td></td>
</tr>
<tr>
<td>Curacao</td>
<td>0.213*</td>
<td>0.094*</td>
<td>0.246**</td>
<td>0.020</td>
<td>0.078</td>
<td>0.079*</td>
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<tr>
<td>Panama</td>
<td>0.106</td>
<td>0.338**</td>
<td>0.424**</td>
<td>0.008</td>
<td>0.233**</td>
<td>-0.020</td>
<td></td>
</tr>
<tr>
<td>Belize</td>
<td>0.139**</td>
<td>0.318**</td>
<td>-0.032</td>
<td>0.275**</td>
<td>0.078*</td>
<td>0.390**</td>
<td></td>
</tr>
<tr>
<td>Bahamas</td>
<td>0.104*</td>
<td>0.477**</td>
<td>0.053</td>
<td>0.102*</td>
<td>0.091*</td>
<td>0.018</td>
<td></td>
</tr>
<tr>
<td>Turks &amp; Caicos</td>
<td>0.074*</td>
<td>0.385**</td>
<td>-0.007</td>
<td>0.063*</td>
<td>-0.030</td>
<td><strong>0.223</strong></td>
<td></td>
</tr>
<tr>
<td>Florida</td>
<td>0.099*</td>
<td>0.157*</td>
<td>0.042</td>
<td>0.106*</td>
<td>0.126*</td>
<td>0.097*</td>
<td></td>
</tr>
</tbody>
</table>

* $P < 0.05$, ** $P < 0.01$, bold = significant after sequential Bonferroni adjustment.
Figures

Figure 1. Sampling locations of *A. cervicornis*. Sampling sites in the Florida Keys and inset map of the greater Caribbean with sampling locations from Vollmer and Palumbi (2007). Numbers correspond to site names in Table 1. Rectangle in Caribbean map indicates the location of the Florida Keys.
Figure 2. Haplotype network of native (n1 – n19) and introgressed (i1 – i14) mtDNA haplotypes found in Florida, Western Caribbean, Greater Bahamas, and Eastern Caribbean regions. The haplotype sequences have been submitted to GenBank under accession numbers GQ863966-GQ863998. Circle is drawn proportional to the number of times each haplotype was observed in the Caribbean. * Indicates haplotype designated as ancestral in TCS network.
Evolution of Caribbean \textit{Acropora} growth forms

Sometime before 3 million years ago, a coral larva with a novel mutation colonized a shallow patch of sea floor. Probably it was successful in this new habitat because, as an adult colony, it had a robust morphology and could withstand the high energy waves that would have fragmented its parent species. Gradually, through many generations involving sexual reproduction and inbreeding, and the accumulation of additional mutations, the parent and daughter species became the distinctive looking corals that we now refer to as \textit{Acropora cervicornis} and \textit{A. palmata}. However, even millions of years later, these species are not completely isolated. Although \textit{A. palmata} has evolved some gametic incompatibility, \textit{A. cervicornis} has evolved no such barrier (Fogarty \textit{et al.} 2012). The two species still share enough similarity to hybridize, producing \textit{A. prolifera}, with occasional introgression of \textit{A. palmata} alleles into the \textit{A. cervicornis} population.

In chapter 2, I asked the question: in two closely related but distinctive looking coral species, what genes are responsible for constructing their highly different morphologies? Using one of the most cutting edge genetics tools available for looking at gene expression in non-model organisms, ‘next-generation’ Illumina RNA sequencing, I discovered that the answer is complicated. My approach to determining the genes involved in growth form was based on the idea that genes involved in growth should be differentially expressed between the two functionally different regions of the \textit{Acropora}}
colony, the apical tips (including the axial polyp) vs. branch bases. Comparing transcription both within colony and between species indicated that there is a large degree of genetic division of labor (DOL) within the Caribbean *Acropora*. Over 2200 transcripts were differentially expressed (DE) between base and tip within colonies, and a similar number were DE between species. Over 300 transcripts showed gene expression patterns dependent on both of these factors, indicating genes that may be involved in determining the differing morphologies between species.

Most of the base vs. tip gene expression differences were conserved between species, indicating that many genes are used in the same way to establish within-colony DOL, for both species. These include genes involved in developmental signaling (Wnt, Notch and BMP), carbonic anhydrase, ion transport, extracellular matrix (ECM) and putative skeletal organic matrix production. That these gene expression patterns are shared between species indicates that other *Acropora* corals may share these same modes of genetic regulation of growth. Further studies in Indo-Pacific *Acroporas* could reveal whether corals in this much more diverse species complex, which may have diverged from the Caribbean *Acropora* about 12 million years ago (van Oppen *et al.* 2001), share similar genetic DOL.

