Cellular Mechanisms for Nitrogen Transport in the Coral-Algal Symbiosis

Rationale: Coral reefs provide services totaling $10 trillion despite covering only ~0.3% of the ocean floor[1]. Their evolutionary success relies on the association between coral animals and symbiotic algae. Corals provide shelter and nutrients for symbionts which in turn supply sugars and O_2 to their hosts[2]. Corals host symbionts within the symbiosome, an intracellular space defined by the coral-derived symbiosome membrane. This membrane is thought to allow corals to regulate delivery of nutrients to the symbiont but the specific transport mechanisms are mostly unknown. Alarmingly, human-caused ocean warming, acidification, and eutrophication disrupt this symbiosis leading to the expulsion of symbionts (known as 'bleaching'), decreased coral fitness, and death[2]. However, the lack of mechanistic knowledge of healthy symbiosis impairs our ability to understand why bleaching occurs, identify resilient and vulnerable species, and design conservation strategies. The mechanisms that deliver nitrogenous molecules (N_m) to symbionts are particularly important. Healthy corals must provide symbionts with enough N_m for the repair of photosystem proteins and other basic functions; however, excess N_m could result in symbiont overgrowth and bleaching[2]. Thus, corals must possess yet unidentified mechanisms to regulate N_m delivery to symbionts.

I propose to study the mechanisms controlling N_m delivery to symbionts and to characterize responses to environmental stress in two coral species with differential susceptibility to eutrophication. In other animal models, NH_3 moves across membranes *via* Rhesus channels (Rh). When paired with an acidification pathway, NH_3 gas combines with H^+ to form NH_4^+ that is trapped on the other side of a membrane[3]. Coral Rh is an ideal candidate for transporting NH_3 across the symbiosome membrane for N_m delivery for three reasons: (1) an "Rh-like" gene is upregulated in anemones upon symbiont acquisition[4], (2) corals acidify the symbiosome using V-H^+-ATPases, which would favor NH_4^+ trapping in the symbiosome[5], and (3) NH_4^+ is symbionts' preferred N_m source[6]. **I hypothesize** that (1) corals supply N_m to symbionts *via* Rh in the symbiosome membrane, (2) N_m supply is controlled *via* transcriptional and translational Rh regulation and changes in Rh localization, and (3) future ocean conditions can bypass the Rh pathway resulting in bleaching. **Preliminary Results:** I cloned the first coral Rh from *Acropora yongei* (ayRh) (MH025799), developed anti-ayRh antibodies, and confirmed ayRh protein expression *via* Western Blots. I found that ayRh is more abundant in the symbiosome membrane during daytime compared to the night *via* immunofluorescence microscopy (IFM) (Fig 1).

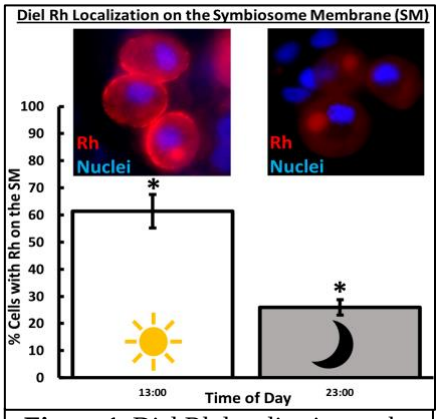

Figure 1. Diel Rh localization to the SM (n = 50) (p = 0.0246*).

Aim 1: Establish ayRh Transport and Function. ***In vitro:*** I will express recombinant ayRh in *Xenopus* oocytes and measure its transport kinetics. Oocytes injected with ayRh cRNA or scrambled cRNA (controls) will be incubated with the radiolabeled NH_3/NH_4^+ analog [^{14}C]methylammonium, and uptake rates will be measured with a gamma counter. Since some vertebrate Rhs can also transport CO_2[7], I will determine ayRh CO_2 permeability by measuring CO_2-induced changes in oocyte pH with the pH-sensitive dye SNARF1. I will run statistics in R and Prism™. I predict that ayRh is NH_3- and not CO_2-permeable, supporting my hypothesis of Rh-mediated N_m delivery. If ayRh transports both, I will adjust my hypothesis and explore the role of Rh in providing carbon and N_m for symbiont photosynthesis and metabolism. ***In vivo:*** I will explore the correlation between Rh abundance and N_m transport rate in isolated coral cells hosting

symbionts[5]. I will measure Rh abundance by Western Blot and NH_4^+ uptake rates from seawater using spectrophotometry. I predict a direct relationship between Rh abundance and capacity for N_m transport. All materials are already available in my collaborating and host labs.

