Evaluating the temporal stability of stress-activated protein kinase and cytoskeleton gene expression in the Pacific reef corals *Pocillopora damicornis* and *Seriatopora hystrix*

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**A B S T R A C T**

The gastrodermal tissues of anthozoans that harbor endosymbiotic *Symbiodinium* experience light-dependent fluxes in dinoflagellate derived photosynthetic compounds that have the potential to impact the osmotic homeostasis of the animal. To explore this unusual osmoregulatory scenario, genes encoding proteins that play highly conserved roles in osmoregulation (mitogen-activated protein kinases, [MAPKs]) and the maintenance of the cytoskeleton (*α*-actin, tropomyosin, and *α*-tubulin) were quantified over diel cycles using quantitative real-time polymerase chain reaction. The expression of MAPK genes in *Pocillopora damicornis* increased at night, while the expression of cytoskeleton genes in *Seriatopora hystrix* decreased. The increase in *P. damicornis* MAPK expression may reflect host osmolyte production in response to reduced osmotic pressure at night. The concomitant decrease in expression of genes encoding cytoskeleton proteins at night is consistent with this interpretation, indicating reduced production of spatially demanding proteins under periods of intracellular crowding. However, the latter interpretation is confounded by the fact that molecularly-inferred *Symbiodinium* densities were found to be significantly greater at night in both corals, so changes in cytoskeletal gene expression may also reflect crowding to accommodate the greater density of these endosymbionts. In addition to providing insight into an unusual physiological attribute of photosynthetic endosymbioses, this study represents the first to measure gene behavior in field and cultured corals with a method that considers the dual-compartment nature of the associations.

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1. Introduction

During the day, the osmotic equilibrium of cnidarian gastrodermal cells housing endosymbiotic dinoflagellates (genus *Symbiodinium*) is influenced by *Symbiodinium* photosynthesis and the production and translocation of osmolytes such as glycerol into the host cell's cytoplasm (Muscatine, 1967; Muscatine and Cernichiari, 1969). Conversely, *Symbiodinium* derived osmolytes cease to be produced and translocated at night, and pools are consequently respired (Gates and Edmunds, 1999). Thus, there is potential for host gastrodermal cells to undergo diel fluctuations in volume (Mayfield and Gates, 2007; Fig. 1). Given that even small changes in cell volume have pervasive impacts on macromolecular structure and function (Hochachka and Somero, 2002), such volume deviations have important implications for cell physiology in endosymbiotic cnidarians (Lang et al., 1998).

Cell volume changes are regulated by a group of signal transduction molecules called mitogen-activated protein kinases (MAPKs; Cowan and Storey, 2003), which control the initiation and termination of osmolyte production pathways (Kultz and Burg, 1998). Thus, the expression of this highly conserved class of genes is temporally dynamic and environmentally responsive (Kultz, 2001). We therefore hypothesize that coral MAPK mRNA levels will reflect subtle changes in cell volume that occur over the course of a day as rates of photosynthesis in *hospite* are altered in response to the light cycle. Specifically, host MAPK expression may increase at night to stimulate osmolyte production pathways to compensate for the lack of photosynthetically-derived osmolyte efflux from the *Symbiodinium* in the dark.

Cell volume changes also alter the structure of the cytoskeleton (Hoffmann and Pedersen, 1998), as well as the expression of the genes and proteins that compose this complex protein scaffold (Chowdhury et al., 1992; Henson, 1999). Cytoskeletal proteins such as actin (the dominant protein of microfilaments), tropomyosin (an actin-binding protein), and tubulins (responsible for microtubule construction)
Fig. 1. Schematic of hypothesized diel variation in coral gastrodermal cell volume associated with the photosynthetically-derived osmolytes fluxes from Symbiodinium. During the daytime, cells expand as the osmolarity increases due to the translocation of photosynthetically-derived osmolytes from Symbiodinium into the host cytoplasm. At night, as photosynthetic activity ceases, the holobiont respires Symbiodinium derived osmolytes, which triggers host regulatory activities to re-establish osmotic equilibrium.

