UNIVERSITY OF CALIFORNIA, SAN DIEGO

Characterization of sodium potassium -ATPase and vacuolar proton -ATPase in three coral species from two different clades

A Thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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The Thesis of Sidney Olivia Perez is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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University of California, San Diego

2014

DEDICATION

I dedicate this thesis to my family, friends and colleagues, especially my parents, Gabriela and Jose Perez for their abundant love and encouragement throughout my scientific studies and life endeavors; my brother Tristan who, with his free spirit and confidence, inspires me to continue to pursue new things; I also give special thanks to my friends who have provided me with joy and memories throughout the stressful times of my studies.

Additionally, I would like to especially recognize the members of the Tresguerres lab for their guidance and help in this project and in life– Dr. Martin Tresguerres for his mentorship, constant encouragement when experiments were tricky, and of course for giving me the opportunity to conduct meaningful research; Dr. Katie Barott, for her mentorship, enthusiasm for answering all of my many questions, and providing inspiration to further challenge myself, scientifically; and to the rest of the Tresguerres lab for many laughs and memories, both in and out of the lab. I will *always* remember your support, wisdom, camaraderie and shared times.

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LIST OF ABBREVIATIONS

CCM	Carbon concentrating mechanism
CA	Carbonic anhydrase
CO	Coelenteron
DIC	Dissolved inorganic carbon
HRP	Horse radish peroxidase
NBC	Sodium bicarbonate co-transporter
NGS	Normal goat serum
NKA	Sodium potassium –ATPase
ROS	Reactive oxygen species
SK	Skeleton
SW	Seawater
VHA	Vacuolar proton –ATPase

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ABSTRACT OF THE THESIS

Characterization of sodium potassium -ATPase and vacuolar proton -ATPase in three coral species from two different clades

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Professor David Holway, Co-Chair

The movement of ions across a cell membrane is critical to a wide variety of physiological functions across all phyla, however, little research has been conducted on ion transport in Cnidarians. Understanding coral physiological mechanisms is of increasing interest, however, due to mounting environmental stress. Two key ion transport dependent processes are calcification and photosynthesis by the endosymbiotic algae. This study utilized fluorescent immunostaining to localize two key ion transport

proteins in three species of scleractinian corals: *Acropora yongei*, *Stylophora pistillata*, and *Pocillopora damicornis*. Proteins investigated included vacuolar hydrogen –ATPase (VHA) and sodium-potassium ATPase (NKA). VHA was localized to the intracellular symbiosome membrane of endodermal cells, providing supporting evidence for its participation in a carbon concentrating mechanism (CCM). This localization was conserved in all three species of coral. VHA was also localized in the aboral endoderm, and was more abundant in the growth tips of corals, suggesting it may play a role in facilitating rapid calcification by aiding in pH regulation. NKA was observed in the calcifying epithelium of all three species. However, it was found in the apical membrane of the oral ectoderm of *A. yongei*, only. The similar localization of NKA in the calcifying tissue suggests it plays a conserved role in calcification, while the differences in location between other tissues indicate that corals have evolved diverse ion transport mechanisms for other functions. These differences may lead to differential responses to environmental stressors.

INTRODUCTION

Coral reef status and health

Coral reefs support one of the most diverse ecosystems on our planet, and are likely the most diverse of all marine habitats [1]. In addition to the intrinsic value corals have in laying the foundation for an entire ecosystem, they also provide a multitude of benefits to humans including but not limited to: providing tourism and recreational income for many nations, physical structure and protection to coastlines from storms, large potential for medical research, historical and climate records, and a massive seafood market [1, 2]. Since the 1980's concerns regarding the health of coral reefs have escalated with increased consciousness of anthropogenic stresses such as climate change, pollution and overfishing [1, 3]. The general consensus stands that corals as a whole will not fare well; several reports estimate that one-third of all corals are at high risk of extinction by the end of this century, unless they can adapt [4, 5]. These predictions are staggering and beg the question as to whether all coral species are equally susceptible to environmental change, as well as what biogeochemical mechanisms govern differential vulnerability. Understanding which corals are most subject to environmental stress and *why* can help streamline costly and labor-intensive conservation efforts.

Many efforts have been, and continue to be, conducted toward evaluating coral biodiversity and susceptibility. Most of these assessments are ecological surveys based on descriptive science, or observational studies, that require ample man-power [1, 5, 6]. While data from such studies are certainly valuable and critical for raising awareness of the coral health state, garnering public support, and driving political change toward conservation measures, they elucidate little as to the physiological reasons for differential

susceptibility to anthropogenic stress. On the other hand, knowledge of physiological differences at the cellular and molecular level in various species of corals can help predict which corals will be most vulnerable before "symptoms" of decline may even be observable in the field.

In 2005, Downs and colleagues encouraged the scientific community to shift the current paradigm of coral health assessment from a descriptive science to one that incorporates investigative and mechanistic science [6]. This is a conclusion that has subsequently been reached by several other scientists [7, 8]. Downs analogized this approach to the methods used in evidence-based medicine and he stressed the importance of implementing such studies in sparking mitigatory action supported by robust scientific evidence (reviewed in [6]). The application of mechanistic studies in conjunction with field studies would provide a powerful combination of tools to predict and identify which corals are in danger of health decline, diagnose vulnerable populations as being "ill," understand why the decline in health may be occurring, and take action in the field to rectify and prevent it.

An example of the potential application of mechanistic studies lies in recent findings in our laboratory suggesting species-specific mechanisms for bicarbonate (HCO_3^-) uptake from the seawater [9]. Differences in HCO_3^- uptake may confer differential responses to ocean acidification, an anthropogenic phenomenon in which an increase in CO_2 is resulting in a decrease in pH, and a shift in the availability of HCO_3^- and CO_3^{2-}. Knowledge of which species will be most susceptible to ocean acidification can help drive political action toward communities with the highest abundance of vulnerable species. While this is only an example and speculative, in the incredibly

diverse class of Anthozoa there are bound to be many species-specific mechanisms of coral physiology, some of which may govern differential physiological responses to environmental stressors. Without a more thorough understanding of widespread and species-specific mechanisms, we cannot predict, understand or interpret data collected on coral reef health that are necessary to implement mitigative policies. Thus, more research must be conducted to understand how corals work on the cellular level, both as a whole class of organisms, and as individual species.

One aspect of cellular physiology that is well studied in other organisms, but not in the phylum Cnidaria is ion-transport, which is critical for most physiological processes. Specifically, in corals ion transport is essential for the central physiological activities of photosynthesis and calcification [10, 11]. Thus, this thesis will outline some of the known physiology of corals, recent research strides in elucidating important cellular mechanisms, and the involvement of ion-transport, as is relevant to this study.

Coral anatomy, physiology and terminology

Corals belong to the phylum Cnidaria, and are composed of hundreds of stinging, small identical polyps. Each polyp is connected to one another through gastrovascular canals that make up the coelenteron, otherwise known as the gut (Fig. 1). The coelenteron is the site for uptake of the coral's symbionts, dinoflagellates of the genus *Symbiodinium*. The symbionts are commonly called zooxanthellae, and being algal cells, they photosynthesize to provide up to 95% of carbon used for energy by the coral [10].

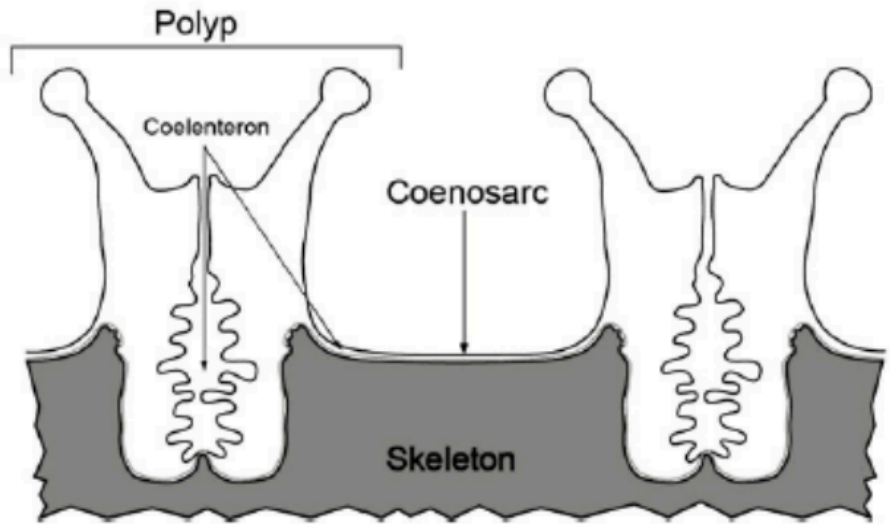

Figure 1: Basic anatomy of a coral

Adapted from [12]. Two adjacent polyps connected by gastrovascular canals, forming the coelenteron. Tissue between polyps is termed the coenosarc.

Furthermore, corals are diploblastic, meaning they are comprised of only two germ layers: the ectoderm and endoderm. On either side of the coelenteron exists an ectodermal and endodermal cell layer, making up four cell layers in total. The two cell layers adjacent to the seawater are the oral ectoderm and oral endoderm; the two cell layers adjacent to the secreted exoskeleton are the aboral endoderm and aboral ectoderm (Fig. 2). The oral ectoderm is associated with nutrient uptake, endodermal layers harbor the zooxanthellae, and the aboral ectoderm is composed of calcifying cells that precipitate the skeleton. The aboral ectoderm is also known as the calicoblastic epithelia, and will be referred to as such in this thesis. These calicoblastic cells are responsible for forming the foundation of coral reef ecosystems in the form of calcium carbonate ($CaCO_3$) structures [11] that can be visible from outer space. The two physiological

processes of photosynthesis and calcification are central to coral health and will be discussed further.