In the Caribbean *Acroporas*, I found many genes DE between species; however, I did not focus on differences only occurring between species because they are less likely to be involved specifically in growth and morphology. In addition, differences found only between species samples may be attributable to environmental differences between the habitats where the samples were collected.
Focusing on genes that were expressed differently both within the colony as well as between species, I found that developmental signaling, bicarbonate transport and ECM may be involved in the divergent growth forms of *A. cervicornis* and *A. palmata*. In particular, a number of genes with potential roles as Wnt pathway regulators were upregulated in tips of either species. I hypothesize that Wnt regulation is highly important for determining polyp development and that specific genes, including $\text{cthrc1}$, $\text{apolipophorins}$, $\text{lrp1B}$, $\text{fzd6}$ and $\text{sox9}$, may be particularly important in maintaining the single or few axial polyps per branch in *A. cervicornis* versus multiple fused axial polyps in *A. palmata*. Furthermore, the bicarbonate transporter $\text{SLC4A10}$ may be important in determining $\text{CaCO}_3$ deposition differences between these species. ECM proteins identified in this analysis may also be important regulators of skeleton formation, as a few have previously been isolated from coral skeleton (*hemicentin*, *protocadherin Fat 2* and *S. esculenta* cephalotoxin) or are involved in biomineralization in barnacles (*lectin BRA-3*). However, due to the large number of DE genes and the scale at which I observed differential expression (organismal level rather than for distinct tissues), further research is needed to explore the roles of these candidates in corals.

It will be particularly interesting to determine whether any of these same genes are involved in determining the morphotype of the hybrid *A. prolifera*, which exhibits a range of intermediate colony forms, but more closely resembles its paternal parent (Vollmer & Palumbi 2002). One possibility may involve activation or inhibition of Wnt signaling by localized maternal Wnt or Dkk RNAs during development, which has an important role in vertebrate axis specification (Tao *et al*. 2005; Cha *et al*. 2008). Localized maternal Wnt and fzd RNAs appear to be critical in proper axial patterning in
the hydrozoan *Clytia* (Momose *et al*. 2008), and possibly in *Hydra* (Fröbius *et al*. 2003), indicating that Wnt maternal effects on development of multicellular organisms are an ancient mechanism of genetic regulation. For Indo-Pacific *Acropora*, divergent morphologies do not correspond with apparent evolutionary history, based on the nuclear *Pax-C* intron and mitochondrial putative control region, which may be the result of hybridization or convergent evolution of growth form (van Oppen *et al*. 2001). Through comparative gene expression experiments, it would be interesting to determine whether similar growth forms result from differential expression of the same or different sets of genes.

Going forward, I suggest two research avenues that can be conducted with currently available techniques that can shed additional light on genetic evolution and control of growth form in these colonial, calcifying organisms. 1. Evaluation of gene expression in a similar manner (branch bases vs. tips) for additional *Acropora* species, including the Caribbean hybrid, *A. prolifera*, as well as Indo-Pacific *Acropora*, and 2. Sequencing of candidate genes, including up-stream regulatory regions and intronic sequences to evaluate mutations that may affect their expression differently between species. A third potential research direction is aquarium-based experimental manipulation of gene expression in *A. cervicornis* fragments, including inhibition of the Wnt signaling pathway. Such experiments may be possible with the development of appropriate techniques of RNA interference for colonial corals. Corals and other anthozoans appear to have the molecular tools to carry out RNAi-mediated inhibition of targeted genes (Grimson *et al*. 2008; Liew *et al*. 2014; Moran *et al*. 2014), and RNAi manipulation of gene expression has been successful in the solitary polyps of the symbiotic anthozoan
anemone *Aiptasia* (Dunn *et al.* 2007a; b). However, no such experiments have yet been conducted in colonial corals, to the best of my knowledge.

**Daily patterns of gene expression in the coral host**

Based on coral growth literature indicating significant differences in how corals calcify in day compared to night, in chapter 3 I examined differences in gene expression in *A. cervicornis* between day and night, with a focus on differences that also varied between the different regions of the colony (branch bases vs. tips). The full-transcriptome sequencing method provides a broad picture of how many processes are occurring within the coral at the same time. While this study was conceived to evaluate the genetic effect of light-enhanced calcification (LEC), results indicated that differences between day and night include differential expression of genes involved in circadian and light-response (photoreceptors, DNA photo-lyase, and GFP-like proteins), ROS response, nitrogen and lipid metabolism, and carbohydrate transport. Compared to the vast differences in gene expression found between branch bases and tips, there was a relatively modest transcriptional difference in gene expression between noon and mid-night.

The most intriguing findings of this study were revealed through the transcripts showing an interaction effect between the main factors of the statistical model (colony position and time of day). I found that the magnitude of gene expression difference between day and night for some genes varies between branch bases and tips, suggesting that the coral experiences day and night differently at different points within the colony. In particular, multiple circadian-related genes (*Amcry1* and *tef*) show greater up-regulation of expression during the day in branch tips than bases, which may be related to
the effect of *Symbiodinium* on the oxidative state of the cells. The iron homeostasis and oxidative stress response gene *hebp2* exhibited a similar expression pattern to *Amcry1* and *tef*, supporting a possible relationship between oxidative state and circadian regulation in corals, which has been proposed previously (Levy *et al.* 2011). The other point of interest is genetic evidence that the coral is using the nitrogen metabolism gene *glutamine synthetase* to restrict nitrogen availability to *Symbiodinium* in the branch bases during the day.