2.1. Gene isolation

As such, cytoskeleton gene expression decreases in periods of cell shrinkage (Ovadi and Saks, 2004; Koivusalo et al., 2009), to reduce the production of these bulky proteins and to allow biochemical reactions to occur unobstructed immediately before regulatory volume increases (RVI; Minton, 2001). Likewise, an increase in cytoskeleton gene expression and protein production occurs as cells swell during the RVI. We therefore hypothesize that the expression of β-actin, tropomysosin, and α-tubulin in corals will decrease at night in response to cell shrinkage as osmolyte efflux from Symbiodinium ceases in the dark (Fig. 1).

To test these hypotheses, degenerate primers were designed to isolate three members of the stress-activated protein kinase 2 (SAPK2) subfamily of MAPKs from corals, including p38, the animal homolog of the yeast high-osmolality glycerol 1 (hog1) gene, which is known to function directly in osmoregulation (Mao et al., 2004). The expression of these genes was measured over diel cycles in *Porites compressa* sampled directly from one Hawaiian fringing reef. In a companion study, the expression of three genes encoding dominant cytoskeletal proteins, β-actin, tropomysosin, and α-tubulin, were measured over diel cycles in *Seriatopora hystrix* housed in 50 kl flow-through aquaria in Taiwan. Gene expression was measured using an endosymbiosis-specific quantitative real-time polymerase chain reaction (qPCR)-based assay (Mayfield et al., 2009). We observed significant increases in MAPK expression in *P. damicornis* colonies sampled at night, with nocturnal decreases in cytoskeleton gene expression occurring within S. hystrix. However, nocturnal elevation of *Symbiodinium* density was also measured with our qPCR assay in both coral species, suggesting that intracellular crowding, in addition to decreased osmotic pressure, could explain such gene expression patterns.

2. Materials and methods

2.1. Gene isolation

A cetyltrimethylammonium bromide protocol (Dempster et al., 1999) was used to extract genomic DNA from 20 mg fragments of the Hawaiian corals *Porites compressa*, *Montipora capitata* and *P. damicornis* collected at 1 m depth on snorkel from reefs adjacent to Coconut Island, Kane‘ohe Bay, O‘ahu, Hawai‘i (21° 26.2′ N, 157° 47.6′ W). Readers are referred to Stimson et al. (2001) for a thorough characterization of these reefs.

To design the degenerate MAPK primers for PCR (referred to as SAPK2-F2, SAPK2-R2, and SAPK2-R3 in Table 1), two protein and nucleic acid alignments were constructed using published MAPK sequences from a range of organisms (sensu Lee et al., 2003). The first alignment included sequences selected by comparing the *Nematostella vectensis* p38 nucleic acid sequence (Genbank accession no. JG18021) with sequences in NCBI’s Genbank database with BLAST (tblastx) to identify closely related invertebrate sequences. The closest relatives were from *Apis mellifera* (XP_395384), *Aplysia californica* (AAP30859), *Litторина littorea* (AAZ38882), *Suberites domuncula* (CAC80141), *Caenorhabditis elegans* (AA98017), *Anopli- eles gambiae* (XP_320380), and *Aedes aegypti* (EAT39843). The second alignment was created using only vertebrate sequences, as basal metazoan genes often show greater homology to vertebrate genes than with derived model invertebrate taxa such as *C. elegans* (Kortschak et al., 2003). Sequences included were from *Danio rerio* (AAH33937), *Cyprinus carpio* (BA11188), *Xenopus tropicalis* (NP_001005824), *Gallus gallus* (CAG32435), *Mus musculus* (NP_036081), *Rattus norvegicus* (P70618), *Canis familiaris* (NP_001003206), *Boo Taurus* (XP_614274), *Pan troglodytes* (XP_518431), and *Homo sapiens* (18BMA). The primers (p38-C-F1, p38-C-R1, and p38-R1, Table 1) were designed to span regions common to all SAPKPs.