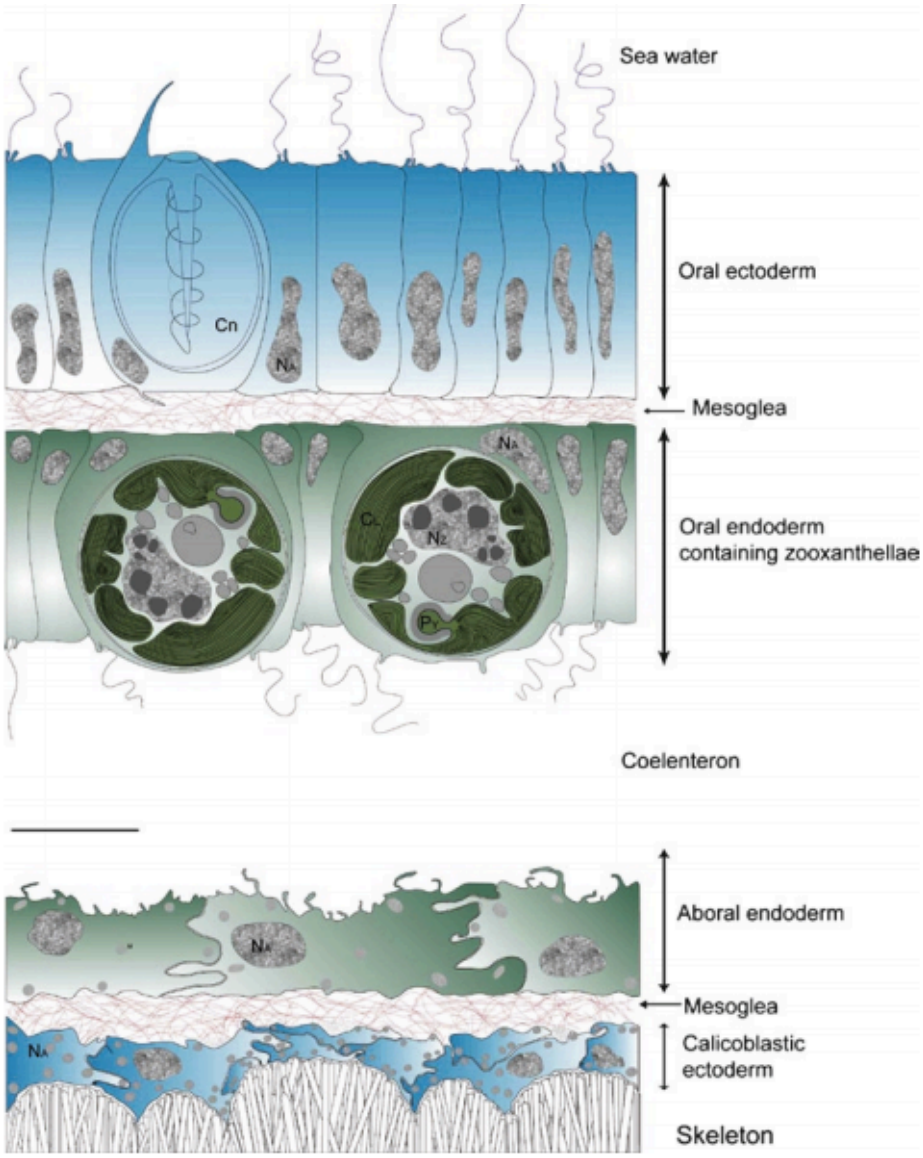

Figure 2: The four cell layers of a coral

Adapted from [11]. Cross-section of coral tissue. The four cell layers include: the oral ectoderm, oral endoderm, aboral endoderm and aboral ectoderm also known as the calicoblastic ectoderm. Ectodermal and endodermal layers are separated by an extracellular matrix called the mesoglea.

Photosynthesis: a relationship between coral and algae

Photosynthesis by the symbiotic algae can supply the majority of energy to the coral through the fixation and translocation of carbon in the form of sugars. Free-swimming zooxanthellae enter with seawater into the mouth of the polyp and are phagocytosed by the endodermal cells adjacent to the coelenteron. The cellular mechanisms of cell-to-cell recognition and phagocytosis that occurs are still being investigated and can be reviewed here [10].

Through phagocytosis, the *Symbiodinium* are taken up and surrounded by an extra host-derived membrane termed the "symbiosome membrane" in addition to the coral plasma membrane (Fig. 3, Fig. 4). The inorganic carbon used by the zooxanthellae for photosynthesis must be transported through several membranes from the coral to the algae. Conversely, for the coral to utilize the sugars produced by *Symbiodinium*, the fixed carbon molecules must be translocated through these barriers from zooxanthellae to host.

Photosynthesis: a requirement for enriched CO_2

The molecule of inorganic carbon used by the algal cells for photosynthesis is carbon dioxide (CO_2). However, the first enzyme in photosynthesis, RubisCO, has an inherently low affinity for CO_2, likely because it evolved in an atmosphere with higher levels of CO_2 [13]. Speciation of dissolved inorganic carbon (DIC) is driven by the reversible reaction $CO_2+H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- + H^+ \Leftrightarrow CO_3^{2-} + H^+$ and its equilibrium is pH dependent (Fig. 5). The first reaction (conversion between CO_2 and H_2CO_3) is

catalyzed by the enzyme carbonic anhydrase (CA), and therefore this enzyme can facilitate both photosynthesis and calcification. In the ocean (pH ~8.1), as well as in

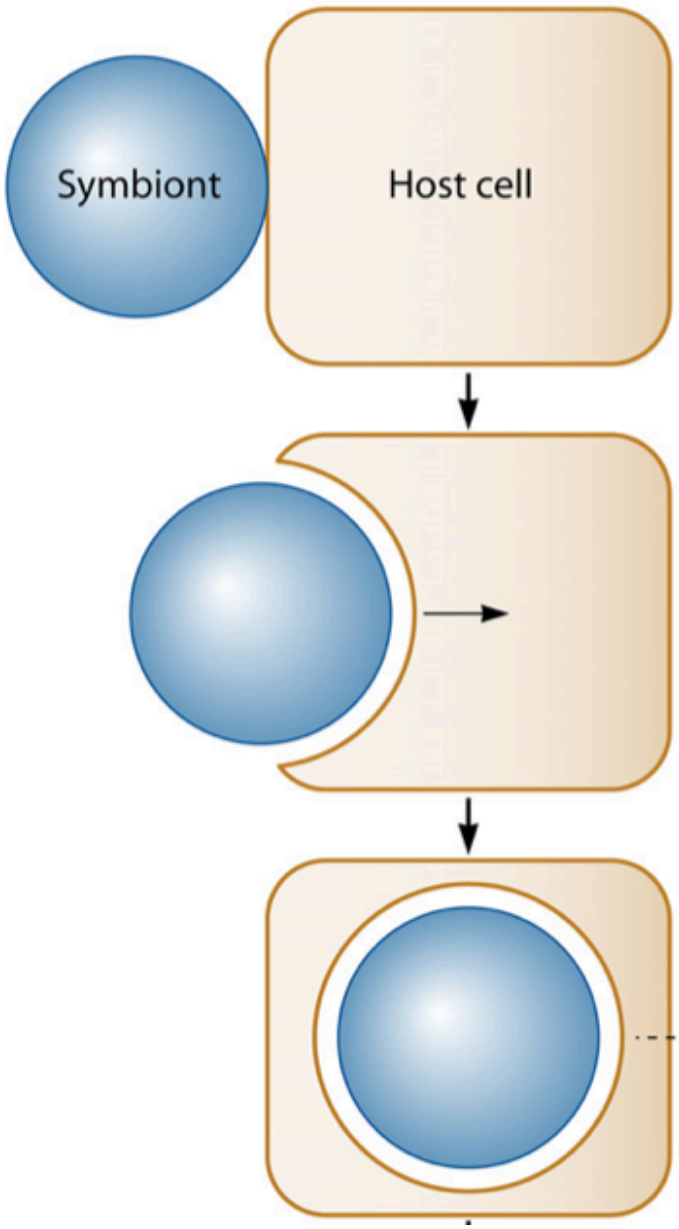

Figure 3: Phagocytosis of *Symbiodinium* and generation of symbiosome membrane

Adapted from [10]. Through the phagocytosis of a symbiont a portion of the plasma membrane in contact with the symbiont is invaginated and pinched off to form the symbiosome. The symbiont is thus surrounded by a host-derived symbiosome membrane and coral plasma membrane.

most biological fluids (pH ~7.1-7.4), DIC is predominantly present as bicarbonate (HCO_3^-). It is not surprising, then, that HCO_3^- from seawater is a major source of DIC utilized for photosynthesis in Cnidarians [14, 15, 16]. However, because HCO_3^- is a charged molecule, it cannot diffuse through the multitude of membranes that exist between seawater and symbionts, and it likely requires

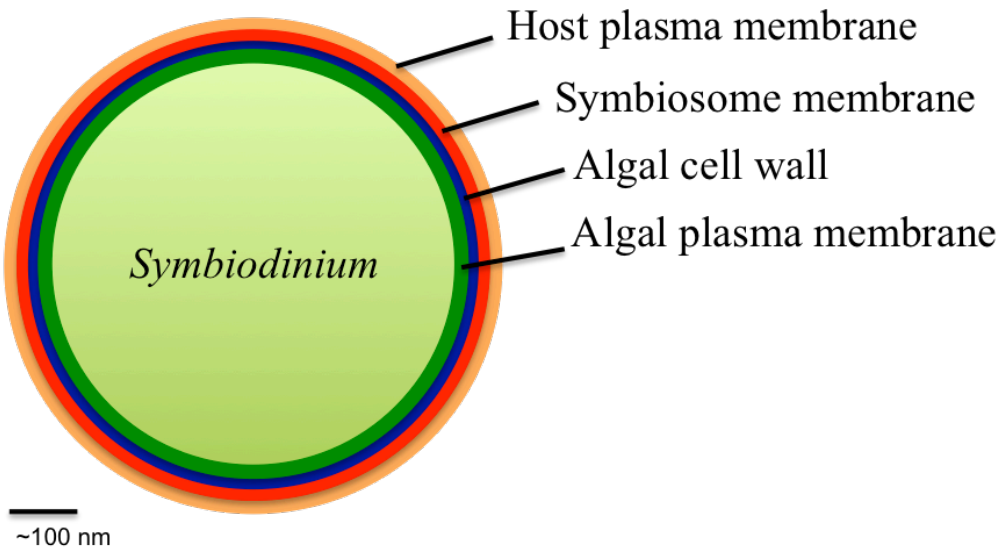

Figure 4: Membrane structures surrounding symbionts

A symbiont is surrounded by it's own algal plasma membrane, the algal cell wall, a symbiosome membrane, and finally the coral host plasma membrane, all of which are in a proximity of ~100nm.

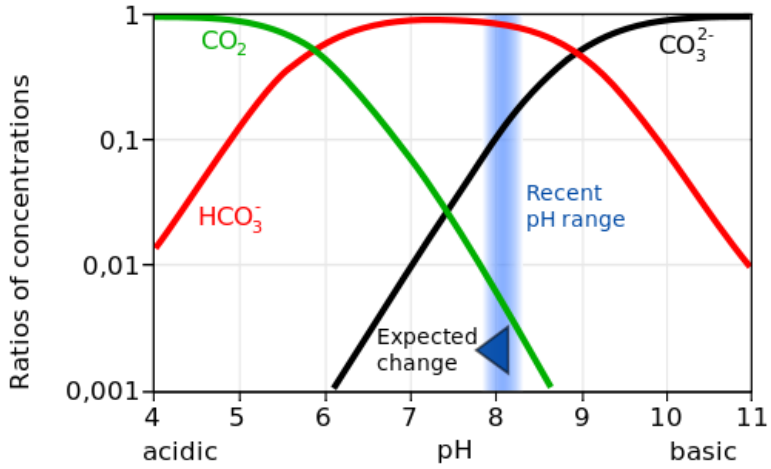

Figure 5: Speciation of DIC depicted in a Bjerrum plot

Taken from Wikipedia (Karbonatsystem_Meerwasser_de.svg). The species of DIC include carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}). Relative abundance is driven by the pH-equilibrated reaction: $CO_2+H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow HCO_3^- +H^+ \Leftrightarrow CO_3^{2-}+H^+$. Acidic conditions favor CO_2, while basic conditions favor CO_3^{2-}. The blue band depicted above represents ambient seawater pH (pH ~8.1).

membrane ion-transporter proteins to enter the coral epithelia. Furthermore, there are no known CO_2 pumps to concentrate the substrate for photosynthesis. Thus, HCO_3^- in the cell must ultimately be converted to CO_2 for use by RubisCO in photosynthesis.