Aim 2: Characterize Coral Rh Regulation. I will expand on my preliminary results (Fig.1) to identify mechanisms that regulate Rh abundance in the symbiosome membrane. In addition to *A. yongei*, I will work with *Stylophora pistillata*, which is more resilient to N_m eutrophication[8]. This comparative approach may unveil species-specific mechanisms that confer resilience in polluted oceans. Transcriptional and translational Rh regulation will be tested using qPCR and Western blotting in coral samples taken during day and night timepoints. Rh's subcellular localization and dynamics will be assessed in unprecedented detail *via* IFM on a super-resolution confocal microscope. Building on preliminary experiments, I will sample every three hours over a two-day period. Furthermore, I will use the highly specific photosynthesis inhibitor DCMU to determine if the presence of Rh in the symbiosome membrane depends on photosynthetic activity or simply on the presence of light[2]. I will automate IFM data collection and quantitative analysis with ZEN™ software; I will use my coding experience to create custom workflows to achieve high throughput and bias reduction during analysis. I will run statistics in R and Prism™. I predict the Rh pathway is present in both coral species, that Rh trafficking to and away from the symbiosome membrane depends on photosynthetic activity, and that Rh mRNA and protein abundance will remain relatively constant reflecting basal turnover rates.

Aim 3: Establish Rh Responses to Stress. To determine the effects of future ocean conditions on Rh, I will grow *A. yongei* and *S. pistillata* in three conditions: (1) control, (2) elevated N_m (10 μM NH_4Cl), and (3) elevated N_m and CO_2 (10 μM NH_4Cl, 1000 μatm CO_2). I will collect samples at 12:00 and 24:00 daily over a 70-day period (10 days of control, 30 days of treatment, and 30 days of recovery in control conditions) and rapidly analyze Rh expression and subcellular localization as described above; this method will also allow me to quantify symbiont density to estimate bleaching. Additionally, I will study symbionts' photobiology using respirometry and PAM fluorometry and genotype symbionts to explore potential effects of symbiont strain. I will run statistics in R and Prism™. I predict the Rh pathway will be initially downregulated in both experimental treatments. I also predict that corals in elevated N_m and CO_2 conditions will undergo the highest degree of bleaching due to larger loss of host control over symbiont growth; these effects will be more pronounced in eutrophication-sensitive *A. yongei*. Finally, I predict the Rh pathway will gradually return to normal during recovery and reestablishment of symbiosis.

Intellectual Merit/Broader impacts: This study will characterize a novel N_m transport mechanism in coral symbioses and develop much-needed biomarkers to evaluate species-specific vulnerability to environmental stress and early detection of bleaching. It also has the potential to reshape our understanding of coral symbioses by establishing a novel diel regulatory mechanism that traffics proteins to and from the symbiosome membrane. I am well qualified to conduct this research based on my experience with IFM, molecular biology, coral biology, and computer science. In my PhD, I will continue to mentor undergraduates through my tutoring program, many of whom are Latina females, and I will expand my program to low income high schools. Results from my project will be presented to the scientific community through peer-reviewed papers and conferences, and to the general public in youth activities, lectures, and exhibits through Sally Ride Science and the Birch Aquarium (which hosts 450k visitors annually). My career goal is to be an R1 professor and these activities will shape my future outreach and education programs.

References: (1) *Global Environ Change* 2014, 26, 152-158. (2) *Microbiol Mol Biol R*, 2012, 76, 229-261. (3) *Transfus Clin Biol* 2006, 13, 85-94. (4) *G3-Genes Genom Genet* 2014, 4, 277-295. (5) *PNAS* 2015, 112, 607-612. (6) *Mar Biol* 1983, 167, 157-167. (7) *Membranes* 2017, 7, 61. (8) *Mar Biol* 2000, 19, 103-113.