Coral Skeleton Density Banding:
Biotic Response to Changes in Sea Surface Temperature

By

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Thesis
Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Geology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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ABSTRACT

Density bands in the CaCO$_3$ (aragonite) skeleton of scleractinian corals are commonly used as chronometers, where crystalline couplets of high and low density bands represent the span of one year. Isotopic analysis of these density bands provides a sensitive reconstructive tool for paleoclimatology and paleoecology. However, the detailed biotic mechanisms controlling coral skeleton aragonite nucleation and crystallization events and resulting skeletal growth rate remain uncertain. The coral tissue organic matrix, composed of macromolecules secreted by the calicoblastic ectoderm, is closely associated with skeletal precipitation and is itself incorporated into the skeleton. We postulate that density banding is primarily controlled by changes in the rate of aragonite crystal precipitation mediated by the coral holobiont response to changes in sea surface temperature (SST). To test this hypothesis, data were collected from coral skeleton-tissue biopsies (2.5 cm in diameter) extracted from four species of *Montastraea* growing on the fringing reef tract of Curaçao, Netherlands Antilles. Annual mean variation in SST on Curaçao range from 29° in mid-September to 26° C in late February. Samples were collected at strategic time periods spanning the 3° C annual variations in SST. Our nanometer-scale optical analyses of skeletal morphology have revealed consistent changes between high- and low-skeletal density bands, resulting in an 11% increase in the volume of aragonite precipitated in high-density skeletal bands. The re-localization and/or change in abundance of mucus, carbonic anhydrase (a molecule that catalyzes the hydration of carbon dioxide), calmodulin (a calcium-binding protein) and the change in density of gastrodermal symbiotic dinoflagellates has permitted estimates of seasonally-fluctuating carbon allocation by the coral holobiont in response to changing environmental conditions.
Acknowledgments

This project was funded by the Office of Naval Research (N00014-00-1-0609), two Leighton Awards and one Roscoe Jackson Fieldwork Scholarship from the UIUC Department of Geology, as well as by the Earth and Society Initiative in Disease Emergence and Ecosystem Health scholarship.

I would like to thank the members of the Fouke Lab, Amanda Oehlert, Alan Piggot, and Phil Miller for their assistance as my labmates and friends. In addition, the aid and expertise of Scott Robinson, Darren Stevens and Leilei Yin at the Beckman Institute of Advanced Technology, Mayandi Sivaguru, Glenn Fried, Donna Epps and Debbie Piper at the Institute for Genomic Biology, and Rob Sanford and Marilyn Whalen at the Department of Geology was essential to the success of the project. I must thank my adviser, Professor Bruce W. Fouke for the opportunity to work on this project and for his hard work and dedication to the project and to his students. Finally, I thank my family for their helpful conversations, encouragement, and interesting perspectives on this research.
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Scleractinian corals, or reef-building corals, serve as an ecological cornerstone for tropical and sub-tropical reef ecosystems (Veron, 2000). A universal characteristic of coral skeletal growth is the formation of density bands, micrometers to centimeters in thickness, where a crystalline aragonite couplet of high- and low-density bands constitutes one year of growth (Carricart-Ganivet et al., 2000; Knutson et al., 1972). Because of their observed regularity, coral skeletal density bands provide growth chronometry and thus serve as sensitive tools for the reconstruction of recent and ancient climate, sea surface temperature (SST) and ecology (Barnes and Lough, 1996; IPCC, 2007). Since the observations of annual density banding, studies have ensued to extract climatological data, such as SST, salinity, sediment loads, even light intensity or solar radiation from coral skeleton (Chalker and Taylor, 1975; Klein et al., 1993; Lough and Barnes, 2000; Muscatine et al., 1984). Furthermore, isotopic analyses of coral skeletal banding has also served as a primary data source for the Intergovernmental Panel on Climate Change (IPCC) reports, which predict an increase in global surface temperature of greater than 4°C over the next century (IPCC, 2007). Although coral skeletons are invaluable archives of climatological data and used ubiquitously as such, the influence of environmental factors on the precipitation of the aragonitic skeleton has not yet been deciphered because the exact mechanisms driving crystal growth remains unknown (Allemand et al., 2004; Cohen and McConnaughey, 2003).

Because coral skeletogenesis is not yet fully understood, the process of forming density bands within the skeleton is also controversial. High-density bands (HDB) are
thought to form during higher seasonal SST (July-September) while low-density bands (LDB) form during lower seasonal SST (October-June; Barnes and Lough, 1993; Carricart-Ganivet, 2007; Carricart-Ganivet et al., 2000). The thickness of each band is proportional to the amount of time within which they precipitate, resulting in thicker LDBs commonly punctuated by thinner HDBs (Carricart-Ganivet, 2004; Carricart-Ganivet, 2007; Cruz-Piñón et al., 2003). However, the regularity of band formation is somewhat controversial, where sub-annual bands have been observed in instances of extremely high SST (Worum et al., 2007). Although the change in density is an obvious qualitative observation through radiography density bands had yet to be quantitatively defined until this study (Allemand et al., 2004; Barnes and Lough, 1993; Carricart-Ganivet, 2007; Knutson et al., 1972; Swart et al., 1995).

Scleractinian corals are largely colonial invertebrates composed of polyps, the functional unit of a colony, which are each closely genetically related, if not identical (Veron, 2000). These hermatypic corals house photosynthetic dinoflagellates, called zooxanthellae, of the genus *Symbiodinium* (Chalker et al., 1986; LaJeunesse et al.). It is this relationship that both allows the coral to effectively resist degradation by wave action and borers and restricts the coral to shallow seas where sunlight is intense and SST remains above 18°C for the majority of the year, roughly ±32° latitude (Cohen and McConnaughey, 2003). Although ahermatypic corals also precipitate a skeleton through a similar process, the photosynthetic algae are implicated as contributors in skeletogenesis (Cohen and McConnaughey, 2003; Veron, 2000).