It has been hypothesized that algae, including *Symbiodinium,* have evolved carbon concentrating mechanisms (CCM's) to enrich for the CO_2 necessary to photosynthesize [17]. The principle of a CCM can be described by the pH driven equilibrium for DIC speciation. A reduced pH drives the reaction in favor of the speciation to CO_2 (Fig. 5). Thus, the hypothesis proposes that the acidification of intracellular compartments, such as the symbiosome, results in the concentration of DIC as CO_2, catalyzed by the enzyme carbonic anhydrase [17, 18, 19, 20]. Both this newly concentrated DIC and CO_2 from the

cellular respiration of mitochondria can diffuse through membranes, and are further concentrated by other CCM's until they reach Rubisco in the chloroplasts.

Venn and colleagues first measured the intracellular pH of a coral cell containing zooxanthellae and found the area immediately surrounding the symbionts to be acidic (pH <6) [20]. However, the exact pH could not be measured due to limitations in the range of pH sensitivity of their study. Recent research in our laboratory has resulted in a more accurate estimation of the pH surrounding the symbionts as low as pH ~4 [21]. Additionally, we used immunofluorescence techniques to localize the protein V-type H^+-ATPase (VHA) around the perimeter of zooxanthellae in two species of corals, *Acropora yongei* and *Stylophora pistillata.* These two species represent two clades of corals: Robusta (to which *S. pistillata* belongs) and Complexa (to which *A. yongei* belongs). These clades are estimated to have diverged between 250 and 400 million years ago [22, 23, 24].

VHA is an ion-transporting proton (H^+) pump that utilizes ATP-hydrolysis to pump hydrogen ions against a concentration gradient. VHA has been shown to pump H^+ ions against a gradient as high as ~1000 fold in plants and as such is commonly responsible for the acidification of intracellular compartments [21]. Furthermore we demonstrated that when *A. yongei* and *S. pistillata* were treated with a specific inhibitor of VHA, bafilomycin [22], the pH around the symbionts became less acidic and photosynthesis rates were inhibited. This work provides direct evidence for the hypothesis that the coral is able to actively acidify the symbiosome space as a CCM in order to promote photosynthesis. However, the close proximity of the coral plasma

membrane, symbiosome membrane, algal cell wall and algal plasma membrane (~100nm) made it difficult distinguish the specific intracellular localization of VHA in coral tissues.

Work in this thesis includes the validation of anti-VHA antibodies utilized in our previous study. In addition, this study further explores the intracellular localization of VHA in isolated coral cells. Finally, a third species and second representative of the clade Robusta, *Pocillopora damicornis,* was also investigated to determine whether the role of VHA as part of a CCM is further conserved.

Calcification: generation of a microenvironment to promote skeleton precipitation

Calcification by the calicoblastic cells results in the largest bioconstructed structures on the planet [11]. This biomineralization by the coral is an exercise of biological control that favors the relatively rapid mineralization of $CaCO_3$, as defined by the precipitation of calcium ions (Ca^{2+}) and carbonate ions (CO_3^{2-}) to form solid $CaCO_3$. Thus, both of these ions must be present at the site of calcification and transported through several cell layers from either the seawater or coelenteron.

In order to facilitate this mineralization, it is hypothesized that the calcifying cells generate a specialized microenvironment within the extracellular matrix beneath the calicoblastic layer, called the subcalicoblastic medium (Fig. 7). While the mechanisms of calcification are still debated (reviewed in [11]) and generally unknown, some recent studies have made progress in elucidating potential mechanisms.

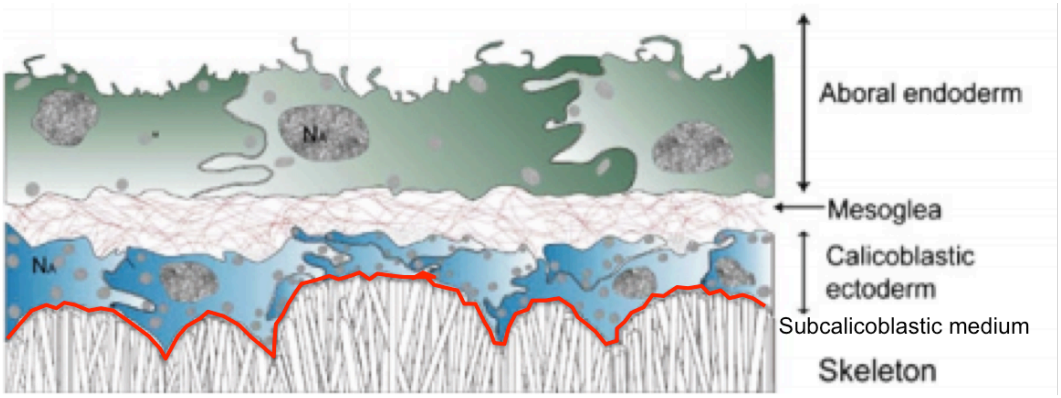

Figure 6: The subcalicoblastic microenvironment beneath calicoblastic cells

Adapted from [11]. The extracellular microenvironment (red) exists between the calicoblastic ectoderm and skeleton; this is the site of calcification.

Metabolic CO_2 from the respiration of mitochondria is believed to supply the majority of DIC required for calcification [11], and recently mitochondria have been abundantly localized in calicoblastic cells [9]. This DIC from respiration is likely converted to HCO_3^-, driven by the physiological pH of the cells (pH ~7.4) and catalyzed by carbonic anhydrase [11, 19]. DIC transport into the subcalicoblastic medium remains unknown. Interestingly, Venn and colleagues measured the extracellular pH of the subcalicoblastic medium and found it to be ~0.5 pH units higher than seawater (pH ~8.1) while the intracellular pH of the calicoblastic ectoderm was typical of cells (pH ~7.4) [26]. This increase in pH supports the hypothesis that the calcifying cells are actively regulating the extracellular pH of this microenvironment to facilitate the conversion of

DIC to CO_3^{2-} (Fig. 5). Furthermore, this reaction appears to be catalyzed by an extracellular carbonic anhydrase enzyme in the subcalicoblastic medium [27].

In order to maintain a more basic pH in the subcalicoblastic microenvironment, the calicoblastic cells are likely removing protons from the extracellular medium and translocating H^+ into the coral calicoblastic cells. However, the intracellular pH of the calicoblastic epithelia does not differ from other cells, indicating that protons are further translocated, likely into the coelenteron and perhaps neutralized [27, 28]. Interestingly, VHA was occasionally localized in the aboral endoderm cells of *A. yongei* tissue (Barott personal observations) (see Fig. 2 for anatomy of tissue layers). Thus, VHA could be providing a mechanism to translocate protons from the coral tissue into the coelenteron to facilitate pH regulation at the subcalicoblastic medium and promote calcification. This idea is further explored in this thesis.

Calcification: zones of rapid $CaCO_3$ precipitation

In 2011 Jokiel reviewed the idea that corals have evolved morphologies to segregate their branches into "zones" of rapid photosynthesis and rapid calcification. He proposed that the generation of these zones allows for more efficient direction of inorganic carbon transport to these two important processes [29]. The tips of corals are zones of rapid calcification, and the middle of coral branches are zones of rapid photosynthesis. Thus, I hypothesized that the occasional aboral endoderm localization of VHA would be more abundant in the growing tips of a coral branch where proton removal would be more necessary to facilitate the rapid calcification.

Barott et al. [9] also localized the enzyme Na^+/K^+ -ATPase (NKA) in *A. yongei* and *S. pistillata*. NKA is important for ion-transport in a wide variety of physiological

processes across all phyla. Utilizing the energy from hydrolysis of ATP, NKA maintains an electrochemical gradient across the membrane of a cell by exporting more positive ions (three Na^+) than it imports into the cell (two K^+), and thus also maintains a low concentration of Na^+ inside the cell. NKA can consume a considerable portion of the metabolic energy generated, and has been shown to use as much as 80% of ATP in developing sea urchins [30]. One physiological function of NKA is its involvement in secondary active transport, in which the transport of ions, and nutrients such as glucose and amino acids are coupled to the import of Na^+[31].

NKA localization to the oral ectoderm was present apically in *A. yongei*, however, not in *S. pistillata* (see Fig. 2 for anatomy)[9]. These results suggest a species-specific mechanism for ion transport, likely having to do with nutrient or DIC uptake. For example, the sodium gradient generated could be used for the secondary active transport of HCO_3^- from seawater by a Na^+/HCO_3^- -Cotransporter (NBC), which was also localized in the oral ectoderm of *A. yongei*, but not *S. pistillata* [9]. These differences may be related to the fact that *A. yongei* and *S. pistillata* have so many years of evolution between them. Such results illustrate that physiological differences among coral taxa have implications for predicting how corals will cope differently under environmental stress.

Interestingly, NKA was also abundantly localized to the basolateral membrane of calicoblastic cells of *A. yongei* and *S. pistillata* [9]. This finding suggests that NKA energizes calcification in both species, perhaps through the use of electrochemical gradients to import ions, DIC, or organic matrix proteins.

This thesis explores how NKA expression in a third species, *P. damicornis* compares to localization of *A. yongei* and *S. pistillata*. I hypothesized that localization would be similar to what is observed in *S. pistillata*, as the two species belong to the same family (Pocilloporidae). Additionally, this study, explored potential differences in NKA localization of the calicoblastic cells present in the growing tip of a coral branch where calcification is occurring more rapidly, compared to the middle.

Thesis aims

Previous research on the mechanisms of photosynthesis and calcification in corals has highlighted the importance of ion-transporting proteins in these processes. Ion transport is important for pH regulation and the transport of nutrients and ions through several epithelial layers to the sites of photosynthesis and calcification. Recent research has shed light on the involvement of VHA in photosynthesis as part of a CCM [21], and the likely involvement of VHA and NKA in calcification [9]. This thesis aims to expand on these results by further investigating these proteins in *A. yongei*, *S. pistillata*, and *P. damicornis,* which are representatives of two clades that have hundreds of millions of years of evolution between them.

This study used immunolocalization to further investigate 1) the intracellular localization of VHA 2) whether VHA of the aboral endoderm may be more abundant in the growing tip of a coral that could facilitate the precipitation of $CaCO_3$, 3) whether the localization of NKA differs or is more abundant in the growing tip, and 4) whether the patterns of NKA and VHA localization are similar or differential in *P. damicornis* compared to *S. pistillata* and *A. yongei*. This study will lead to a deeper understanding of the potential roles of these proteins in the essential processes of photosynthesis and

calcification. Furthermore it will contribute to the rapidly expanding and exciting research of ion-transport in corals.

