Gene expression of four carbonic anhydrase paralogs across the symbiotic states of the temperate sea anemone, *Anthopleura elegantissima* (Brandt 1835)

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Abstract

Anthropogenic CO_2 emissions are shifting the global climate equilibrium, causing widespread losses in biodiversity. Anthozoan cnidarians are some of the species most vulnerable to environmental change. Environmental stress causes corals and sea anemones to expel their endosymbiotic algae, which constitute a primary source of nutrition for some Anthozoa. Carbonic anhydrase (CA) proteins are important contributors to endosymbiont photosynthesis by regulating dissolved inorganic carbon forms within the cell, contributing directly to host nutrition. This study used real-time quantitative polymerase chain reaction to investigate the differential gene expression of four different CAs in the temperate sea anemone, *Anthopleura elegantissima*, across three symbiotic states: animals harboring the chlorophyte *Elliptochloris marina*, those harboring the dinoflagellate *Brevolium muscatinei*, and aposymbiotic animals. This investigation found that both symbiotic states upregulated CA expression compared to the aposymbiotic state. Of the four CAs investigated, two were found to be differentially expressed between *B. muscatinei*- and *E. marina*-harboring anemones. This research supports findings of symbiont-induced CA gene expression, contributing to the understanding of symbiont-host cell physiological interactions and nutrient flow. Such studies are crucial to understand adaptive potential in anthozoan and symbiont species

Introduction

Anthozoan CA proteins concentrate inorganic carbons within the cell for endosymbiont photosynthesis. CA enzymes catalyze the interconversion between bicarbonate to carbon dioxide. However, increasing warm temperature abnormalities generate toxic reactive oxygen species in intolerant endosymbionts, causing the host Anthozoa to eject its endosymbionts in a process known as "coral bleaching.' This threatens the stability of tropical coral reef ecosystems because corals depend highly on the nutrients provided by their endosymbionts.

Animals living in coastal temperate systems, like *Anthopleura elegantissima* and its endosymbionts, are adapted to dramatic fluctuations in light, temperature and pH. In cool, low-light conditions, *A. elegantissima* harbors the chlorophyte *Elliptochloris marina*. Alternatively, *A. elegantissima* harbors the more productive dinoflagellate *Brevolium muscatinei*, which is better adapted to warmer, high-light conditions. Living in rocks and crevices where there is no light, the high nutrient environment allows *A. elegantissima* to exist without its symbionts.

Hypothesis

Based on DIC demand and productivity, it was hypothesized that:

- The highly-productive *B. muscatinei* anemones would express significantly more CAs than less-productive *E. marina* anemones.
- That symbiotic anemones would express significantly more CAs compared to aposymbiotic anemones.

Results

Figure 1: Heatmap depicting relative fold change (2-ΔΔCt) across Aele CAs genes (AeleCA1, AeleCA4, AeleCA6, AeleCA9). Green anemones are surrounded by a green box, and brown anemones samples are surrounded by a brown box. Fold change ranges from 0.0 (white) indicating no change in gene expression and 9.0 (red) indicating large change in gene expression from the aposymbiotic state.

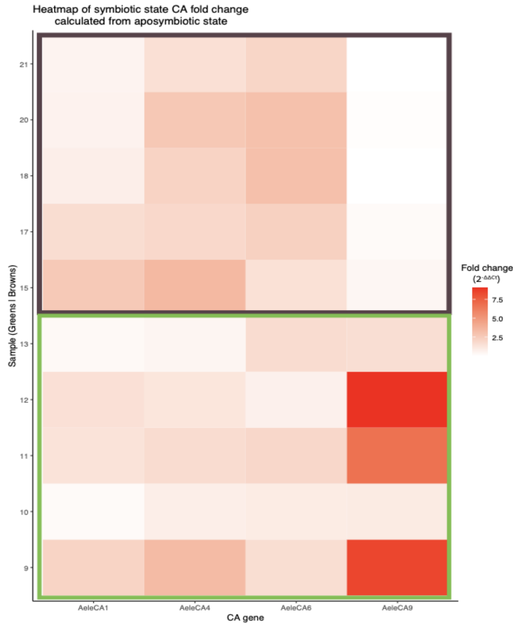
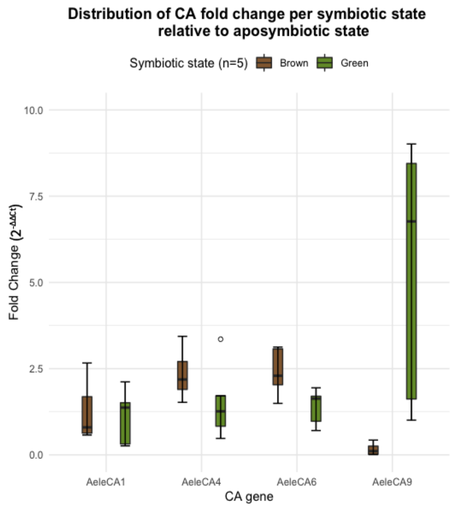