Coral polyps are interconnected via tissue called the coenosarc. Coral tissue is composed of three layers: ectoderm, a connective tissue layer called mesoglea,
endoderm (Fig. 4,5). The oral ectoderm is in direct contact with seawater, houses mucocytes, which excrete mucus to form a surface layer of mucus, and cnidocytes, which can be used as a form of defense or for passive carnivory (Peters, 1983). Oral endoderm, or gastroderm, houses the endosymbionts, the zooxanthellae.

Oral tissue and aboral tissue are separated by a space, the coelenteron (Peters, 1983). The aboral ectoderm is also called the calicoblastic epithelium, a thin layer of long, interdigitated cells that serve as the second barrier between the skeleton and the seawater, anchored to the skeleton by specialized cells called desmocytes (Allemand et al., 2004; Moya et al., 2008). This layer has been postulated to actively transport ions transcellularly or paracellularly, supplying the skeleton with the needed components for growth. Desmocytes have been implicated in the secretion of the organic matrix. An organic extracellular matrix (ECM) has been observed by Helman et al. 2008, who suggested this ECM, composed of proteoglycans, collagens, and adhesive glycoproteins, aided in intercellular adhesion, as well as cell-skeleton adhesion (Helman et al., 2008). Helman et al. identified acidic amino acids and sulfated polysaccharides as the major components the organic matrix, which is thought to be the organic template for the three-dimensional structure of the skeleton, possibly facilitating nucleation of aragonitic crystals. The organic matrix is undoubtedly a major controller of the rate and ultrastructure of the aragonite skeleton, but the nature and magnitude of its influence on the skeleton is poorly understood. In addition, the effect of abiotic environmental factors on the organic matrix composition and abundance has yet to be documented. It is secreted by the calicoblastic epithelium and is itself incorporated into the skeleton, comprising 0.01% of the dry weight of the skeleton (Helman et al., 2008; Veron, 2000).
Abiotic and Biotic Influence on Aragonite Precipitation

Coral skeletons have been argued to be primarily the product of either: 1) abiotic processes in SW such as temperature, and carbonate saturation state; or 2) biotic processes such as organic matrix, where all skeleton crystal bundles are encased in mucus and active transport of ions occurs across the calicoblastic epithelium, which creates a sink for respired carbon dioxide (CO₂; (Chalker and Taylor, 1975; Cohen and McConnaughey, 2003). The skeleton is composed of acicular bundles of aragonite, precipitating below the epithelium, called sclerodermites (Cuif and Dauphin, 1998; Gladfelter, 1983). The diameter of the aragonite needles ranges from several hundred nanometers to a few micrometers, and grow together in a three-dimensional fan formation around a center of calcification that have been found to be rich in sulfated polysaccharides and of organic origin (McConnaughey). The arrangement of these crystal bundles is similar to that of common inorganically grown spherulitic crystal morphologies (Barnes, 1970; Gladfelter, 1983; Gladfelter, 1984). This spherulitic morphology is consistent with crystal growth that occurs rapidly from a large temperature change. However, the coral skeletal system is isothermal, implicating a large change in saturation state of aragonite at the skeleton-tissue interface (Cohen and McConnaughey, 2003). This physicochemical model is further supported by the variation in needle width, where wider needles are exhibited by slower growing genera, and narrower needles precipitate from the faster growing genera of corals (Barnes, 1970). Other evidence of abiotic influence on coral skeletogenesis is that increased temperature increases the calcification, which could be an inorganic explanation for the increase in
skeletal density during warmer SST (Reynaud et al., 2007). These observations support the possibility of some characteristics of the coral skeleton are permitted through inorganic processes.

