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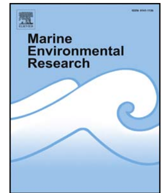

Salinity stress results in differential Hsp70 expression in the *Exaiptasia pallida* and *Symbiodinium* symbiosis

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ABSTRACT

Abiotic factors affect cnidarian-algal symbiosis and, if severe enough, can result in bleaching. Increased temperature and light are well characterized causes of bleaching, but other factors like salinity can also stress the holobiont. In cnidarian-dinoflagellate systems, the expression of host genes, including heat shock protein 70 (Hsp70), changes due to thermal and light stress. In this experiment, we characterized to what extent salinity stress affects Hsp70 expression in the holobiont by simultaneously measuring host and symbiont Hsp70 expression in response to up to 8 h of hypo- and hypersaline conditions in the sea anemone *Exaiptasia pallida* and its intracellular symbiont *Symbiodinium minutum*. We show that *E. pallida* Hsp70 expression increases (6-11-fold) at high salinities whereas *Symbiodinium* Hsp70 expression shows little change (1.4–2.6-fold). These data suggest that cnidarian Hsp70 response is similar across multiple abiotic stressors further validating the Hsp70 gene as a biomarker for abiotic stress.

1. Introduction

Symbiotic relationships between cnidarians and their intracellular dinoflagellate partners, *Symbiodinium* sp. form the basis of reef environments (Muller-Parker et al., 1997). While corals may be the most prominent organisms in many reef systems, other cnidarians are more amenable to culture and experimentation aimed at understanding the relationship between cnidarians and dinoflagellates (Weis et al., 2008a). The sea anemone *Exaiptasia pallida* lives in a mutualistic symbiosis with *Symbiodinium* sp. and has recently been suggested as an ideal model cnidarian-dinoflagellate system (Lehnert et al., 2012; Voolstra, 2013).

Maintaining a healthy symbiosis at the cellular level is a delicate process (Davy et al., 2012; Weis, 2008b). Any stress that alters the osmotic relationship between a cnidarian and its symbiont may result in cellular responses, including the production of heat shock proteins (reviewed in Sørensen et al., 2003). Heat shock proteins (Hsps) are responsible for aiding in and directing the folding, assembly, and transport of cellular proteins under normal and stress conditions; Hsp70 is one evolutionarily-conserved, stress-inducible member of this group of chaperones in the cellular cytosol (Sørensen et al., 2003). Given the sensitivity of animal and dinoflagellate Hsp70 to a range of stressors, including chemicals and nutrients (e.g. Guo and Ki, 2012; Guo et al., 2015; Rosic et al., 2014), Mukhopadhyay et al. (2013) advocate for its utility in environmental monitoring.

The response of Hsps to short-term thermal stress has been documented in both marine invertebrates and dinoflagellates. In general, Hsps increase with sublethal temperature elevation in cnidarian-dinoflagellate systems (e.g. Choresh et al., 2001; Hofmann and Somero, 1995; Snyder and Rossi, 2003). For example, Black et al. (1995) showed that *E. pallida* produce higher levels of Hsps 68 and 72 after short-term thermal stress.

Cnidarian-dinoflagellate symbioses and coral reef habitats are threatened by a number of abiotic stressors in addition to rising sea surface temperatures, including increasing ocean acidity and changing salinity (Bindoff et al., 2007; Hoegh-Guldberg et al., 2007). Ocean salinity is maintained by large scale, long-term equilibrium between freshwater fluctuation, the oceans' currents, and mixing processes (Durack and Wijffels, 2010). However, episodic salinity stress may become of increasing importance as climate change events lead to increased rainfall or evaporation (Curry et al., 2003; Durack and Wijffels, 2010). As global temperatures rise, some areas of the ocean are freshening, while salinity has been increasing in shallow waters where corals live, including over the range of *E. pallida* (Bindoff et al., 2007; Boyer et al., 2005). Heavy rains and outflows from flooded rivers can periodically cause days to weeks of hyposaline conditions in coastal marine environments (Coles et al., 1992; Downs et al., 2009).