PCRs (50 μl) comprised 1 unit (U) *Immolase*® polymerase (Bio- line), 250 μM each dNTP, 2.5 mM MgCl₂, 1× Immunobuffer® (Bio- line), 500 nM each primer (Table 1), 15–100 ng DNA template, and in some cases, 1 μl⁻¹ bovine serum albumin (BSA). Gene fragments were amplified in two rounds of PCR. The first round conditions comprised 10 min at 95 °C (required to activate the “hot-start” Immolase) followed by 35 cycles of 95 °C for 60 s, 50 °C for 75 s, and 72 °C for 75 s and a final 10 min extension at 72 °C. One microliter of the product amplified in the first round of PCR (including controls) was used as template in the second round of PCR using an identical setup for the reaction, an annealing temperature of 55 °C, and denaturing, annealing, and extension durations of 30 s.

PCR products were visualized on agarose gels using ethidium bromide and gel electrophoresis and appropriately sized bands were excised from the gel with a razor blade. Each gel slab was weighed and excised from the gel with a razor blade. Each gel slab was weighed and excised from the gel with a razor blade. Each gel slab was weighed and excised from the gel with a razor blade. Each gel slab was weighed and excised from the gel with a razor blade. Each gel slab was weighed and excised from the gel with a razor blade.

Table 1

<table>
<thead>
<tr>
<th>Primer name</th>
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<th>Tm (°C)</th>
<th>AA sequence</th>
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<td>WSVGCIM</td>
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<td>WSVGCIM</td>
</tr>
</tbody>
</table>

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Table adapted from Mayfield et al. (2010).
the amplicons from the agarose. The DNA fragments were cloned into a TOPO™-TA dual promoter vector (Invitrogen), and clones containing positive inserts were identified using PCR and M13 vector primers, sequenced, and assigned an identity by comparison with sequences in NCBI using BLAST (tblastx). The sequences for the genes isolated were also compared with those in “CnidBase” (www.cnidbase.org) and “SymBioSys” (sequoia.ucmerced.edu/SymBioSys), using BLAST to identify putative cnidarian homologs.

2.2. P. damicornis natural experiment

Fragments (~100 mg) from five colonies of P. damicornis were collected across a small (~3000 m²) fringe reef on the leeward side of Coconut Island, Hawai’i (coordinates listed above) at 05:45, 06:30, 12:30, 18:45, 19:30 and 23:00 on three non-cloudy days in May 2008 (27–28 °C and 32 psu). No colony was sampled more than once to avoid the potential impact of sampling stress on coral gene expression. The sampling times were selected to encompass a range of osmolyte flux states in the corals (Fig. 1) associated with Symbiodinium photosynthesis, from initiation (sunrise), to maximum (midday), to cessation (sunset), and to inactive (night). Coral fragments were immediately frozen in liquid nitrogen and stored at −80 °C until processed.

2.3. S. hystrix aquarium experiment

S. hystrix specimens (~1000 cm³ colonies, n = 30) were collected at 6–10 m on SCUBA from Wanlitong, Taiwan (22.03°N, 120.72°E) in March 2008 and acclimated in duplicate 50 kl (a TOPO™-TA dual promoter vector (Invitrogen), and clones containing positive inserts were identified using PCR and M13 vector primers, sequenced, and assigned an identity by comparison with sequences in NCBI using BLAST (tblastx). The sequences for the genes isolated were also compared with those in “CnidBase” (www.cnidbase.org) and “SymBioSys” (sequoia.ucmerced.edu/SymBioSys), using BLAST to identify putative cnidarian homologs.

After homogenization of the coral tissues in the RNA and DNA spike-inoculated TRizol, the pestle was rinsed with an additional 500 µl TRizol, and this wash was added to the initial TRizol/coral homogenate. This mixture was transferred to a microcentrifuge tube and the RNA and DNA extracted as in Mayfield et al. (2009). DNAs were assessed for quantity and quality on a NanoDrop spectrophotometer (Thermo Scientific) and on etidium bromide-stained 1% agarose gels, respectively, and diluted 5-fold prior to qPCR.

A DNase treatment was performed on 10 µl RNA according to manufacturer’s instructions (Promega), and the DNA-free RNA was precipitated with sodium acetate and isopropanol. The RNA pellet was washed once with 75% ethanol and re-suspended in 20 µl DEPC-treated water. RNAs were assessed for quantity and quality with a NanoDrop spectrophotometer and on etidium bromide-stained 1% agarose gels, respectively.