METHODS

ANIMALS

Colonies of *Stylophora pistillata*, *Pocillopora damicornis*, and *Acropora yongei* were obtained from the Birch Aquarium in La Jolla, CA in October 2013. In addition, some colonies of *A. yongei* were donated by the Dimitri Deheyn laboratory at the Scripps Institution of Oceanography (SIO). All animals were maintained in flow-through aquaria at SIO with seawater warmed to 26°C. Lighting conditions consisted of a 12h/12h day/night cycle using AquaticLife Dual Lamp T5 HO fixtures. A moonlight that simulates the phases of the moon was also installed. Tanks were cleaned every other day to remove algae on the aquarium glass or found growing on corals. Specimens were allowed to acclimate for at least one week before sampling.

ANTIBODIES

Primary antibodies for protein detection by western blot and immunohistochemistry included: rabbit polyclonal anti-Na^+/K^+-ATPase α-subunit (NKA_α) antibodies, and rabbit polyclonal anti- V-type H^+ -ATPase B-subunit (VHA_B) antibodies.

SDS-PAGE AND IMMUNOBLOTTING

Antibodies, used in this thesis and previous studies, were validated for all three coral species for specificity by Western blot [9, 21].

Coral branches from each species colony were snapped into approximately 3.8 cm (1.5 inch) fragments immediately before protein content was to be analyzed by Western blot. Tissue was then blasted from the skeleton using a pressurized airbrush in a volume of 5 mL homogenization buffer. The homogenization buffer consisted of S22 buffer

(450mM NaCl, 10mM KCl, 58mM $MgCl_2$, 10mM $CaCl_2$, 100 mM HEPES- pH 7.8) supplemented with a protease inhibitor cocktail (SIGMA-ALDRICH) and PhosSTOP EASYpack phosphatase inhibitor cocktail (Roche). Additionally, *A. yongei* tissue slurry, but not *S. pistillata* or *P. damicornis*, was passed through a 21G1 needle to aid the break up of mucus. The resulting tissue slurries of *A. yongei*, *S. pistillata* and *P. damicornis* were separately labeled "Crude Homogenate 1" and kept on ice for further processing.

Tissues for the anti -NKA_α Western blot analysis required further optimization: the crude homogenate 1 was fractionated by differential centrifugation in order to enrich for membranes and the associated membrane proteins. To do this, zooxanthellae were first pelleted from ~1 mL of the crude homogenate 1 at 500 rcf for 15 min, at 4°C. The supernatant containing host cell membranes and cytoplasm was removed and centrifuged at 21,130 rcf (maximum of available centrifuge) for 30 min, at 4°C. This pelleted the cell membranes and associated proteins (Fig. 8). Membranes were then resuspended in a volume of 100 µL homogenization buffer.

All tissue samples prepared for Western blot analysis were subjected to a Bradford protein concentration assay (BioRad). Briefly, standards of various concentrations (0.14 µg-μL^{-1}, 0.28 µg- μL^{-1}, 0.56 µg- μL^{-1}, 0.7 µg- μL^{-1}, and 0.9 µg- μL^{-1}) of bovine serum albinum (BSA) were used to produce a standard curve plotting concentration against light absorbance via a spectrophotometer. Coral sample concentrations were calculated using the slope of the standard curve and absorbance values of BSA standard concentrations.

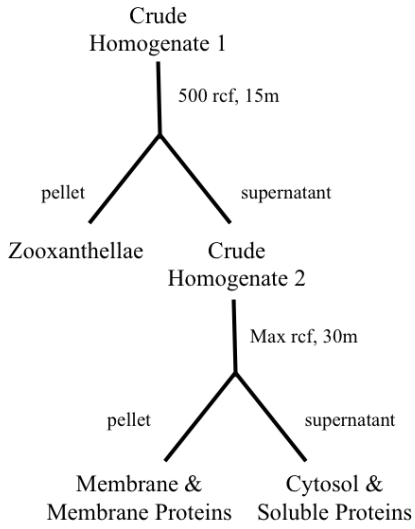

Figure 7: Schematic of differential centrifugation of coral tissue

Protocol of centrifugation steps to enrich for membranes and membrane proteins such as NKA

For rabbit anti-VHA_B Western blot analysis, 1-5 µg were used for protein detection, and for rabbit anti- NKA_α blots, 5-20 µg were used for protein detection. Samples were denatured in a 1:1 solution of sample buffer consisting of 95% 2X Laemmli buffer (BioRad) and 5% beta-mercaptoethanol (BME). Those samples designated for VHA detection were then pre-incubated at 70°C for 15 min, while those designated for NKA detection were pre-incubated at room temperature for 15 min. This incubation was conducted in sample buffer in order to induce protein denaturing. Denatured samples were then run on a 10% polyacrylamide SDS-PAGE gel at 60V for approximately 15 minutes, or until dye had run through stacking gel. The voltage was then adjusted to ~180-200 V until completion. Samples were run along side BioRad Precision Plus Protein Pre-stained Standards Dual Color to determine relative protein molecular weight.

Upon completion of electrophoresis, the separated proteins in the gel were transferred to a methanol-activated PVDF membrane using TurboBlot transfer stacks and transfer buffer (BioRad). The membrane was then blocked for one hour on a room temperature (RT) shaker in blocking buffer (5% powdered fat-free milk in Tris-Buffered Saline + 0.1% Tween detergent (TBS-T)). Blocking buffer was then replaced with primary antibodies diluted in blocking buffer (anti -NKA_{α} 0.04 μg ml^{-1}; anti -VHA_B .0015 μg ml^{-1}) and the membrane incubated in the appropriate antibodies overnight at 4°C on shaker. Unbound primary antibody was removed by three TBS-T washes, 10 minutes each on a RT shaker. Secondary goat anti-rabbit antibody conjugated with Horse Radish Peroxidase (HRP) (BioRad) was then diluted in blocking buffer (1:10,000) and incubated with the membrane for one hour on a RT shaker. Unbound secondary antibody was removed by three, 10-min washes in TBS-T, on RT shaker. The membrane was developed using 1.5mL of a 1:1 solution from enhanced chemi-luminescence kit (GE Healthcare) and bands were visualized using the BioRad Chemidoc Imaging system.

A pre-absorbed peptide control for the customized VHA_B antibodies was also generated to further ensure specificity of protein recognition; primary antibodies were pre-incubated with 400-fold excess VHA_B peptide (0.006 μg ml^{-1}) overnight, at 4°C. This was calculated using the molecular weight of a typical antibody, (composed of two heavy chains, and two light chains), ~150 kDa and the molecular weight of the peptide, ~1.7 kDa.

IMMUNOLOCALIZATION

In order to visualize the localization of proteins within coral tissues, the validated antibodies were used in immunolocalization studies.

Immunohistochemistry

Corals branches were broken into ~2.5 cm (1 inch) fragments and immediately fixed in 3% paraformaldehyde in S22 buffer, and incubated overnight at 4°C on shaker. Samples were then decalcified in Ca^{2+}-free S22 buffer supplemented with 0.5M EDTA for 7-14 days. Decalcification buffer was changed daily until skeleton was fully dissolved. *A. yongei* tissues were dissected with a scalpel to separate the "tip" from the "middle" of a branch. The tip was considered the top 0.5 cm of a given branch, where zooxanthellae seemed more absent and the middle was the base of the fragmented branch.

Samples for all species (N=3 for *A. yongei*, N=3 for *S. pistillata* and N=6 for *P. damicornis*) were dehydrated and prepared for paraffin embedding by 10-min incubations in a series of increasing ethanol concentrations (70%, 95%, 100%) followed by three incubations in xylene. Tissues were then subjected to three, 30-min incubations in paraffin, embedded in paraffin and allowed to set in the wax for 1-2 days at RT.

Tissues were then sectioned at 7μm thickness using a rotary microtome (Kedee). Tissue sections were floated on a drop of water on microscope slides and incubated on a hotplate at 40°C for approximately one minute. Slides were then placed on top of a paper towel and the hotplate temperature was lowered to 30°C. Slides were left overnight to allow the wax and tissue to affix to glass.

Prior to immunostaining, samples were deparaffinized and tissue rehydrated by 10-min incubations in xylene (3 times), followed by hydration in a decreasing ethanol series (100%, 95%, 70%). Next, tissues were further hydrated and permeabilized in 0.2% triton-x detergent in Phosphate Buffered Saline (PBS-T) for 10 min. Using a hydrobarrier pap pen (Invitrogen) a small square was drawn around each tissue section,

and samples were incubated in 70-100 μL blocking buffer (2% normal goat serum (NGS) + 0.02% keyhole limpet hemocyanin (KLH) in PBS-T) within the drawn square for one hour at RT.

Primary antibodies were diluted in blocking buffer (anti-NKA_{α} 2 μg ml^{-1}; anti-VHA_{β} 3 μg ml^{-1}) and 70-100 μL applied over tissues, overnight at 4°C in a humidified chamber (humidity was maintained using wet paper towels on bottom of chamber). Unbound primary antibody was washed off by a single five-min wash in PBS and tissues were re-permeabilized for five minutes in PBS-T. The hydrobarrier was redrawn and tissues were incubated for one hour at RT in the secondary antibody (goat anti-rabbit conjugated with Alexa-555 fluorphore; Invitrogen) diluted in blocking buffer (4 μg ml^{-1}). Secondary antibody was then pipetted off, and nuclei were stained using Hoescht DNA stain (1 mg ml^{-1}) for five min. Slides were washed in PBS for five minutes to remove excess stain. Finally, the hydrobarrier was removed, slides mounted with fluorogel (Electron Microscopy Solutions) and a coverslip, and then sealed with nail polish.

NKA and VHA expression and localization in the growing tip vs. middle of *A. yongei* branches was examined via immunohistochemistry. Initial microscope observations revealed that NKA appeared to be localized to the basolateral membrane of all calicoblastic cells, and to the apical membrane of all oral ectoderm cells, thus no further analysis was conducted (N=3). On the contrary, VHA appeared to more often be localized to the aboral endoderm in the tip samples as was previously observed (Barott personal observations). In order to more closely examine VHA expression frequency in the aboral endoderm in the tip vs. middle, 89 images were taken over several preparations (N=3) in which all four cell layers could be observed, at a similar light exposure. Next,

the images were examined for localization to the aboral endoderm in a blind manner, in order to avoid bias.

Secondary antibody controls were used to eliminate the possibility of non-specific binding of the secondary antibody; controls were incubated in blocking buffer lacking secondary antibody. Sections were examined using a fluorescence microscope (Zeiss AxioObserver Z1) and some images were captured using structured illumination (Zeiss Apotome 2). The secondary antibody was visualized using the microscope's Cy3 channel and nuclei visualized by the DAPI channel. Control images were taken with the same exposure settings used for the corresponding anti-NKA_{α} and anti-VHA_{β} treatments. Images were adjusted, for brightness and contrast only, using Zeiss Axiovision software.