The effects of changing salinity on marine invertebrates are highly variable. The anemone *Bunodosoma cavernata* survived a wide range of salinities over two weeks' duration (Benson-Rodenbough and Ellington,

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Table 1
Sequence information and optimized conditions for *Exaiptasia pallida* and *Symbiodinium minutum* primers used in qPCR.

Target Gene	Direction	Primer Sequence (5′-3′)	Tm (°C)	GC Content (%)	Product size (bp)	Reference
Aiptasia pallida						
Hsp70	Forward	TCCTCCAGCACAGAAGCAAG	57.1	55.0	118	This study
	Reverse	GACACGAGCGGAACAGATCA	57.3	55.0		
RPL11	Forward	AGCCAAGGTCTTGGAGCAGCTTA	60.6	52.1	125	(Lehnert et al., 2013)
	Reverse	TTGGGCCTCTGACAGTACAGTGAACA	61.5	50.0		
RPS7	Forward	ACTGCAGTCCACGATGCTATCCTT	60.2	50.0	125	(Lehnert et al., 2013)
	Reverse	GTCTGTTGTGCTTTGTCGAGATGC	58.6	50.0		
Symbiodinium minutum						
Hsp70	Forward	TTTGAGGAGCTGTGCATGGACTACTT	60.1	46.1	120	(Leggat et al., 2011)
	Reverse	GGAACCACCCACCAGAACCACATCAT	62.7	53.8		
β-actin	Forward	TGGACAACGGAAGCGGAATG	58.0	55.0	80	(Leggat et al., 2011)
	Reverse	GCCAACAATGGATGGGAAAACT	56.2	45.4		
SAM	Forward	GCCTACATTTGCCGACAGATG	56.4	52.4	101	(Rosic et al., 2011b)
	Reverse	AATGGCTTGGCAACACCAAT	56.0	45.0		

1982). Some corals have demonstrated a high tolerance to short-term, broad salinity fluctuations (e.g., *Porites furcata*; Manzello and Lirman, 2003), but coral reefs are generally believed to be stenohaline (Coles et al., 1992). Biomass, oxygen flux, and bleaching, which is defined as loss of symbiont density or pigmentation, were unaffected by short term (hours) salinity reduction in *Stylophora pistillata* (Hoegh-Guldberg and Smith, 1989), but longer duration (weeks) of slight salinity changes reduced gross photosynthesis:respiration rates (Ferrier-Pagès et al., 1999). Hyposaline conditions resulted in bleaching in *Zoanthus sociatus*; all individuals survived hours of hypo- or hypersaline conditions, but two days of exposure to experimental salinities caused death (Soares and Sousa, 2011).

Some effects of changing salinity on Hsps have been reported in a range of marine invertebrates. Downs et al. (2009) found that 24 h of hyposaline conditions (28, 24, and 20 ppt) resulted in tissue damage, symbiont loss, and increased protein levels of cnidarian Hsp70 and dinoflagellate Hsp60 in *S. pistillata*. Salinity stress reduced the accumulation of Hsps in a bivalve (Werner, 2004). At the level of mRNA, changing salinity did not affect Hsp70 expression in a sponge (López-Legentil et al., 2008) but lowered salinity increased Hsp70 expression in oysters (Zhao et al., 2012). To date, response to salinity stress by production of Hsp70 has not yet been investigated at the level of transcription in cnidarians.

The characterization of biomarkers of early stress like Hsps can assist in our ability to understand and predict the effects of environmental changes in marine organisms (Choresh et al., 2001). In this experiment, we aimed to determine if short-term salinity stress elicits changes in the transcription of genes encoding heat shock proteins at the level of transcription, focusing on simultaneous expression of Hsp70 orthologs in both *E. pallida* and its endosymbiont. By detecting transcriptional responses preceding bleaching in cnidarian-dinoflagellate symbioses such as those investigated here, we may gain the ability to detect physiological stress before it is grossly and ecologically apparent.