2.4. P. damicornis RNA/DNA extractions

RNAs and DNAs were extracted from sub-samples (60–80 mg) of the coral fragments using a modified TRIzol® (Invitrogen) protocol. The samples were first homogenized using a mortar and pestle in 500 µl TRIzol spiked with 10 pg exogenous somatolactin (SL) RNA (created by in vitro transcription from a plasmid containing a SL gene fragment and T7 RNA polymerase as in Mayfield et al. (2009)) and DNA (a serially-diluted SL PCR product). The RNA spike controls for inadvertent variation in extraction and reverse transcription efficiencies, as well as pipetting discrepancies, that may bias gene expression data (Bustin, 2000; Bower et al., 2007). The other hand, the DNA spike is required to normalize the number of Symbiodinium hsp70 genome copies quantified with qPCR, resulting in the generation of the symbiont molecular proxy (SMP), mRNA expression levels are normalized to this value to control for potentially different quantities of Symbiodinium RNA in total RNA extracts (Mayfield et al., 2009). Failure to consider the dye-coupling material nature of these endosymbioses may lead to gene expression artifacts arising from differential densities of Symbiodinium among samples.

2.5. S. hystrix RNA/DNA extractions

For the S. hystrix samples, RNA and DNA were extracted as above, except that TRI-Reagent™ (Ambion) was used instead of TRIzol, and 50 and 10 pg SL RNA and DNA spikes, respectively, were added to the coral fragments submerged in TRI-Reagent. DNase-treatments and RNA/DNA quality assessment and dilutions were performed as above.

2.6. cDNA synthesis

P. damicornis RNA (~500 ng) was converted to cDNA using a “high capacity” kit (Applied Biosystems) and 400 ng oligo-dt primers following the manufacturer’s instructions. The cDNAs were diluted 2-fold in DEPC-treated water prior to qPCR. Likewise, approximately 600 ng S. hystrix RNA was converted to cDNA with MMLV reverse transcriptase (Epigenetics Technologies, manufacturer’s recommendations) and diluted 2-fold prior to qPCR.

2.7. Quantitative real-time PCR of P. damicornis nucleic acids

qPCR was used to quantify the number of copies of P. damicornis p38, bmk1, erk2, Symbiodinium hsp70, and the spiked SL RNA and DNA. SYBR® Green I was utilized for qPCR (20 µl) of all cDNAs after small amplicon primers (Table 2) were optimized to verify absence of secondary products. Power SYBR® Green mastermix (Applied Biosystems) was used with the primer concentrations listed in Table 2. For each sample and gene, reactions were performed in triplicate on a Step One Plus™ qPCR machine (Applied Biosystems). Thermocycling conditions comprised initial incubations at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 59 °C for 1 min for the MAPKs, while qPCR of Symbiodinium hsp70 and SL were performed as described in Mayfield et al. (2009). A melt curve analysis was performed to test for nonspecific amplification products by incubating the reactions for 10 s at 0.5 °C increments between 60 and 90 °C. MAPK gene expression was normalized to the SL spike, the SMP, and total RNA as in Mayfield et al. (2009) with data pooled over the triplicate days.

2.8. Quantitative real-time PCR of S. hystrix nucleic acids

qPCRs (20 µl) were performed in triplicate for each sample and gene using 4, 1, and 2 µl cDNA with β-actin (HM147127), tropomyosin (HM147128), and α-tubulin (HM147129) primers (concentrations found in Table 2), respectively, with Power® SYBR Green mastermix on an Applied Biosystems 7500 real-time PCR machine. The original sequences from which the primers were designed were obtained from an unpublished S. hystrix expressed sequence tag (EST) library courtesy of Prof. L.H. Wang of NMMA. With the β-actin reactions, 1 µl 100X BSA was added. The thermocycling conditions were as follows: 50 °C for 2 min and 95 °C for 10 min for 1 cycle each followed
by 33, 31, and 35 cycles of 95 °C for 15 s and 60, 61.5, or 61.5 °C for 60 s for β-actin, tropomyosin, and α-tubulin, respectively. Gene expression was normalized as above and pooled across the duplicate aquaria and triplicate sampling days. One-way ANOVA was performed to assess the impact of time on SMP and gene expression for both corals, and Tukey’s post-hoc tests were used to test for differences to that of the canonical TEY phosphorylation motif, TGY (Fig. 2C). All organisms chosen for the alignment possess the TGY phosphorylation signature.