Other models propose the biological mediation of the precipitation of aragonite. The secretion of an organic matrix, a framework of glycoproteins, aspartic- and glutamic-rich proteins, and sulfated polysaccharides, by the calicoblastic epithelium may employ either inhibitory or facilitative control over nucleation events through molecular constituent variation (Helman et al., 2008). The presence of an organic substrate has been shown to increase the rate of aragonite precipitation both in mollusks and hot spring systems (Cohen and McConnaughey, 2003; Kandianis et al., 2008). Another indication of biologically controlled calcification is a light-enhanced calcification model, where diurnal cycling in the rate of precipitation of coral aragonite show an increase in calcification during the day, and a decrease, but not cessation, during the night. A Ca\(^{2+}\)-ATPase pump facilitates the transport of calcium ions (Ca\(^{2+}\)) across the calicoblastic epithelium, which shuttles Ca\(^{2+}\) into the calcifying space in exchange for two protons. This influence was supported through the observation of decreased [Ca\(^{2+}\)] when the Ca\(^{2+}\) ATPase enzyme was inhibited (Kingsley and Watabe, 1985). This pump is light activated and shuts down during the night. However, it has been implicated that the availability of Ca\(^{2+}\) in this process in a non-rate-limiting step, where changes in calcium concentrations instigate little change in the rate of precipitation in of coral skeleton (Tambutte et al., 1996). The Ca\(^{2+}\) cycling offers additional evidence for biotic control, where the saturation state of aragonite in the calcifying space beneath the calicoblastic epithelium can reach up to 100x that of normal SW, allowing precipitation of aragonite in
corals at an increased rate by a similar factor. Additionally, the metalloenzyme carbonic anhydrase (CA) has been implicated as a mediator of a rate-limiting step in a carbon-limited system, as coral skeletogenesis may be, by facilitating the hydration of CO$_2$ into HCO$_3^-$ (Tambutte et al., 1996). CA plays a similar role in many other organisms, exhibiting facilitative effects during calcification processes (Erez, 1978; Goreau, 1963; Jackson et al., 2007; Tambutte et al., 1996). Lastly, the isotopic composition of the coral skeletons indicates kinetic fractionation of oxygen and carbon, as opposed to fractionation due to isotopic equilibrium with SW (McConnaughey et al., 1997). Both $^{13}$C and $^{18}$O are depleted in the skeleton with respect to SW (Swart et al., 1995; Swart et al., 2004). The exchange of oxygen between H$_2$O and HCO$_3^-$ may be the slowest step in crystal-SW equilibrium and oxygen may fail to equilibrate during the rapid precipitation of aragonite (McConnaughey et al., 1997). The difference in isotopic composition between oxygen in the CaCO$_3$ of the skeleton and the H$_2$O of the SW implies that the carbonate ion was not supplied by SW, nor was there time enough to reach equilibrium (Adkins et al., 2003). $^{13}$C is also depleted in the skeleton with respect to SW, containing much less $^{13}$C than aragonite in equilibrium with dissolved inorganic carbon (DIC) in SW, likely due to the kinetic discrimination during conversion of CO$_2$ to HCO$_3^-$ (Adkins et al., 2003; McConnaughey et al., 1997; Swart et al., 1995). These observations suggest a biological influence on the precipitation of aragonite in the coral animal.

The purpose of this study was to quantify the effect of a biological response of corals to increased seasonal SST, an abiotic environmental factor, within the context of elucidating the process of density band formation in coral skeleton. First, the change in the ratio of aragonite to pore space between high- and low-density bands in Montastraea
*Faveolata*, a primary reef builder in the Caribbean Sea, was quantitatively defined for the first time. Second, the change in the abundance of 1) zooxanthellae, 2) endodermal and ectodermal mucus, 3) calmodulin (a calcium binding protein), and 4) the metalloenzyme CA was measured over a 1ºC seasonal SST change. Zooxanthellae and mucus within the coral tissue can be used to implicate changes in trophic strategy, carbon cycling, and availability of nutrients in the biotic micro-environment influencing coral skeletal growth (Anthony and Fabricius, 2000). The abundance and localization of calmodulin was quantified to describe the usage and abundance of calcium available for skeletogenesis, a process that has been suggested to be a passive and non-rate-limiting step (Tambutte et al., 1996). Finally, the change in abundance and localization of carbonic anhydrase was quantified over varying seasonal SST. In previous work, CA has been localized in the calicoblastic epithelium, but had yet to be quantitatively analyzed in terms of abundance or response to changing environmental or seasonal parameters until this (Moya et al., 2008; Tambutte et al., 2007a; Tambutte et al., 2007b). Lastly, a digital elevation map was constructed with the spatial resolution of two micrometers, resulting in a detailed three-dimensional digital rendering of polyp architecture. This technique was developed uniquely for this project. Each of the described components of the tissue was localized and quantified over seasonal changes in SST and analyzed in the conceptual framework of mechanistic control of skeletogenesis, as it is influenced by the coral response to changing seasonal SST.
CHAPTER 2
MATERIALS AND METHODS

Geobiological Setting

Curaçao, Netherlands Antilles, situated approximately 65km north of Venezuela in the Southern Caribbean Sea and just outside the hurricane belt, provides a 70-kilometer fringing reef tract that is optimal for in situ experimentation. Playa Kalki (12º06’33.44”N, 68º57’14.85”W), the sample site for this experiment, represents a location of pristine waters and is located on the northwest sector of the island (Fig 1). At 10m WD, photosynthetically active radiation is 18-22% at this site (Klaus et al., 2007). SST varies seasonally by 3ºC, ranging from approximately 26-29ºC, with a mean annual temperature of 27.5ºC ± 0.5ºC.

Experimental Design and Field Sampling

Skeleton-tissue biopsies (2.5cm in diameter, 2-3cm in depth) were collected in triplicate from Montastraea faveolata colonies that were “apparently” healthy and no smaller than 1 m in diameter. Apparently healthy corals were those that exhibited no discoloration of tissue or any obvious signs of disease such as necrotic tissue or fungal infection. All biopsy samples were taken from colonies at 10 to 12 m water depth (WD) within the reef crest sedimentary depositional facies on the leeward coral reef track of Curaçao (Frias-Lopez et al., 2002); Fig 2). This consistent contextual strategy within facies ensures that each coral head chosen for sampling has experienced a similar suite of physical, chemical, and biological environmental parameters during the course of their lifetimes.
Based on our qualitative observations across the island, the reef crest facies is also the environment where *M. faveolata* is most abundant. Each skeleton-tissue biopsy was collected by gently hammering a 2.5-cm diameter stainless steel C·S. Osborne & Company arch punch No. 149 (http://www.csosborne.com) approximately 2 cm into the surface the each coral head (Fig. 3). Considering each massive *M. faveolata* coral head as a globe, samples were consistently taken from a position that was at approximately 45° North latitude. The biopsy was transferred from the arch punch to sterile 50 ml polypropylene centrifuge tubes that were filled with seawater at depth. Biopsies were then stored at depth in these sealed seawater-filled centrifuge tubes for the duration of the sampling SCUBA dive, a time span that varied between 5 to 30 minutes. Upon return to the surface at the end of the dive, the triplicate biopsies were immediately immersed in one of the three fixatives and stored temporarily on ice. The fixatives included 70% ethanol, formalin (10% of 37% stock formaldehyde, 90% deionized water) and Carnoy’s solution (volumetric ratio of 6:3:1 of ethanol: chloroform: acetic acid). Those biopsies fixed in Carnoy’s solution were placed in an ice bath for 6 to 8 hours, after which the fixative was decanted and replaced with 70% ethanol, while those stored in formalin and ethanol were kept in a dark cooler. Biopsies remained in theses fixative while being shipped in a dark cooler from Curaçao to the University of Illinois.