Immunocytochemistry

To further examine VHA symbiosome immunostaining in more detail, isolated coral cells were prepared from *A. yongei* branches using a soft bristled toothbrush and 50 mL of filtered sea water (FSW), 0.2µm. Cells were then filtered through a 40µm cell strainer to help break up and remove mucus. Next, 1mL aliquots were pelleted at 3,000 rcf for 4 min at 4°C, resuspended in 4% paraformaldehyde in S22 buffer, and fixed for 15 min on ice. Cells were again pelleted, fixative removed and the cells concentrated in S22 buffer (~10mL of cells in fixative were resuspended in 500 µL of S22). Then, a square was drawn on glass slides using hydrobarrier pap pen, ~5µL cells spread within this square, and allowed to air-dry. Several layers of cells were applied to the slide depending on the density determined by microscope examination. Once affixed to slides, isolated cells were permeablized in PBS-T for 10 min. Samples were then blocked for 1 hour at RT with 2% NGS + 0.02% KLH in PBS-T.

Cells were probed with the primary anti-VHA_B antibody diluted in blocking buffer (3 $\mu g\ ml^{-1}$) overnight at 4°C in a slide box lined with damp paper towels, to prevent the drying out of samples. Primary antibody was removed with a 5 min wash in PBS. The cells were then incubated in secondary antibody (4 $\mu g\ ml^{-1}$), and stained with Hoescht to visualize DNA, as described previously in tissue section staining. Secondary antibody controls were treated as above except no primary antibody was used. Immunofluorescence was visualized and captured as described, above.

Analysis of VHA staining in isolated cells was conducted by random examination of nearly 300 cells (288 cells) from the preparations (N=3). Symbiosome staining, as characterized by a red fluorescent ring surrounding the zooxanthellae, or lack of symbiosome staining was recorded in three categories: 1) isolated zooxanthellae with one observable algal nucleus 2) a single zooxanthellae within a coral host cell containing two observable nuclei (one algal nucleus, one host nucleus), or 3) two zooxanthellae within a single coral host cell containing three observable nuclei (two algal nuclei and one host nucleus). Zooxanthellae were detected by their shape and green autofluorescence. Hoescht staining revealed nuclei, where the algal nucleus was distinguishable from coral by its particular shape (caused by permanently super-coiled chromosomes).

RESULTS

NKA and VHA expression in *A. yongei*, *S. pistillata* and *P. damicornis*

Optimized immunodetection of NKA and VHA via Western blot confirmed protein expression of VHA_B and NKA_α. For all three species, VHA Western blots recognized a ~55 kDa protein (Fig 9B) which is the predicted size calculated from the cDNA sequence for VHA in *A. yongei* [21] and BLAST searches against transcriptome data available for *S. pistillata* and *Pocillopora*. The epitope for the VHA antibodies is 100% conserved in *A. yongei*, *S. pistillata* [21], and *P. damicornis* based on available transcriptome data (http://cnidarians.bu.edu/PocilloporaBase). Furthermore, the 55 kDa band was not present in the peptide pre-absorption control blots for any of the three species (Fig. 9B). Western blots with the anti-NKA_α antibodies conceded a ~110 kDa band (Fig 9A) in all three species which is the predicted size for the NKA α subunit in corals based on available transcriptome data. Furthermore, the epitope for the NKA_α antibodies is highly conserved in *A. yongei*, *S. pistillata* and *P. damicornis* (Table 1). These results indicate that the antibodies used in this study specifically recognize VHA_B and NKA_α in corals.

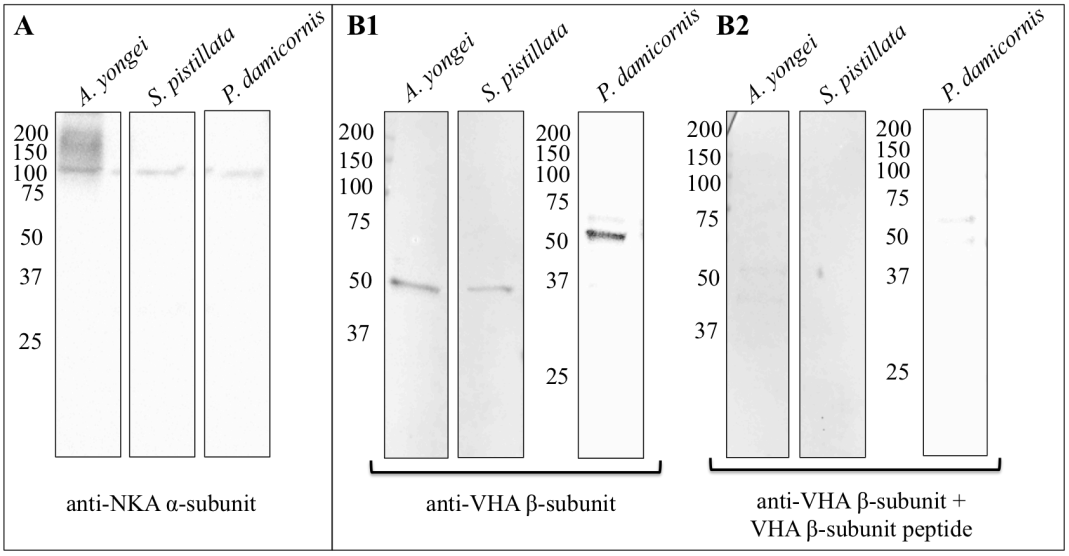

Figure 8: Immunoblot detection of Na+/K+ -ATPase (NKA) and V-type H+ -ATPase (VHA) in corals

A) NKA B1) VHA B2) peptide pre-absorption control showing lack of signal

Table 1: Epitopes used for anti-NKA alpha, and anti-VHA B antibodies

VHA_B epitope was 100% conserved, and NKA_α epitope was highly conserved in all three species of coral

Target protein	**Immunogen**	**Immunogen size** (a.a.)	***A. yongei* Immunogen % Identical (% similar)**	***S. pistillata* Immunogen % Identical (% similar)**	***P. damicornis* Immunogen % Identical (% similar)**
Na^+-K^+ ATPase (α subunit)	aa 551-850 of human NKAα1 (#)	300	87% (97%)	87% (97%)	93% (96%)
V-H^+-ATPase (B subunit)	AREEVPGRRGFPG Y (*)	14	100%	100%	100%

VHA immunolocalized in aboral endoderm and around symbionts

Previous research has shown VHA localization immediately surrounding symbionts as well as strong evidence of VHA's role in the acidification of the symbiosome space in *A. yongei* and *S. pistillata* [21]. To determine whether similar localization was present in *P. damicornis* six tissue samples were examined via immunohistochemistry along with samples of *A. yongei* and *S. pistillata*. VHA localized around *Symbiodinium* cells in the endodermal layers in all three species (Fig. 10 A-C). However, this "symbiosome staining," while observed in some *P. damicornis* (Fig. 10C), in others (sampled in May and June of 2014) appeared less abundant or difficult to distinguish against the red autofluorescence of symbionts, and the signal bleached quickly (Fig. 10D). VHA was also occasionally localized in the aboral endoderm of *A. yongei* and in one sample of *P. damicornis* (Fig. 10 A and E), consistent with a study currently under peer review [9].

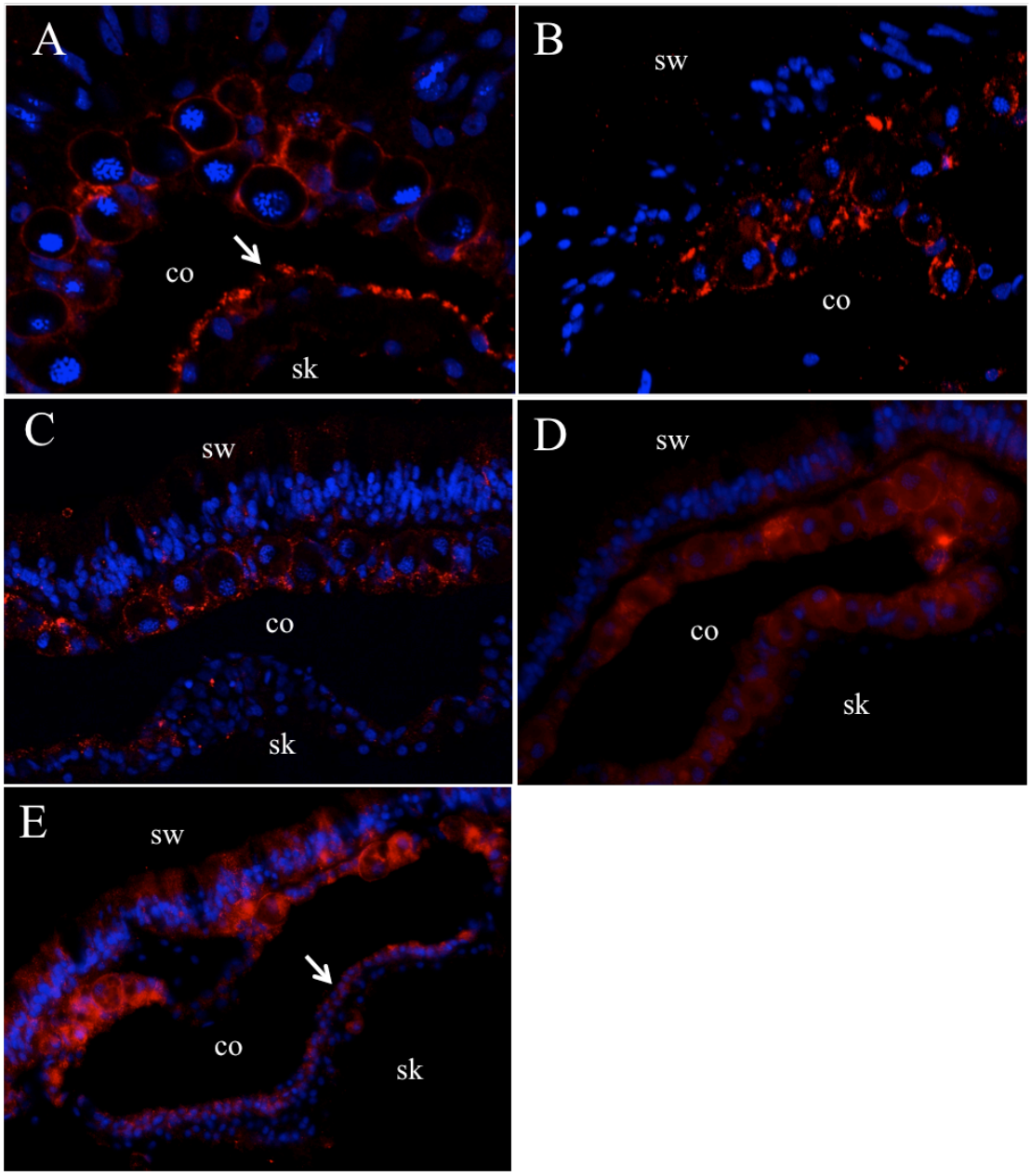

Figure 9: VHA in *A. yongei*, *S. pistillata*, and *P. damicornis* tissues

VHA localization surrounding symbionts in (A) *A. yongei* (B) *S. pistillata* and (C) *P. damicornis*. Symbiosome staining was less obvious in some *P.* damicornis samples (D). VHA in the aboral endoderm of some samples of (A) *A. yongei* and (E) *P. damicornis* indicated by arrows. VHA in red, nuclei in blue; sw= seawater side, co= coelenteron, sk= skeleton side