Figure 2: Boxplot depicting fold change between brown (*B. muscatinei*) and green (*E. marina*) anemones across Aele CA genes. Boxplots include the mean sample fold change and standard error at 5 samples of each symbiotic state and gene. Significant gene upregulation from aposymbiotic state was detected in all green and brown Aele CAs ($P < 0.05$) with the exception in brown's AeleCA9 ($P = 0.056$). Brown anemones significantly upregulated AeleCA6 gene expression compared to greens ($P = 0.034$). Green anemones significantly upregulated AeleCA9 gene expression compared to browns ($P = 0.034$).



Tables: Significant differences (all denoting increased gene expression) are noted by the asterisk (*). Slight significant increased gene expression was noted in brown anemone AeleCA9. (top) Statistical significance report of CA genes using a one-way t-test comparing symbiotic brown and green anemone gene expressions to aposymbiotic anemone gene expression. (bottom) Summary table of two-sided statistical analysis tests performed between brown *B. muscatinei*-harboring anemone CA gene expression and green *E. marina*-harboring anemone CA gene expression.

Gene	State	One-way t-test P-value (df = 4)
AeleCA1	brown	0.017*
	green	0.018*
AeleCA4	brown	0.001*
	green	0.019*
AeleCA6	brown	<0.001*
	green	0.002*
AeleCA9	brown	0.056
	green	0.017*

Gene	Welch two-sample t-test [P-value, (df)]
AeleCA1	0.781 (7.905)
AeleCA4	0.215 (6.957)
AeleCA6	0.034* (7.430)
AeleCA9	0.037* (4.018)

Discussion and Implications

The effect of symbiotic state on CA gene expression

Significant upregulation was found in all symbiotic CAs respective to the aposymbiotic state ($P < 0.001$). RT- qPCR results indicate Differential gene expression varies between green and brown symbiotic states. AeleCA1 and AeleCA4 were upregulated in symbiotic anemones ($P<0.001$), and expression did not vary between symbiont types ($P = 0.034$). This indicates that these proteins increase DIC transport but are not particularly associated with either symbiont. AeleCA6 was upregulated in both symbiont-harboring anemones, but upregulated significantly more in brown anemones, likely because *B. muscatinei* is more productive than *E. marina* ($P = 0.034$) (Bergschneirder and Muller-Parker, 2008). But AeleCA9 are not significantly upregulated in brown anemones compared to aposymbiotic anemones, indicating that it may not contribute significantly to brown anemone DIC transport ($P = 0.056$). However, AeleCA9 showed significant upregulation in *E. marina*-harboring anemones and is likely critical in the *E. marina* DIC transport system, suggesting that AeleCA9 is located on the symbiosome membrane internal to the *E. marina*-harboring *A. elegantissima* host cell ($P = 0.017$).