**Radiography**

All biopsies were first first radiographed, in solution with tissue intact, to create a three-dimensional morphological map of coral skeleton density banding in each sample. Then, the skeleton of the biopsies fixed in ethanol was sectioned into five millimeter sections
using a rock saw, after all tissue had been removed by washing each biopsy in a 10% bleach solution. The sections of the aragonitic skeleton were radiographed using a large-animal digital x-ray instrument – need to add manufacturer and model name/number – at the Radiology Department of the Large Animal Clinic at the School of Veterinary Medicine of the University of Illinois, Urbana, IL. The biopsy was placed onto an x-ray focus plate at a 101.6 cm (40 in) focal film distance. Each biopsy was x-rayed at 70 killivolt-pascals (kVp) and 640 milliamps (mA) for an exposure time of 2 milliseconds (ms), which optimized contrast in the skeleton. These settings were determined by first radiographing three sections in triplicate at a variety of exposure times, powers and focal distances to bracket the optimal settings. The settings used were selected optically by determining which exhibited the best contrast between skeleton and background, and which preserved the best clarity of the digital image.

Decalcification and Sectioning of Soft Tissue

Classical histology techniques were combined with immunohistochemical methods to quantify the density of zooxanthellae and mucocytes (or mucin inclusions) in the gastroderm and ectoderm. These techniques are described in detail in Piggot et al. (2009) and briefly summarized here (Piggot et al., 2009). Biopsies were treated with 0.5 mM neutral buffered ethylenediaminetetraacetic acid (EDTA), which is a chelating agent used to decalcify coral tissue fixed in Carnoy’s solution and Formalin. Biopsies were placed in a beaker filled with EDTA and set on a shaker table to perturb the solution, which was changed every 12 hours until exclusively soft tissue remained. Once decalcified, the biopsies were rinsed in MilliQ water for 24 hours and then stored in 70%
EtOH at 4°C. After decalcification, the tissue was dehydrated through a series of abbreviated washes in ethanol and xylenes and then infiltrated with paraffin wax (Leica ASP 300 Tissue Processor, Leica Microsystems, Bannockburn, IL). The infiltrated biopsy was subsequently trimmed with a sterile scalpel and oriented with oral cavity upright and secured the ectoderm of the base of the polyp to a plastic processing cassette (Surgipath Medical Industries Inc., Richmond, IL) via slightly cooled paraffin. The base of each of the multiple polyps was gently pressed against the cassette, while the paraffin cooled and adhered each polyp to the cassette, to ensure that all were vertically aligned with one another. The polyps were then embedded in paraffin blocks to prepare them for 5-micron thick transverse and vertical tissue sectioning using a Leica RM2255 rotary microtome, during which the depth into the polyp was measured and recorded by the microtome during sectioning. This was done by establishing a depth of “0 µms” at the moment when the tissue was first optically detectable, via careful observation, in the sections of paraffin. Once established, the depth into the polyp was tracked and recorded as it was sectioned further. Ten to twenty histology sections were made, collected from 740 µm to 860 µm, bracketing the vertical tissue depth of 800 µm within each polyp biopsy, which is just above the coenosarc in M.faveolata (~1000 µm below the top of polyp). Sections were mounted onto glass histology slides (manufacturer) and stored in the dark at 4°C until further processing.

Immunohistochemical Staining

Mounted tissue sections were then dewaxed in a series of Histoclear™ (similar to xylenes) washes, before being rehydrated in 100% ethanol and phosphate buffered saline
solution (PBS). Slides were then incubated in a blocking buffer, and stored in the dark for 2 hours, after which they were rinsed with PBS. Wheat germ agglutinin (WGA) conjugated with Alexa Flour 647 was then applied to the slides for 30 minutes before being rinsed for 5 minutes in PBS. Coverslips were applied to the slides using an anti-fade mounting medium (Prolong Gold). The slides were stored at 4°C until data collection (see Piggot et al. 2009 for detailed protocol).

In addition to the Piggot et al. (2009) protocol, carbonic anhydrase, and the calcium binding protein (CaBP) were also identified using fluorescent labeling of each targeted structure in the mucus and tissue micro-layers of each polyp (Piggot et al., 2009). The anti-body used to label the alpha-carbonic anhydrase (anti-STPCA; Genscript, Piscataway, New Jersey, USA), which is a peptide derived from the coral *Stylophora pistillata* (Moya et al., 2008). The CaBP, also called calmodulin, was labeled using a standard antibody (anti-CALM; Invitrogen USA), to target membrane-bound calmodulin in Scleractinians. Both primary antibodies were applied as a 1:200 dilution. The secondary antibodies for the anti-STPCA and anti-CALM were goat-anti-rabbit AlexaFluor350 and goat-anti-mouse AlexaFluor 546, respectively, and were applied as a 1:200 dilution. Coverslips were applied to the slides using an anti-fade mounting medium (Prolong Gold), and the slides were then stored at 4°C in the dark until microscopic analyses.