VHA is localized on symbiosome membrane, not host or *Symbiodinium* membranes

Because of the close proximity of the coral plasma membrane, the symbiosome membrane, the algal cell wall and algal plasma membrane, further examination of whether the observed VHA symbiosome staining was localized to the symbiosome exclusively, was necessary. Immunocytochemistry on isolated *A. yongei* cells revealed that VHA was found around the perimeter of *Symbiodinium* in symbiosis with a coral cell. 86% (88/102) of cells examined with one or two algal cells surrounded by a coral cell contained VHA staining around the symbiont, but not extended around the host nuclei (Fig. 11C-F). This indicates that VHA is not present in the coral plasma membrane in which it would be observed around the external perimeter of the host nuclei. In contrast, only 11% (21/186 cells) of the zooxanthellae cells not in symbiosis (lacking host nucleus) contained observable VHA staining in the perimeter of the algal cell (Fig. 11G-H). This pattern indicates VHA is not in the symbiont (algal plasma membrane or algal cell wall). Furthermore, of this 11%, staining was generally less interconnected, sometimes surrounding only half the algal cell and could be a result of partial symbiosome membrane remaining intact due to insufficient dissociation during cell preparation. The observed symbiosome staining on these isolated cells provides supporting evidence that VHA is indeed localized to the symbiosome exclusively and not the coral host membrane, nor the zooxanthellae cell.

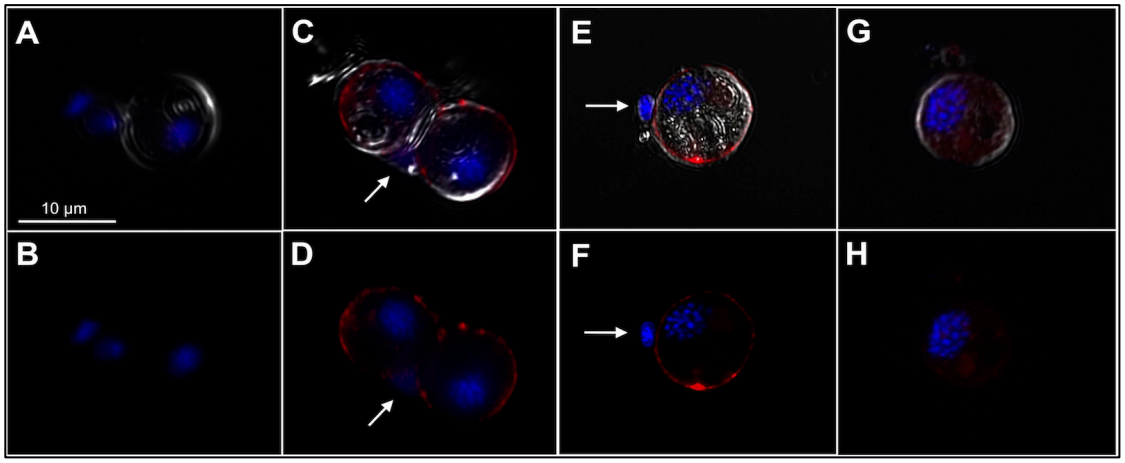

Figure 10: Immunolocalization of VHA in cells isolated from the coral *A. yongei*

A-B) Secondary antibody control, C-D) Coral cell containing two *Symbiodinium* cells, E-F) Coral cell containing a single *Symbiodinium* cell, and G-H) *Symbiodinium* cell separated from the host. Arrows indicate host nuclei. Anti-VHA_B antibody shown in red, nuclear staining is shown in blue. Top row includes DIC image merged with corresponding fluorescence image in bottom row.

Frequency of VHA localization to the aboral endoderm increases in the tip

To further investigate the VHA localization in the aboral endoderm the tips and middles of *A. yongei* branches were examined. Of the images examined from tissue sections examined from the tip, almost all (96%; N=3) exhibited some, or strong localization of VHA to the aboral endoderm (Fig. 11A and 11C). In contrast, of the images examined from tissue sections from the middle of a branch, less than half (40%; N=3) exhibited some or strong localization of VHA to the aboral endoderm (Fig. 11B and 11C). Since the tips of a branch calcify at a more rapid rate compared to the middle of a branch, these results suggest that VHA is more abundantly expressed in the aboral endoderm at the tip of a coral in order to facilitate rapid precipitation by removing H^+ away from the site of calcification (figure 12).

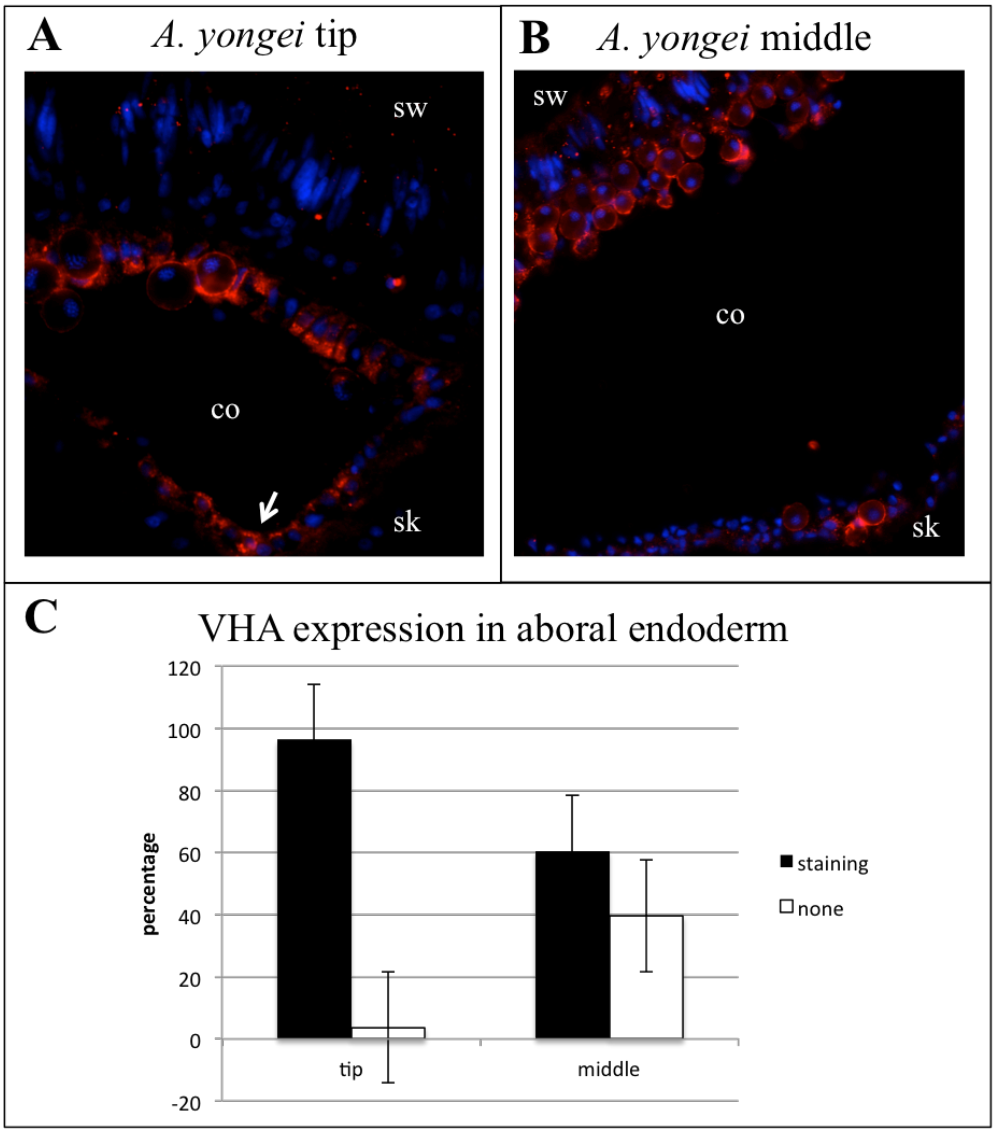

Figure 11: VHA of the aboral endoderm in the tips and middles of *A. yongei* branches

A) VHA immunolocalization of the aboral endoderm of *A. yongei* branch tip B) Lack of VHA localization to the aboral endoderm in the middle of a branch. sw= seawater side, co= coelenteron, sk= skeleton side. C) Frequency of aboral endoderm localization (black bar) in the tip vs. middle of branches (N=3).

NKA localization in calicoblastic layer of *A. yongei*, *S. pistillata* and *P. damicornis*

Consistent with previous studies on *A. yongei* and *S. pistillata* [9], NKA was localized in the calicoblastic epithelium of all three species (Fig. 12) indicating a potential role in calcification.

In *A. yongei* only, NKA was also localized apically in the oral ectoderm (Fig. 12A) consistent with previous studies, suggesting a species-specific mechanism- perhaps for uptake of DIC and nutrients [9].