### 3. Temporal variation in SMP

The SMP varied significantly over time (Fig. 3A) in P. damicornis (1-way ANOVA, F4,89 = 15.76, p < 0.001). Specifically, colonies collected at 23:00 had higher SMP values than those collected during the day (12:30 vs. 23:00 Tukey’s post-hoc test, p < 0.05); the SMP was approximately 2-fold higher at 23:00 than at 12:30 and did not change significantly between 5:45 and 19:30 (1-way ANOVA, F4,74 = 0.85, p = 0.51). The S. hystrix SMP (Fig. 3B) also differed significantly over time (1-way ANOVA, F4,85 = 44.62, p < 0.001) with a significant 4-fold increase at 21:00 over 12:00 and 18:00 levels (Tukey’s post-hoc test, p < 0.05).

### 3.3. Temporal variation in P. damicornis MAPK gene expression

Both erk2 and p38 mRNA levels were below the levels of qPCR detection (data not shown). However, bmk1 was detectable, but the ERK5 subfamily of MAPKs. The coral BMK1 amino acid sequences show a high degree of similarity to putative orthologs from A. millepora (100%), A. palmata (100%), and N. vectensis (88%). The canonical ERK phosphorylation motif, TEFY, is conserved in all cnidarians and, of the organisms selected for the alignment, differs only in the mussel.

<table>
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<th>Primer name</th>
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### Table 2

Quantitative real-time PCR primers. PD = Pocillopora damicornis. SH = Seriatopora hystrix.

Both erk2 and p38 mRNA levels were below the levels of qPCR detection (data not shown). However, bmk1 was detectable, but the ERK5 subfamily of MAPKs. The coral BMK1 amino acid sequences show a high degree of similarity to putative orthologs from A. millepora (100%), A. palmata (100%), and N. vectensis (88%). The canonical ERK phosphorylation motif, TEFY, is conserved in all cnidarians and, of the organisms selected for the alignment, differs only in the mussel.

The R2 primer set yielded a 157 bp fragment from P. damicornis (FJ858778) that is most closely related to erk2 (Fig. 2B). The corresponding protein, ERK2, is a member of the ERK1 subfamily. The P. damicornis ERK2 protein sequence is identical to that of A. millepora and very similar (98%) to that of N. vectensis (Fig. 2B). The canonical TEY phosphorylation motif is conserved in these anthozoans.

The primers designed using the second, vertebrate weighted design (p38-C-F1 and p38-R1/C-R1, Table 1) were used in a nested design (p38-C-R1 in place of p38-R1 in the second round of PCR) and amplified a 195–386 bp fragment of p38 (hog1), a SAPK2, from P. damicornis, M. capitata and P. compressa (FJ858782, FJ858780 and FJ858781, respectively). The P. damicornis, M. capitata and P. compressa p38 amino acid sequences are 93.2%, 91.4% and 91.4% similar, respectively, to that of the putative N. vectensis p38 sequence (Fig. 2C). All organisms chosen for the alignment possess the TGY phosphorylation signature.
and mRNA levels varied significantly over the course of the day (Fig. 4, 1-way ANOVA, $F_{5,89} = 4.32$, $p = 0.02$). There was a statistically significant 3-fold spike in expression at 23:00 over 12:30 expression levels (post-hoc test, $p < 0.05$).

3.4. Temporal variation in S. hystrix cytoskeleton gene expression

Levels of $\beta$-actin expression in S. hystrix varied significantly over time (Fig. 5A, 1-way ANOVA, $F_{4,89} = 24.39$, $p < 0.001$), with the most notable change being the 4-fold decrease at night (post-hoc test, $p < 0.05$) relative to 12:00 levels. Tropomyosin expression (Fig. 5B) also varied significantly over time (1-way ANOVA effect of time, $F_{4,89} = 9.75$, $p < 0.01$), with a significant 3.5-fold decrease at 21:00 relative to 12:00 levels (post-hoc test, $p < 0.01$). Similarly, $\alpha$-tubulin was expressed at significantly different levels over time (Fig. 5C, 1-way ANOVA effect of time, $F_{4,89} = 52.96$, $p < 0.001$) with a 7.5-fold decrease at 21:00 relative to 12:00 levels (post-hoc test, $p < 0.01$).