*Immunohistochemical Characterization and Quantification of Mucus and Tissue Layers*

The triple-labelled tissue sections were then imaged using a Zeiss Axiovert M 200 microscope (Zeiss, Obercochen, Germany) and Axiovision software (version 4.6).
operating with an AxioCam MRC5 (Zeiss) camera. The distributions of zooxanthellae, CA, N-acetylglucosamine (mucus), and CaBP (calmodulin) in the tissue layers were spatially mapped and quantified using Imaris image analysis software (version 5.8, Bitplane Inc., Minneapolis, MN). A total of three polyps per sample could be analyzed with each stain application. The intensity threshold was manually chosen to optimize the selection of the immunohistochemical target and exclusion of what may be considered background fluorescence. The Imaris software was then used to automatically select all regions of the image exhibiting the specifically selected range in fluorescence intensity. The total abundance of each compound was determined by quantifying the total area that exhibited the specified pixel intensity threshold and was normalized to total polyp area, measured in Axiovision software (version 4.6).

Digital Elevation Mapping of Polyps

*M. faveolata* polyps selected for digital elevation mapping were decalcified, processed and infiltrated in the same manner as during histological preparation. The one exception is that the polyps were then embedded in an opaque red wax to decrease reflected fluorescence surrounding the embedded polyp tissues during imaging. The wax and tissue block were secured onto a mount facing a Ziess Stereoscope (1.5x Objective). this allowed an image with up to 2 μm spatial resolution to be taken of the re-emitted fluorescence from the tissue and zooxanthellae exposed on the block face. The block was then sectioned in 1 μm sections and imaged with fluorescence after each section, until thousands of sections taken through the entirety of each polyp was sectioned and photographed. The Imaris software compiled the ~2500 images, digitally reconstructed
the polyp, and performed 3-D digital rendering of the surface topography (Fig.8). The details of the surface topography and interior structure could then be observed and measured with a spatial resolution of 2 \( \mu \text{m} \), providing the required ultra high-resolution 2-D and 3-D spatial context for the measurements and observations made in this study. This technique was developed explicitly for this project and is the first to supply a 3-D digital reconstruction of the soft tissue of an entire polyp.

**Characterization and Quantification of the Skeletal Meso-Structure**

Skeleton-tissue biopsies fixed in 70\% ethanol were cut into 5mm sections and baked at 105\(^\circ\)C for 12 hours. The skeleton section was then analyzed using a radiograph micro-computed tomography instrument (Xradia Micro-BioCT), reconstructing a three-dimensional digital image of the skeletal section. The x-ray source and detector settings were optimized for the greatest contrast, high-resolution imaging, and high-throughput analyses. This was done by placing each sample 80 mm from the 0.5 magnification camera lens and 100 mm from the x-ray source, allowing a 30 mm field of view and a pixel size of 30.0 \( \mu \text{m} \), where specifications were manually chosen to allow the highest resolution possible while keeping the entire sample in the 30 mm field of view. For high-contrast images, the energy was set at 90kV and the power at 2 watts, with an exposure time of 1 second. The tomography was conducted at a 0.5\(^\circ\) rotation step interval, resulting in a total of 721 images per sample (Fig 12).

The proprietary software used by Xradia reconstructed the images into a three-dimensional object that was then visualized and reconstructed using Amira 5.0 imaging software. High-and low-density skeletal bands shown in the reconstruction were
manually cropped into separate images, according to the location of the corresponding bands shown clearly in the two-dimensional radiographs of each sample. Each region of interest was manually selected and the total volume of the selected three-dimensional space was recorded. All selected high- and low-density regions were treated with the same selection protocol. Next, the aragonite and associated pore space in each skeletal sample were digitally identified and labeled. Aragonite was identified as the voxels, three-dimensional pixels (1 pixel cubed), exhibiting absorption above a threshold that could be manually adjusted to accurately identify the skeleton versus the background or pore space. The voxels below the designated absorption threshold were labeled pore space and those above were labeled aragonite. The ratio of the volume of aragonite to pore space was then automatically quantified using the Amira “measure labeled material” function and recorded. The abundance of aragonite was then normalized to the recorded total volume of the successive high- or low-density bands comprising the coral skeleton. This permitted changes in coral skeletal density between high- and low-density regions to be quantified for the first time.
CHAPTER 3
RESULTS

Samples collected in May 2008, when SST was measured at 27.0°C (±0.03°), in 12 m water depth (WD) at Playa Kalki exhibited the mean area of tissue composed by zooxanthellae, normalized to the tissue area of each polyp, was significantly lower (0.04864 ±0.006617, mean ± standard error than samples collected in March 2008 (0.093490913 ±0.002867384), when SST was measured at 26.0 ºC (±0.03ºC) (Fig. 11). The area occupied by mucus exhibited a similar trend, where May 2008 samples displayed significantly less mucus (0.05396±0.003308) than March 2008 samples (0.09104±0.006225). The mucus quantified for this study included two localizations of mucus that have not been previously distinguished. Endodermal mucus was observed in close association with zooxanthellae cells in the oral and aboral endoderm. Mucus was also observed lining the region of the decalcified septa, bounded by calicoblastic epithelium. Mucus from the oral ectoderm was excluded from these quantifications. Next, the abundance of calmodulin in coral tissue showed no statistical difference between sample sets (0.04093±0.01095 in March and 0.05461±0.01121 in May 2008). Calmodulin was localized primarily in the granulocytes within the oral gastroderm, as well as less-concentrated dispersal in the oral ectoderm. Lastly, the expression of carbonic anhydrase increased significantly during warmer months, changing from 0.2735±0.007082 at 26.0°C in March to 0.3925±0.08283 with a SST of 27°C during May. CA was localized in the calicoblastic epithelium, but was also expressed in the coelenteron-facing border of the oral endoderm. Skeletal analyses resulted in the quantification of the 11% increase in the abundance of aragonite from LDBs to HDBs.
(Fig 12). Additionally, construction of an two-micron spatial resolution digital elevation map was achieved, providing a digital rendering of an entire polyp, providing a three-dimensional context for the two-dimensional histology sections used for immunohistochemical analyses.
**CHAPTER 4**
**DISCUSSION**