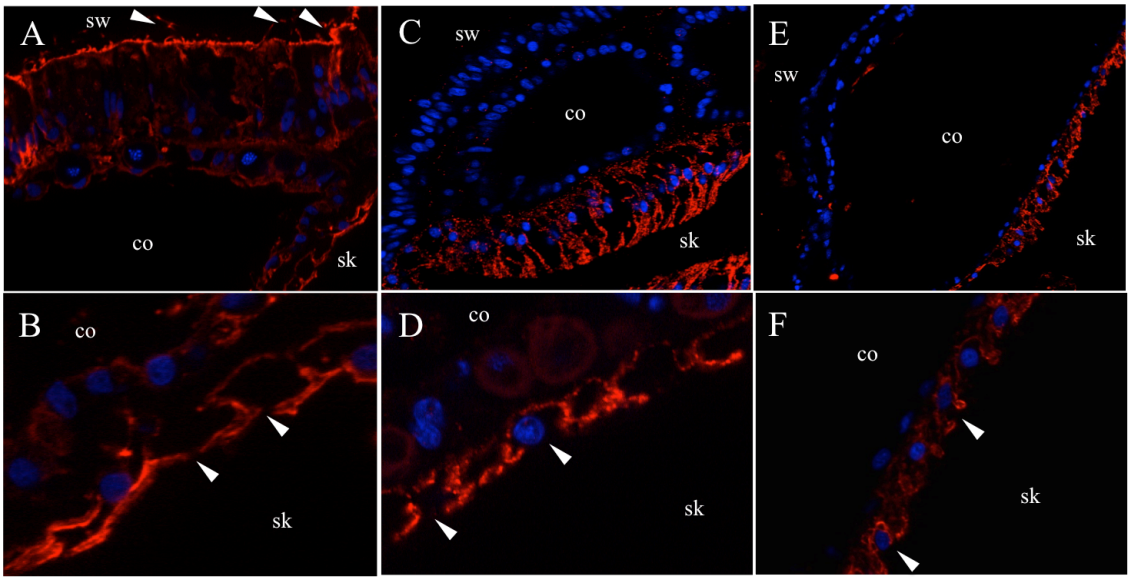

Figure 12: NKA in *A. yongei*, *S. pistillata*, and *P. damicornis* tissues

NKA localization in calicoblastic cells of A. yongei (A) S. pistillata (C) and P. damicornis (E) where it was present in the basolateral membrane, arrows indicating apical pole (B, D, F). NKA also present in the oral ectoderm of *A. yongei*, arrows indicating cilia (A). NKA in red, nuclei in blue; sw= seawater side, co= coelenteron, sk= skeleton side

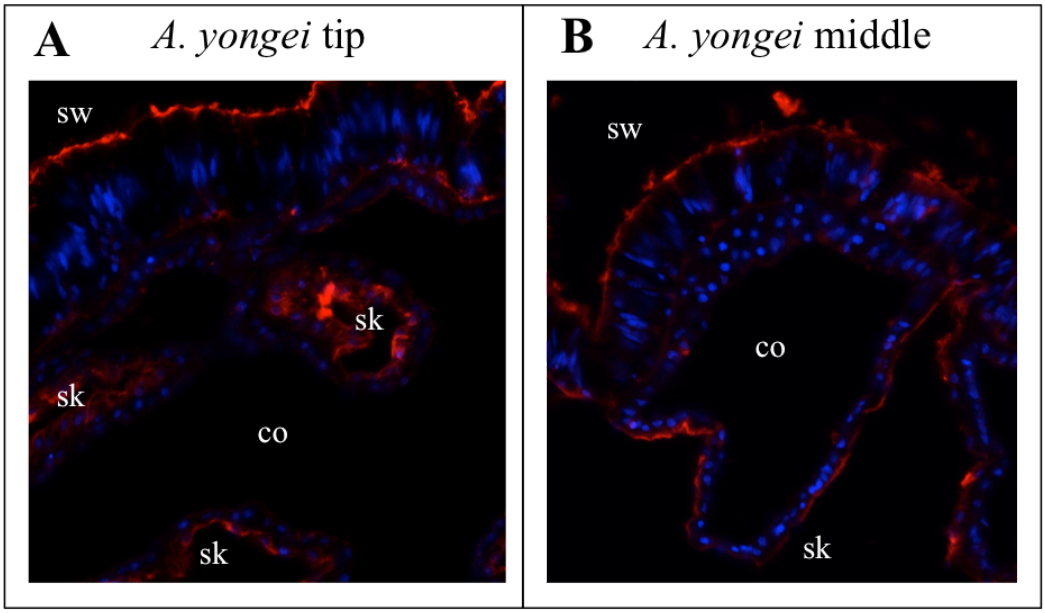

Figure 13: Immunolocalization of NKA in the tips and middles of *A. yongei* branches

NKA localization in the oral ectoderm and calicoblastic cells in tip of *A. yongei* branch (A) and middle of *A. yongei* branch (B). NKA in red, nuclei in blue; sw= seawater side, co= coelenteron, sk= skeleton side

NKA localization does not differ in tip vs. middle of *A. yongei* branches

In order to test the hypothesis that NKA localization may differ in calicoblastic cells at the tip compared to the middle, three *A. yongei* branches were dissected, and examined by immunolocalization. Unlike VHA, NKA did not appear to differ in localization nor abundance in the tip vs. the middle of *A. yongei* branches. Similarly, it did not appear to differ in the oral ectoderm (Fig. 13).

Acknowledgements

This work in part includes material currently under peer-review for publication: Barott KL, Perez SO, Linsmayer LB, Tresguerres M. Differential localization of ion-transporters suggests distinct mechanisms for calcification and photosynthesis between complex and robust corals. *In review in Proceedings of the Royal Society B.* Data from this thesis has also been accepted as contribution for publication in: Barott K, Venn A, Perez S, Tambutte S, Tresguerres M. Coral host cells acidify symbiotic algal microenvironment to promote photosynthesis. *PNAS USA*.

DISCUSSION

VHA is present in the symbiosome membrane of corals, contributing to a CCM

Recently, we described a carbon concentrating mechanism in the corals *A. yongei* and *S. pistillata*, demonstrating that VHA is responsible for the acidification of the symbiosome lumen [21]. This study proposed that the decrease in pH facilitates the speciation of DIC to enrich substrate binding of RubisCO for photosynthesis. Results from this thesis have confirmed the intracellular localization of VHA to the symbiosome membrane in isolated *A. yongei* cells.

The localization of VHA to the symbiosome membrane in concert with results from our previous study [21] provides strong evidence for the participation of VHA as part of a CCM. VHA likely utilizes coral-generated ATP to generate a sharp proton gradient in the symbiosome lumen using H^+ from the host cytoplasm. HCO_3^- in the symbiosome would then be rapidly converted to CO_2 in the presence of carbonic anhydrase [32]. This CO_2 diffuses through several membranes into the chloroplasts where photosynthesis occurs, and where there are likely other CCM's [33]. Here, RubisCO, which inherently has a low affinity for CO_2, has an enriched availability of the substrate to initiate photosynthesis. Furthermore, VHA contribution to a CCM suggests that corals are able to exert some biological control over the symbionts to modulate photosynthesis, although *Symbiodinium* likely also contributes in part to the acidification of the symbiosome lumen, for example using P-type H^+-ATPases [34, 21].

The localization of VHA in the symbiosome membrane in *P. damicornis* is comparable to the observed localization in the closely related *S. pistillata*, and in *A. yongei*. This supports that VHA contribution to a CCM is conserved across coral species

of two distantly related clades. *P. damicornis* and *S. pistillata* are estimated to have diverged from *A. yongei* between 250-400 million years ago [22, 23]. The separation of the complex and robust clades represents one of the deepest splits that exists in Scleractinian corals [24], thus corals likely evolved the utilization of VHA as a CCM early in their evolutionary time.

VHA symbiosome expression observed in *P. damicornis* appeared to decrease and nearly disappear in some tissues that were sampled in May and June of 2014 compared to samples taken in January 2014. Furthermore, *A. yongei* and *S. pistillata* samples that were fixed in May and June, on the same days as *P. damicornis*, still demonstrated clear localization of VHA in the symbiosome membrane. These patterns suggest that, although the use of VHA as part of a CCM may be conserved, its role in photosynthesis and the plasticity of control that a coral may have in regulating VHA is species-specific. While the change in VHA abundance surrounding the symbionts of *P. damicornis* is unclear, few changes in the aquarium environment may offer some explanation.

For example, in the summer of 2014, an unusually strong upwelling occurred along the coast of California, from which seawater was drawn for experiments in this study. Upwelling events typically result in an increase in dissolved nutrients, such as nitrogen and phosphate compounds, as well as $CO_{2,}$, in surface waters. It is well documented that increases in dissolved inorganic nitrogen lead to increases zooxanthellae density due to a higher availability of nitrogen as nutrients for cell division [35, 36, 37, 38, 39]. CO_2 increases have also been linked to increases in symbiont division [40].

Increases in algal density can potentially lead to cell damage by the generation of reactive oxygen species (ROS) [38, 39]. Additionally, increases in nutrients have been correlated with a decrease in calcification rates, potentially due to the greater proportion of photosynthate (sugars) being used by *Symbiodinium* for growth and division, which depletes the photosynthate available to corals for calcification and the generation of ATP [36, 41]. Such drastic physiological changes occurring in both the zooxanthellae and coral likely disrupt the photosynthetic balance between coral and symbiont. An imbalance in photosynthetic exchange could in turn, lead to alterations in VHA regulation and abundance. For example, the coral may downregulate VHA in order to limit the CO_2 available to the symbionts for photosynthesis. Or on the contrary, perhaps a depletion of ATP may leave the coral unable to maintain the energy-costly CCM [38]

My results cannot help determine whether VHA abundance in *P. damicornis* samples was decreased because the coral was actively regulating it, or whether the coral did not have the metabolic energy available to implement it. However, the fact that VHA was still abundantly present in the symbiosome of *A. yongei* and *S. pistillata* samples taken from the same days, shows that coral responses to environmental conditions are species-specific.

Interestingly, the extent to which photosynthesis rates were inhibited by the VHA-specific inhibitor bafilomycin differed between *A. yongei* and *S. pistillata* [21], where net O_2 production decreased by 83% in *A. yongei* but only 30% in *S. pistillata*. To explore the relationship between the extent of which corals utilize VHA as part of a CCM, and the sensitivity of this relationship to environmental stress, it would be interesting to compare: 1) relative VHA abundance by western blot, and 2) inhibited photosynthesis

rates induced by bafilomycin between several species of corals subjected to different environmental conditions, for example: ocean acidification, nutrient enrichment, or increased irradiance and temperature conditions. Results of this study have certainly raised many novel questions regarding the dynamics of coral-algal symbiosis and how different species of corals regulate symbiosis physiology.

VHA increases in the aboral endoderm of the growth tip of *A. yongei* branches

Potential differences in VHA localization were analyzed in the growth tip and middle sections of *A. yongei* coral branches. VHA was found more frequently in the aboral endoderm in the tips of coral branches. These results suggest that VHA activity facilitates calcification by removing H^+ away from the site of calcification, as follows: in order to precipitate $CaCO_3$, corals increase the pH at the site of calcification known as the subcalicoblastic medium [26]. The mechanisms by which H^+ are removed from the subcalicoblastic medium into aboral endoderm are unknown and may differ from species to species. Cells in the aboral endoderm must also get rid of H^+ to preserve their intracellular pH and maintain flow of H^+ ions from the subcalicoblastic medium (Fig. 14). My results suggest that this is achieved by VHA in the basolateral membrane of aboral endoderm cells, which utilize energy from ATP hydrolysis to pump protons from the cytoplasm into the coelenteron.