4. Discussion

4.1. Isolation of three MAPKs from P. damicornis

A degenerate primer approach was utilized to isolate three MAP kinase genes; bmk1, erk2, and p38 from dominant Hawaiian coral species using primers designed from either invertebrate (Fig. 2A, b, yielding ERKs) or vertebrate (Fig. 2C, yielding p38, a SAPK2) SAPK2 amino acid alignments, confirming the highly conserved nature of this gene family. Interestingly, primers designed to an alignment that excluded invertebrate sequences allowed SAPK2 genes to be isolated from the Hawaiian corals, supporting the conclusions of Kortschak et al. (2003) that coral genes are more similar to their vertebrate counterparts than to those from model invertebrates such as C. elegans and D. melanogaster. The coral sequences isolated here were

![Fig. 3. Temporal variation in symbiont molecular proxy (SMP) in Pocillopora damicornis and Seriatopora hystrix. Data are presented as normalized to the 18:45 and 18:00 values for P. damicornis (A, n = 15) and S. hystrix (B, n = 18), respectively. Error bars represent standard error of the mean with the data from the triplicate sampling days having been pooled for each species. Letters above columns represent Tukey’s post-hoc groupings ($p < 0.05$) from statistical tests performed with the raw (before fold change) data.](image1)

![Fig. 4. Temporal variation in field sampled Pocillopora damicornis bmk1 gene expression. Error bars represent standard error of the mean after having pooled data from the triplicate sampling days in May 2008 (n = 15 samples time$^{-1}$). Letters above columns represent Tukey’s post-hoc groupings ($p < 0.05$) from statistical tests performed with the raw (before fold change) data.](image2)

![Fig. 5. Temporal variation in laboratory maintained Seriatopora hystrix cytoskeleton gene expression. Data are represented as normalized to the 18:00 value for $\beta$-actin (A), tropomyosin (B), and $\alpha$-tubulin (C). Error bars represent standard error of the mean after having pooled data from the triplicate sampling days in June 2008 (n = 18 samples time$^{-1}$). Letters above columns represent Tukey’s post-hoc groupings ($p < 0.05$) from statistical tests performed with the raw (before fold change) data.](image3)
always most similar to homologs of MAPKs from other anthozoans, although all exhibit a high degree of similarity with both invertebrate and vertebrate sequences (Fig. 2).

4.2. Diel variation in the symbiont molecular proxy (SMP)

Unexpectedly, randomly selected coral colonies sampled across a small fringing reef (*P. damicornis*) and within 50 kl aquaria (*S. hystrix*) exhibited significant variation in the SMP over time (Fig. 3). Notably, there were approximately 2 and 4-fold DNA spike-normalized *Symbiodinium* hsp70 genome copies at 23:00 and 21:00 compared to high light levels within *P. damicornis* and *S. hystrix*, respectively, potentially suggesting that the *Symbiodinium* divide in hospite at night and that the population is later reduced by expulsion. This behavior is reportedly commonplace in some corals (Titlyanov et al., 1996) but quite rare in others (Hoegh-Guldberg et al., 1987). Baghdasarian and Muscatine (2000) found that *P. damicornis* gastrodermal cells containing dividing *Symbiodinium* were more likely to be expelled from the tissues, thus serving as a means of regulating the population of their dinoflagellate endosymbionts. Rates of expulsion were fairly low in their study (0.05 d−1), though measurements were only made in the day, the period during which the molecularly-inferred *Symbiodinium* densities observed herein (Fig. 3) were low and relatively stable. In fact, mitotic indices of *Symbiodinium* during the day have been reported to be quite low, 1–2% from *P. damicornis* (Hoegh-Guldberg, 1994) and 1–8% from *S. hystrix* (Hoegh-Guldberg and Smith, 1989). In both corals, mitotic indices rose at night, suggesting division to be synchronous (but see Wilkerson et al., 1983, 1988). The fact that our data suggest a much higher rate of *Symbiodinium* turnover than these earlier reports could be accounted for by the temporal disjunction between DNA replication and visible cytokinesis (Fitt and Trench, 1983; Brown and Zanami, 1992) and/or differences in sampling time (Fig. 1).