*Immunohistochemical Staining and Quantification of Molecular and Cellular Components*

Piggot et al. (2009) developed and applied an effective method for immunohistochemical targeting of the cellular constituents of coral tissue *in situ*, followed by quantitative image analysis. This technique does not require the maceration of tissue, nor the destruction of the structure of tissue, but only the careful histological sectioning to the target horizon within the polyp, followed by immuno-labeling with appropriate primary and secondary antibodies. As a result of the analyses conducted in Piggot et al. (2009), a depth of 800 microns from the top of the polyp was chosen in the present study as the polyp tissue depth horizon that is most sensitive to change in SST and irradiance. The image analyses in the present study, which were modified from Piggot et al. (2009), permit an objective selection of immuno-labeled components via a threshold of fluorescent intensity. In the developed technique, the area of all pixels exhibiting the range of selected intensities was totaled to quantify the total area occupied by that particular tissue cell constituent. This methodology has resulted in the first quantitative documentation and comparison of the localization and abundance of spatially *in situ* zooxanthellae cells, mucus, calmodulin (CaBP) protein and carbonic anhydrase.

Zooxanthellae were observed to decrease in abundance from low to high SST, which is consistent with previous research on the response of zooxanthellae densities to changes in SST (Coles and Jokiel, 1977; Marubini and Davies, 1996; Piggot et al., 2009; Rowan, 2004) (Fig. 10). This decrease in the density of zooxanthellae may be associated with a decrease in the rate of photosynthesis executed within the coral tissue, and thus, a
decrease in the usage of respired carbon dioxide. An accumulation of respired CO\textsubscript{2} in coral tissue would then create an increased [CO\textsubscript{2}] gradient across the calicoblastic epithelium, initiating diffusion or active transport of carbon across this membrane. This is consistent with observations that photosynthesis draws down CO\textsubscript{2} concentrations, inhibiting aragonite saturation state in the calcifying space between the calicoblastic epithelium and the skeleton (Smith, 1973). The transport of CO\textsubscript{2} into the calcifying space would result in the accumulation of protons and could then initiate the Ca\textsuperscript{2+}-ATPase, subsequently providing calcium to the site of precipitation and removing protons (Chalker et al., 1986; Kingsley and Watabe, 1985). The possible decrease in nutrient supply to the coral due to decreased phototrophy, which could decrease calcification rates, may be counteracted by either the suggested increase in [CO\textsubscript{2}] or the a transition in trophic dependence from phototrophy to heterotrophy (Anthony and Fabricius, 2000; Piggot et al., 2009). In this scenario, instead of deriving carbon as a product of zooxanthellae photosynthesis, the coral would be forced to gain energy from ingestion of the mucus surface layer containing commensal bacteria and trapped organic matter from SW, as well as carbon derived from passive carnivory. Trophic plasticity in corals is expected to influence calcification of the skeleton, where heterotrophy is correlated with the precipitation of a denser skeleton (Anthony and Fabricius, 2000). This decrease in zooxanthellae may mark a seasonal change in the carbon cycling within the organism, and may cause an accumulation of CO\textsubscript{2} in the tissue, ultimately resulting in more available CO\textsubscript{2} for the precipitation of calcium carbonate (McConnaughey et al., 1997). However, it is possible that this accumulation may be toxic to the coral animal. Therefore, an increase in the precipitation of aragonite could not only be a by-product of
increased availability of carbon, but may also be a seasonal coping mechanism where an increased calcification rate sequesters CO$_2$ from the tissue and returns concentrations to a more optimal state.

Decreases in the next targeted component, N-acetyl-glucosamine in mucus via WGA, is, however, not consistent with previous observations of increased mucus production during increased SST (Piggot et al., 2009; Reitner, 2005). This may be explained through analysis of the localization of the mucus observed in this study (Fig. 9, 10). Because of a lack of fixation of mucocytes in these samples, the mucus that was quantified was primarily associated with both oral and aboral endoderm. Mucus was observed in close association with both zooxanthellae and the calicoblastic epithelial skeleton-tissue interface, suggesting three possible distinct localizations of mucus within the coral tissue, perhaps with three distinct functions. This study describes a decrease in the abundance of mucus in response to an increase in SST. A possible explanation for this data is that mucus associated with zooxanthellae fluctuates correlatively to changes in zooxanthellae densities. Although this has not been documented, this association may suggest the temporary storage of energy in the form of mucus in this endodermal layer, from which zooxanthellae could derive energy if needed (Falkowski et al., 1984; Muscatine et al., 1984). Because this component was shown to fluctuate with zooxanthellae, and are localized in such close proximity to the algae, this may suggest a functional correlation between the two. Mucus associated with regions of decalcified septa and the calicoblastic epithelium was qualitatively observed to increase in response to increased SST, although these observations are currently being substantiated through quantitative analysis. This mucus may be that associated with not only the calicoblastic
epithelium but also the organic matrix secreted by this tissue layer, where increased abundance of mucus in this region may be related to the production of organic matrix and the promotion of nucleation of aragonite during this up-regulation of mucus production in this particular localization.