2. Materials and methods

Exaiptasia pallida used in this experiment were derived from a single individual collected by Dr. Clayton Cook from Walsingham Pond, Bermuda in 1985 and maintained in culture at Hood College. *E. pallida* from Bermuda have been reported to host *Symbiodinium minutum*, formerly known as clade B (LaJeunesse et al., 2012; Thornhill et al., 2013). Sea anemones were maintained on a diet of freshly-hatched brine shrimp and kept in Instant Ocean® artificial seawater at a concentration of 30 ppt. Stock anemones and experimentally-treated anemones were maintained in an incubator with a 12 h light cycle at a light level of 18.66 μmol s^{-1} m^{-2} at 24 °C.

One day prior to the salinity stress experiment, 100 symbiotic anemones were placed into separate 50 ml centrifuge tubes filled with 30 ppt artificial seawater and allowed to acclimate overnight (~16 h). Anemones that failed to attach to the side of the centrifuge tube and/or did not expand fully were replaced during the acclimation period. On the day of the experiment, the 30 ppt water was replaced with 24, 27, 30, 33, and 36 ppt artificial seawater. Five anemones were sampled after 1, 2, 4, or 8 h of exposure at each salinity beginning at 0800. Each polyp was transferred to a 1.5 ml microcentrifuge tube containing 500 μL of RNA*later* (Qiagen) and macerated. Samples were then stored at room temperature for 1-2 weeks before RNA extraction.

RNA was extracted from the polyp using TRIzol® Reagent (Invitrogen) following the manufacturer's guidelines. RNA concentrations were determined using a FLUOstar Omega LVis plate reader (BMG Labtech). Complimentary DNA (cDNA) was synthesized from each sample using a SuperScript® II Reverse Transcriptase kit (Invitrogen). Each reaction was prepared with 1 μL of 10 mM dNTP mix, 1 μL of 0.5 μg/μL oligo (dt)12-18 primer, 250 ng of sample RNA, and DEPC-treated water for a total volume of 10 μL.

Quantitative real-time PCR (qPCR) was performed on the sample cDNA using the Applied Biosystems SYBR® Green assay in 96 well plates. All reactions were run on a 7900HT Applied Biosystems qPCR cycler in Standard mode with the following thermal profile: 95 °C for 5 min, 40 cycles of 95 °C for 30 s, and 60 °C for 1 min, followed by a dissociation stage (95 °C for 15 s, 60 °C for 15 s, 95 °C for 15 s). Reactions in volumes of 25 μL were prepared using 5.0 μL of 5.0 μM forward primer (1 μM), 5.0 μL of 5.0 μM reverse primer (1 μM), and 2.5 μL of 4 ng/μL template with 12.5 μL of SYBR® Green (Invitrogen) for all primer sets. All samples were assayed in triplicate with both host- and symbiont-specific primers; triplicate reactions C_t values were averaged for each sample.

Primers to amplify a portion of the 3′ end of the Hsp70 gene for qPCR in *E. pallida* were designed from the *Aiptasia* Hsp70 EST nucleotide sequence available at AiptasiaBase (http://aiptasia.cs.vassar.edu/AiptasiaBase/index.php) using the Integrated DNA Technologies primer design tool at www.idtdna.com (Table 1). Primers for previously characterized reference genes for *E. pallida*, ribosomal protein L11 (RPL11) and ribosomal protein S7 (RPS7), were designed by Natalya Gallo of the Scripps Institution of Oceanography (Gallo, 2010) and also used by Lehnert et al. (2013); these two genes were determined to be among the most stably expressed in a range of (mostly temperature) stress conditions. *Symbiodinium* sp. Hsp70 primers and the primers for reference gene beta actin (β-actin) were adopted from the work of (Leggat et al., 2011). Primers for the second reference gene for *Symbiodinium* S-adenosyl-L-methionine synthetase (SAM) were adopted from Rosic et al. (2011b). Primers for *E. pallida* were tested against *Symbiodinium* and primers for *Symbiodinium* were tested against *E. pallida* using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) to ensure that there would be no cross-species amplification.

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