Results of this study hint at processes that will require further investigation through analysis of additional sampling time points, as well as evaluation in controlled light conditions. For example, to evaluate circadian regulation of genes requires subjecting the samples to continuous light and continuous dark conditions to rule out the effect of light-regulated expression. Furthermore, to investigate whether the oxygen content of polyps in branch bases compared to tips affects expression of putative circadian genes, characterization of the natural variability of cellular oxygen content during day and night in both branch bases and branch tips is necessary. Additional experiments that manipulate oxygen concentration of the circulating seawater may also reveal transcriptional effects on circadian genes, if such a hypothesis is true.

**Symbiodinium transcription**

In chapter 4, I investigated the gene expression of *Symbiodinium* living within the *A. cervicornis* samples whose gene expression was evaluated in chapter 3. *Symbiodinium* exhibited over 400 transcripts DE between day and night, which is comparable in number to the transcripts DE between day and night in the coral host. However, the magnitude of
expression difference was much lower in *Symbiodinium*, suggesting post-transcriptional regulation. Approximately 50 transcripts also differed in expression based on location within the coral colony, branch base vs. branch tip. This indicates that *Symbiodinium* function differently within the coral, possibly in response to conditions created by the coral host to control algal population growth in branch bases.

Patterns of DE gene expression indicate transcriptional regulation of some photosynthesis genes between day and night. In particular down-regulation of some genes involved in the light reactions of photosynthesis suggest that photoacclimation may occur on a diel basis to prevent overloading of photosystems during light hours. Multiple chloroplast genes with a potential role in thermal tolerance (*FAD5, psae* and *fcpf*) also showed differences in expression between day and night. Interestingly, the *psbA* gene, which codes for the PSII D1 reaction center protein, and is a likely candidate for resistance to thermal stress, was not DE between day and night, but was DE between bases and tips, and was the most highly expressed gene in the dataset. These results suggest that the *Symbiodinium* invest greatly in production of PSII D1, and that daily regulation of this protein under non-stressed conditions is post-transcriptional or post-translational, while transcriptional regulation may be employed under the differing environments of branch bases and tips. *Rubisco* and three reaction center protein genes showed gene expression patterns indicating both within-colony and diel variation. The patterns of expression for these genes suggest that differing environmental conditions within the different regions of the colony contribute to transcriptional regulation of these genes.
As with the transcriptional regulation observed for the host coral, genes whose expression appears to depend on both colony position and time of day may respond to differing environmental factors within the polyps, such as O$_2$ and CO$_2$ concentrations. Therefore, measurements of these intracellular parameters for both day and night in branch bases and tips can inform further gene expression studies for both coral and symbiont. In chapter 3, the expression pattern of *glutamine synthetase* suggested that the coral may limit *Symbiodinium* population growth by restricting nitrogen availability, and that this may be transcriptionally regulated. Experiments looking at gene expression of both coral host and *Symbiodinium* conducted under elevated nitrogen and CO$_2$ conditions may shed additional light on the basis of transcriptional regulation in both organisms.

**Genetic evaluation of Florida Keys *A. cervicornis***

In chapter 5, using a mitochondrial marker, I evaluated the genetic viability of *A. cervicornis* in the Florida Keys, a population that has been heavily impacted by white band disease. The Florida Keys are also the site of the most concerted ongoing restoration efforts for this species. Populations of *A. cervicornis* are regionally isolated across the Caribbean, with the haplotypes that are shared among multiple regions generally being more ancestral, and therefore representing historical gene flow. More derived alleles shared among regions suggest the most recent genetic input to the Florida Keys originated in the Western Caribbean (Panama and Belize) reefs; however, the time scale of this genetic contribution is unknown. Significant genetic differentiation between the Florida Keys and potential up-stream source populations indicates that these reefs must be managed at a local scale with care taken to sustain genetic diversity when implementing conservation and restoration efforts.
Despite the loss of many of Florida’s Acropora cervicornis corals, the remaining population contains relatively high genetic diversity compared to other areas in the Caribbean, even areas with healthy populations, such as Panama. This is a promising sign for ongoing and future restoration efforts, which should attempt to maintain this diversity through propagation of multiple genets. However, it should be cautioned that the genetic effect of the observed dramatic population decline in the Florida Keys may take time to be reflected in measures of genetic diversity. Within the Florida Keys, there do not appear to be significant barriers to gene flow; however, I cannot state this conclusively due to a low sample size at individual reefs. Continued monitoring of the genetic diversity of Florida Keys populations is recommended.
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barnacle Megabalanus rosa. Comparative Biochemistry and Physiology Part B:
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