Results from this study suggest that mucus produced by corals may require distinction between three different localization strategies within the tissue structure and possibly three different functions and compositions. Mucus associated with oral ectoderm it expelled by mucocytes to construct the coral surface mucus layer (CSM). The CSM coats the living coral tissue, acting as a physicochemical barrier between the coral animal and potential threats in the environment, including pathogens, sedimentation, and excess irradiance (Reitner, 2005). This mucus has been shown to increase in response to increased SST (Piggot et al., 2009; Reitner, 2005). Next, the mucus associated with zooxanthellae in the oral endoderm may serve as an energy storage or by-product of photosynthesis. Data from this study show that the mucus found in the gastroderm decreases with increased SST, possibly in correlation to the decreased number of zooxanthellae. Finally, the mucus associated with the skeleton and calicoblastic epithelium may promote precipitation of coral aragonite by lowering the activation energy of nucleation by providing an organic framework on which to grow (Allemand et al., 2004; Helman et al., 2008; Kandianis et al., 2008). Observations from this study suggest further investigation of these distinctly localized mucus constituents to discern the composition and function of the mucus in these regions.

Results from this study are consistent with previous work that indicates calcium availability is a non-rate-limiting step in coral skeletogenesis and that it is actively
transported across the calicoblastic epithelium through the light-activated Ca\(^{2+}\)-ATPase pump (Allemand et al., 2004; Cohen and McConnaughey, 2003; Tambutte et al., 2007a). The abundance of calmodulin, a CaBP, did not change in response to changes in seasonal SST, suggesting that the availability of calcium remains relatively constant seasonally, and may be temperature-independent. If calcium is readily available for skeletogenesis, in may be inferred that an increase in the Sr/Ca ratio in coral skeletons in response to temperature is not a result of decreased calcium concentrations, but perhaps an increase in calcification rate caused by an increase in the saturation state of aragonite. Changes in Sr/Ca in coral skeletons may then have little to do with [Ca\(^{2+}\)] in coral tissue (Cohen and McConnaughey, 2003; Mitsuguchi et al., 2003; Swart et al., 2002).

Finally, the significant increase in carbonic anhydrase during increased seasonal SST is the first quantitative analysis of the enzyme as it responds to changing environmental factors. Using an anti-body developed by the research group at the Centre Scientifique de Monaco, anti-STPCA, produced from an isolated peptide from *Stylophora pistillata* provided the specificity to study this enzyme in corals (Jackson et al., 2007; Moya et al., 2008; Tambutte et al., 2007b). This increase in the expression of CA implies the increase in the concentration of carbonate ions in the calcifying space as a result of facilitated hydration of CO\(_2\), the rate-limiting step in conversion of CO\(_2\) to carbonate. This increase in the rate of the reaction H\(_2\)O + CO\(_2\) ⇌ HCO\(_3^-\) + H\(^+\) could cause the accumulation of protons and the initiation of the Ca\(^{2+}\)-ATPase pump, providing calcium ions to the site of calcification. The increase in the abundance of CA may be a response to the postulated effect of a decreased number of zooxanthellae: an accumulation of CO\(_2\) in coral tissue during times of decreased photosynthesis and increased SST. This
Accretion of CO₂ may warrant a rapid increase in calcification to deplete the [CO₂] in the tissue, and instigates an increase in the abundance the CA enzyme that facilitates this process.

Because CA is indiscriminate with respect to the source of CO₂ it aids in hydrating, CA could be up-regulated in response to increases in both respired CO₂ as well as DIC in the water column (Moya et al., 2008; Smith, 1973; Swart et al., 2004). An alternative explanation for the increase in CA during higher SST is that this response represents the coral’s compensation mechanism for lower partial pressures of CO₂ in the water column. Global seasonal fluctuations in the atmospheric [CO₂] show a decrease during the summer months, associated with lower concentrations of DIC in the water column (NOAA, Mean annual data, Mauna Loa, HI). Newly recorded decreases in global seawater pH as a result of increasing atmospheric CO₂ (National Research Council 2010) may also play a role in this process. Since DIC, as well as respired CO₂, may be incorporated into the skeleton, an increase in the abundance of CA may be a compensation mechanism for decrease carbon availability for aragonite precipitation. This would allow the coral to drive a reaction that may not occur rapidly enough for sustained aragonite precipitation when carbon availability is limited. It may therefore be postulated that an increase in CA correlates with decreased [CO₂] in the ocean. However, this pivotal concept requires significant further research to substantiate these simultaneous effects of increased SST and [CO₂] atm, on coral tissue enzyme abundance and activity.
CHAPTER 5
CONCLUSIONS

Results from this study indicate that coral skeleton density banding is created by a biological response to changes in seasonal SST via enzymatic catalysis of the rate of skeletal aragonite crystal precipitation. These findings are in contrast to previous studies that have interpreted coral skeleton density banding as a solely abiotic thermodynamic response to SST. A decrease in the number of zooxanthellae and the mucus closely associated with these algae in the gastroderm, may cause an accumulation of respired CO$_2$ and ensuing build-up in the gradient of carbon dioxide across the calicoblastic epithelial layer. While this cellular CO$_2$ gradient may enhance calcification during higher SST (producing high-density skeletal bands), carbonic anhydrase also increases in abundance during this time. This enzyme concentration may increase because of an accretion of respired CO$_2$ and thus serve as a coping mechanism to sequester carbon and thus decrease concentrations within the tissue. Increased CA may, however, be also used as a compensation mechanism for decreased partial pressure of CO$_2$ during higher SST. This would maintain the required rates of calcification despite a possible deficiency in DIC available for skeletogenesis. The measured 11% increase in the density of coral skeleton in LDBs further supports this interpretation of enzymatic catalysis. The formation of HDBs during higher SST may in turn be influenced by a decrease in the number and functioning of zooxanthellae during high SST at a time when carbonic anhydrase is found in higher concentrations.
FIGURES

Figure 1: Geographic map of Curaçao, Netherlands Antilles in the Southern Caribbean Sea. Sample site on the fringing reef at Playa Kalki at the northwestern point of the island.