Implications of this study

Tropical corals account for just 1% of the ocean environment, but host 25% of the ocean's biodiversity. Species with low phenotypic plasticity are predicted to be lost as early as 2050 (Patchari et al., 2017). Temperate coastal environments experience the greatest variability in salinity, temperature, CO2 and irradiance. Cnidaria in these temperate environments, like A. elegantissima, have rapid responses to these dramatic daily fluctuations. CAs are indicative of symbiont productivity and host animal growth. CAs are exceptional study proteins because they reflect photosynthetic nutrient output from the symbiont in a host protein. Describing host pathways that are directly related to symbiosis and nutrient uptake is crucial in understanding the stability of evolved anthozoan-symbiont relationships and anthozoan cell growth. Implications of such information can be used to understand symbiont and Anthozoa species most vulnerable to metabolic physiological disruptors, especially in the case of corals. Describing highly productive endosymbionts tolerant to thermal changes may be a key factor for mitigating cnidarian bleaching and loss of coral reef systems as global climate change and ocean acidification threaten ecosystem stability.

Methods

Laboratory work:

- RNA extractions on 20 small, 20 medium, and 20 large brown anemones. RNA extractions were performed by hybridizing the Weis *Aiptasia pallida* TRIzol-Chloroform extraction and the Omega Bio-Tek E.Z.N.A kit RNA column extraction procedures. Genomic DNA was removed using Ambion TURBO's DNA-free protocol. RNA samples were cleaned using an overnight sodium acetate, glycogen, and ethanol incubation treatment.
- Quality of clean RNA was determined via NanoDrop. RNA concentrations were determined with broad range QuBIT fluorescence. cDNA was synthesized from the RNA using a Protoscript II reverse transcriptase kit. cDNA was quantified using HS dsDNA QuBIT fluorescence. Samples were diluted to 7.5ng/µL.
- 10µM concentrations of forward and reverse primers of AeleCA1 (Weis and Reynolds 1999), AeleCA4, AeleCA6, and AeleCA9 samples (Jack Koch), and NADH and G3P (Kitchen et al. 2015) were prepared.

Gene name	qPCR primers [Forward (F), Reverse (R)]	Cell Location
AeleCA1 (Comp7398_c0_seq2)	**F:** 5'-CGC CAA TCA CCG ATC GAC AT-3' **R:** 5'-TTC ACT GGT CTT GCG CGA TA-3'	Cytosolic
AeleCA4 (Comp34611_c0_seq1)	**F:** 5'-TAC AAC AAG ACT GCT GCC GT-3' **R:** 5'-CTT CCG TCC CGT TGA ACC TC-3'	Membrane-Bound (outer)
AeleCA6 (Comp404_c4_seq3)	**F:** 5'-ACC AGA GTT CCT TTG ACC GTC - 3' **R:** 5'-CTG GGG TCA CGT ATC CAA GG-3'	Secreted
AeleCA9 (Comp2502_c1_seq3)	**F:** 5'-TCG ATT CCG TGC TCA AGC TT-3' **R:** 5'-ATC CCT TGG TGT TAG CTG CA-3'	Membrane-Bound or sectreted (?)
NADH	**F:** 5'ATG GGA TTT GCT GGT CCA CT-3' **R:** 5'-TGG GTA GAC AGG TTC ATC GT-3'	
G3P	**F:** 5'-AGA GGC CTT CTT CAC AGC AC-3' **R:** 5'-GTT GGC AAG GTC ATC CCA GA-3'	

- PCR of cDNA samples were run on a 1%TBE agarose gel ensured cDNA synthesis success. Then, cDNA samples were run in triplicate respective to the primers in RT-qPCR with an interplate calibrator, and no template, no primer, and RNA controls. I was unable to finish RT-qPCR due to complications from COVID-19.
- Supplemental calibrated RT-qPCR data was provided based on similar CA laboratory work performed by Jack Koch. Calibrated RT-qPCR Ct data was analyzed to describe gene expression of four carbonic anhydrase paralogs between symbiotic green and brown anemones and aposymbiotic individuals. This was done by calculating fold change through a series of Ct calculations as described by Schmittigen and Livak (2008):

1) $\Delta Ct = (C_{\text{symbiotic state}} - C_{\text{average (NADH and G3P)}})$
2) $\Delta\Delta Ct = (\Delta Ct \text{ of symbiotic sample}) - (\text{average aposymbiotic } \Delta Ct)$
3) Fold change = $2^{-\Delta\Delta Ct}$

- Welch's two-sample t-test was used on the normal data to calculate significance. Fold change values were statistically analyzed in RStudio and ggPlot2.

Acknowledgements

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