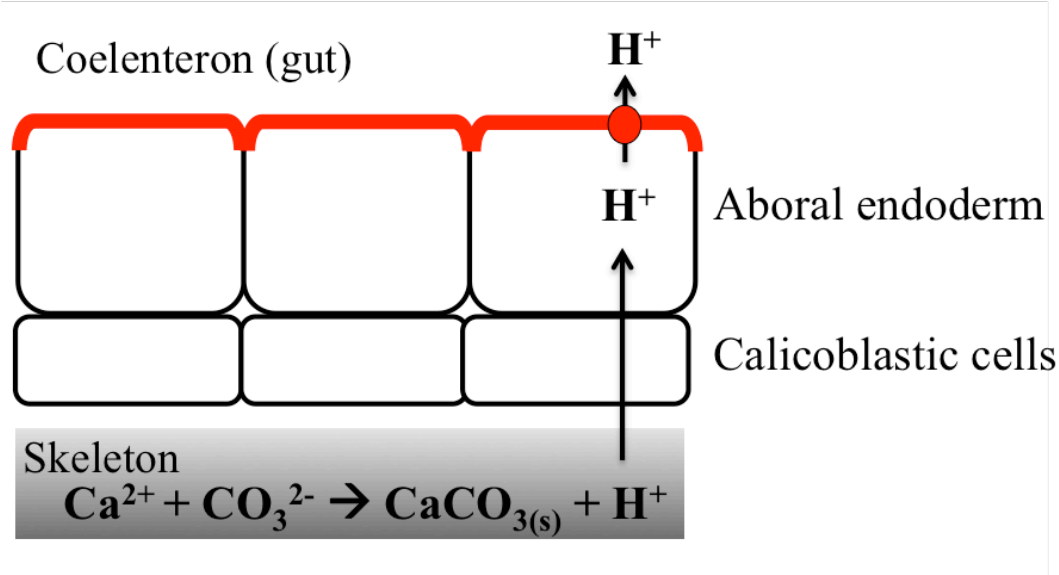

Figure 14: Model of VHA facilitating removal of H+ to facilitate calcification

H^+ removal from the subcalicoblastic fluid is maintained by VHA in order to promote precipitation of $CaCO_3$ at the site of calcification. H^+ is translocated to the aboral endoderm by some unknown mechanism. Here, VHA utilizes ATP to pump protons into coelenteron to be neutralized, and maintain intracellular pH of aboral endoderm.

Differences in the abundance of VHA in the aboral endoderm within a coral branch, as well as the dual localization of VHA in the aboral endoderm and symbiosome, highlight the importance of detailed cellular studies. For example, Kaniewska and colleagues found through transcriptome analysis that VHA mRNA was down-regulated in corals subjected to ocean acidification conditions [42]. They concluded that corals down-regulate VHA under decreased pH stress to mitigate the uptake of CO_2 from seawater, hypothesizing that corals 'shut down' energetically costly processes under environmental stress. However, transcriptome studies only measure the levels of mRNA present in the whole coral sample and cannot discriminate between localization of

proteins in different cell types or intracellular compartments. For example, an increase in VHA mRNA in the aboral endoderm under ocean acidification could be masked by a decrease in VHA mRNA in the oral endoderm (where the symbiosome is). Our laboratory is currently conducting studies to characterize potential VHA changes induced by ocean acidification in these two tissue layers. Furthermore, gene expression is not necessarily directly proportional to protein expression [43], and thus, mRNA abundance may lead to a misrepresentation of how much of a protein is present, let alone active.

Results of this thesis also demonstrate the importance of considering where *within* a coral branch mRNA and protein expression are quantified and analyzed. Protein expression is not uniform throughout a colony of the coral, and thus environmentally induced changes may be more or less prominent in the tip or middle of a coral branch, for example. This study reiterates the importance of determining the specific localization of proteins in coral before subjecting them to environmental stress so that induced regulatory adjustments can be understood.

NKA localized in calicoblastic layer of corals, but species-specific elsewhere

Recently we showed differential localization of NKA between *A. yongei* and *S. pistillata,* where it was localized in the calicoblastic cells of both species; however only in *A. yongei* was the protein found in the apical membrane of oral ectoderm cells [9]. In order to further explore this species-specific difference, a second representative of the clade Robusta was investigated. In *P. damicornis*, NKA was also found abundantly in the calicoblastic cells, and, similar to *S. pistillata*, it was absent in the apical membrane of cells of the oral ectoderm (Fig. 12). These results suggest that the difference in localization of NKA in the oral ectoderm is clade-specific. While, NKA in *A. yongei*

may be involved in the uptake of nutrients or DIC by secondary active transport in the oral ectoderm [9], corals from the clade Robusta (such as *S. pistillata* and *P. damicornis*) likely have evolved different mechanisms for nutrient and DIC uptake.

Localization of NKA to the basolateral membrane of the calicoblastic cells was consistent in all three coral species tested. This suggests that NKA plays an important role in calcification in both Robusta and Complexa. However, since the divergence of the two clades occurred before the appearance of coral skeletons in the fossil record [22], other components of the calcification mechanisms likely evolved independently.

The role of NKA in the calicoblastic cells may be to couple the import of ions, DIC or proteins important for calcification to the subcalicoblastic medium. Interestingly, Kaniewska and colleagues (2011) also found that NKA was up-regulated in corals exposed to ocean acidification conditions in *A. millepora*, suggesting increased NKA abundance and activity enhance calcification [42]. However, *A. millepora* likely exhibits similar NKA localization and cellular mechanisms to *A. yongei,* and thus the detected up-regulation of NKA could be attributed to the oral ectoderm cells. It would be interesting to compare NKA expression changes under environmental stress between the two clades, as even their calcification mechanisms (and the relative involvement of NKA) likely differ.

NKA abundance does not appear to differ in tip vs. middle of *A. yongei* branches

Immunohistochemistry analysis did not reveal an observable difference in abundance or localization of NKA within a branch of *A. yongei*. In the oral ectoderm, this is likely because nutrient and ion uptake is important for all physiological processes throughout the coral colony, though the reasons for those nutrients may differ throughout

the colony. Lack of an observable difference in the calicoblastic cells may be explained by the fact that, although calcification is occurring most rapidly in the growth tips, the entire coral does still undergo calcification and thus may require a significant "baseline" of NKA for maintenance of $CaCO_3$ precipitation.

Furthermore, there are several ways in which cells can regulate protein function and activity, aside from gene expression, for example: post-translational modifications such as the generation of splice variants, phosphorylation or glycosylation commonly occur in the cell and can change the affinity and activity of a protein. Splice variants of NKA result in different affinities for Na^+ in mammalian renal cells [44]. Likewise, different combinations of isoforms of the several NKA subunits (α, β, γ) may alter regulation and activity in different tissues, or locations within a branch.

Moreover, a protein may be expressed, but inactive until it is in the correct cellular compartment. For example, in human alveolar cells subjected to hypoxia, NKA is endocytosed from the membrane and stored in intracellular vesicles where it becomes inactive until restored to normoxic conditions when NKA is re-inserted into the membrane [45]. Finally, other proteins and substrates may be up- or down-regulated to enhance calcification at the tip, for example proteins that transport DIC or nucleation proteins. Certainly, much remains to be studied regarding the role of NKA in calcification among various species of corals.

Conclusions:

The results of this thesis highlight several potential roles for VHA and NKA in photosynthesis and calcification, in three species of corals from two diverged clades. To summarize: the confirmation of VHA in the symbiosome membrane supports previous

strong evidence for the regulation of VHA as part of a CCM. Furthermore, this mechanism is likely conserved in many species, and may be one way in which the host can control photosynthesis. However, its regulation and responses to environmental stress may be species-specific, as suggested by differences in VHA symbiosome localization between the three species examined from the Summer of 2014. This is something that should be further investigated.

NKA and VHA both appear to play a role in calcification, and VHA may be up-regulated to facilitate calcification in the tip. Furthermore, NKA localization differs in the oral ectoderm where it is present in *A. yongei*, only. Additionally, although NKA was present in the calicoblastic epithelia of all three species, it likely evolved independently between the two clades.

This study has demonstrated both similarities in protein expression among species as well as clear differences. I have shown that even within a single branch of a coral can protein expression vary. These results especially highlight how little is known about the involvement of fundamental proteins in photosynthesis and calcification, and how dynamic their involvement likely is.

Similar as to how protein expression and cellular mechanisms are often species-specific, protein expression often differs throughout the life stages of an organism, especially during development- a process known as ontogeny. For example, Reyes-Bermudez et al. (2009) demonstrated differential expression of three coral genes potentially related to calcification between the larval stage, early settlement stage and juvenile stage of the coral *A. millepora* [46]. It would be interesting to see whether NKA and VHA protein expression varied during these life-stages of a young coral in which

settlement, initiation of calcification, and metamorphosis must all rapidly occur in order for a larvae to eventually establish itself as and adult colony. During this thesis, NKA and VHA antibodies have already been optimized by western blot for *P. damicornis* larvae (Appendix), thus, the tools to study this life-stage are in place.

Continued study of the cellular mechanisms of photosynthesis and calcification are crucial for predicting how various species of corals will differentially cope with environmental stress. Thorough studies should be conducted in representative species from the two clades, Robusta and Complexa, so that a clearer picture on how corals differ physiologically can be envisioned. Furthermore, investigations of how these mechanisms differ within a single individual, between the different life-stages of a coral, and between populations that inhabit different field locations should also conducted. Having a fundamental understanding of coral cell physiology will allow for the application of a "mechanistic" approach in assessing coral health; this fundamental knowledge will provide a point of reference as to how corals work under normal conditions so that we can "diagnose" species that are vulnerable to anthropogenic stress, understand *why*, and take action to prevent or at least mitigate their decline in a changing global environment.

APPENDIX

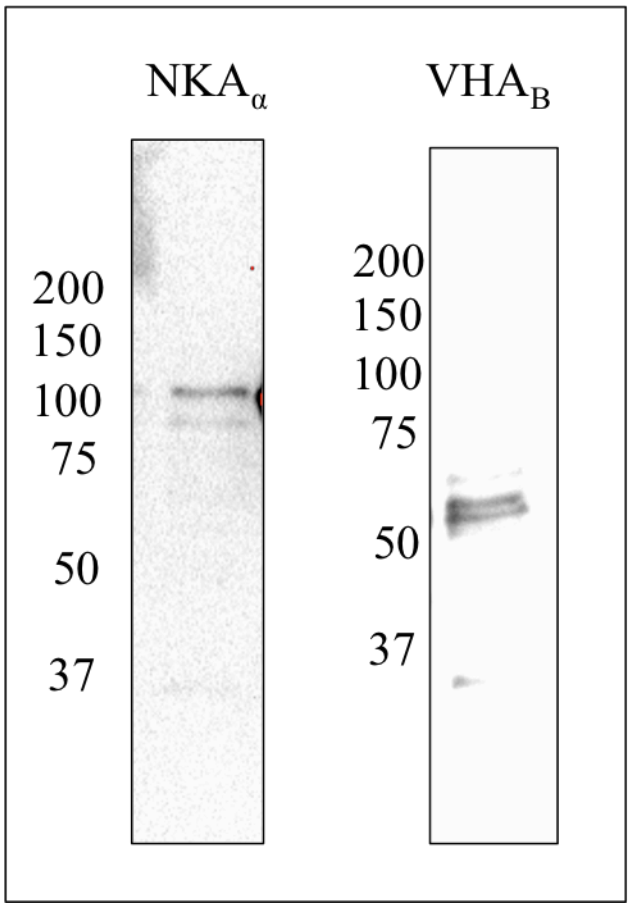

Figure 15: Immunoblot detection of Na+/K+ -ATPase (NKA) and V-type H+ ATPase (VHA) in *P. damicornis* larvae

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