Figure 2: Coral Reef Depositional Facies Model showing the sampled facies, the reef crest.
Figure 3: An \textit{in situ} \textit{M. faveolata} on the reef crest at Playa Kalki. Extraction of coral skeleton-tissue biopsies occurred at 45 degrees latitude on each coral head. Inset: A 2.5 cm diameter skeleton-tissue biopsy.
Figure 4: Modified schematic of a vertical cross-section of a coral polyp situated atop its massive aragonite skeleton, from Veron, 2001. The tissue is composed of three main layers: the ectoderm, which represents the interface with seawater, a layer of connective tissue called mesoglea, and the gastroderm, within which the symbiotic photosynthetic dinoflagellates, called zooxanthellae reside. The coral polyp secretes its calcium carbonate skeleton beneath it through a very thin layer of tissue called calicoblastic epithelium. An organic matrix is also secreted by the organism, composed of largely polysaccharides and is itself incorporated into the skeletal architecture.

Figure 5: Fluorescence microscopy image of autofluorescent coral tissue fixed in 3.7% formaldehyde and demineralized. Image A shows a horizontal cross section of an *M. faveolata* polyp, at a depth of 805ums below the top of the polyp. Image B shows a magnified view of the tissue structure. OE = oral ectoderm; Muc = surficial mucocytes; M = mesoglea; OG = oral gastroderm; Z = zooxanthellae; CE = calicoblastic epithelium; AG = aboral gastroderm; SK = area from where skeletal septum was removed; CO = coelenteron; SW = seawater-tissue interface.
Figure 6: Image A shows a radiograph of the aragonite coral skeleton of *Montastraea sp.*, illustrating the alternation of high and low density regions of the skeleton, where lighter regions are more dense and darker regions are less dense. A crystalline couplet of high and low density bands is hypothesized to represent one year of deposition. Image B shows a vertical cross section, and image C shows a horizontal cross section on the corallite scal, which is about 2mm in diameter. The corallite is bounded by the thecal wall. C = corallite; Col = columella; ED = Extrathecal dissepiments; EnD = endothecal dissepiments; T = Thecal wall; S = septa; Cos = costae.
Figure 7: NOAA Coral Reef Watch satellite SST data for 2001-2010. Piggot et al. 2009 suggest that, because of the flux in the abundance of zooxanthellae and surficial mucocytes that there is a seasonal trophic transition between heterotrophy and autotrophy as the main feeding strategy, where heterotrophy dominates during high SST when zooxanthellae are abundant and mucocytes are few, and autotrophy dominates when zooxanthellae are few and mucocytes are abundant. Also, there is a seasonal transition in the density of the skeleton, when high density aragonite precipitates during high SST and low density aragonite precipitates during low SST.

Figure 8: A digital elevation map (D) produced by manual tomography of a de-mineralized coral polyp (A). Serial blockface imaging (SBFI) is done by slicing the polyp (embedded in red paraffin wax) in one-micron increments (B), imaging the polyp after each one-micron section is removed (B,C), resulting in over 2,000 images as the entire depth of the polyp (2-3mm) is sectioned and imaged. The images are then compiled and processed with image analysis software (Imaris 5.0) to produced a three-dimensional digital rendering of the polyp (D,E). This process allows the analysis of the cellular and molecular components of coral tissue in the context of an entire polyp.
Figure 9: Comparison of the localization and abundance of cellular and molecular components of coral tissue as it changes over time and seasonal SST.

Figure 10: High magnification of oral and aboral tissue (one image is 100 microns across), showing the comparison in the abundance and localization of cellular and molecular components.
Figure 11: Quantitative Analyses of Cellular and Molecular Components of *M. faveolata* Tissue. Results of the tissue analysis at 800ums tissue depth of cellular and molecular components indicate that there is a biological response to changes in seasonal SST: (1) the abundance of zooxanthellae decreased; (2) the overall abundance of the mucus associated the gastroderm and calicoblastic epithelium decreased; (3) the abundance of calmodulin did not change significantly; and (4) the amount of carbonic anhydrase increased as SST increased. All abundances were normalized to the tissue area of each polyp, and error bars represent the standard error for each analysis.
Figure 12: Skeletal analyses of microCT radiography produced the first quantitative characterization of high and low density bands. A 5 mm section of each skeletal biopsy was x-rayed to locate the LDBs and HDBs (A). The serial radiographs taken via microCT were then compiled and three-dimensionally rendered (B). Aragonite and pore space could then be digitally labelled according to absorption thresholds of each material through image analysis (C). The abundance of aragonite was then compared between LDBs and HDBs. Results show an 11% increase in the abundance of aragonite of LDBs to HDBs (D).

Figure 13: NOAA Coral Reef Watch satellite SST data for 2001-2010 is coupled with the data from this study, the data from Piggot et al 2009, and the hypothesis that HDBs form during high SST, and LDB form during low SST.


ecological dominance and genetic diversification of coral endosymbionts in the genus *Symbiodinium*. *Journal of Biogeography*, 37, 785-800.785-800