It is also possible that the significant nocturnal increase in SMP does not result in cell division of *Symbiodinium*, as they may remain in the S phase of the cell cycle due to nutrient limitation or host-mediated growth arrest, degrading the un-utilized DNA after several hours. A sustained period in the S phase was not, however, noted in *Symbiodinium* populations within the anemone *Aiptasia pulchella* (Smith and Muscatine, 1999). Additional work is needed to fully elucidate exactly how division occurs in endosymbiotic *Symbiodinium*; however, these data highlight the temporally dynamic nature of nucleic acid composition in endosymbiotic organisms and emphasize the importance of normalizing for this parameter in gene expression studies.

4.3. Diel variation in *P. damicornis* MAPK expression

As coral gastrodermal cells are not uniformly spherical and are distorted and shrunk considerably upon immersion in traditional fixatives (e.g., paraformaldehyde), cell volume is technically difficult to measure in these anthozoans. Instead, gene candidates were chosen based on their function in vertebrate and invertebrate models in order to yield insight into the hypothesized light-driven changes in coral gastrodermal cell volume (Fig. 1).

4.3.1. bmk1

*bmk1*, whose respective protein is highly conserved across both invertebrate and vertebrate taxa (Fig. 2A), was the only MAPK with a detectable mRNA level in *P. damicornis*, and its expression co-varied with the SMP (Figs. 3A and 4), suggesting that this gene may be involved in regulating the *Symbiodinium* population, as has been suggested by Rodríguez-Lanetty et al. (2006). However, if the ancestral role of *bmk1* is conserved and this gene functions in the stimulation of osmolyte production, as is the case for many MAPKs (Kultz, 2001), then the nocturnal peak lends support to the hypothesis that host osmoregulatory response pathways are active during periods when there is no translocation of photosynthe from the *Symbiodinium*.

4.3.2. erk2 and p38

ERK2 is in the ERK1 subfamily of MAPKs and has a variety of functions including regulation of proliferation, differentiation, cell survival, and osmotic signaling (Kultz, 2001). ERK2 also stimulates transcription factors necessary to produce proteins involved in cytoskeletal structure, such as Myc and Ras (Qi and Elion, 2005), indicating that it is an important protein to monitor under varying osmotic pressures. That said, after designing nine primer sets that all successfully amplified genomic DNA and carrying out extensive PCR trouble-shooting, erK2 mRNA expression was undetectable. Likewise, the protein encoded by p38 plays a key role in osmotic homeostasis by activating osmolyte production pathways (Winkler et al., 2002), but no p38 mRNA expression was detected. Failure to detect mRNAs of both these genes with a variety of primer sets that successfully amplified genomic DNA suggests that the respective proteins are controlled post-transcriptionally or that the genes are only expressed during stressful conditions.

Given the many signal transduction pathways that MAPKs control, the nocturnal increase in *bmk1* expression could not be confidently linked to a specific phenotype. The elevated *Symbiodinium* densities detected at night and the concomitant increase in intracellular crowding also confounded the interpretation of the increased nocturnal expression of this gene, a phenomenon discussed in greater detail below. To examine this in more detail, genes encoding the dominant protein components of the cytoskeleton: β-actin, tropomyosin, and α-tubulin were quantified in *S. hystrix*, with the rationale that a change in their expression would be indicative of a change in cell shape or volume (Koivusalo et al., 2009).

4.4. Diel variation in *S. hystrix* cytoskeleton gene expression

The already crowded state of most cells is exacerbated under periods of reduced intracellular osmotic pressure (Goodsell, 1993; Pedersen et al., 2001) and unless appropriate space is rapidly generated, the numerous biochemical reactions that take place inside the cell will cease to occur due to, for example, the lack of macromolecular access to free water molecules (Garner and Burg, 1994). Cells respond to reduced intracellular osmotic pressure by accumulating compatible solutes from the external environment, by initiating osmolyte production pathways to re-establish ambient cellular osmolarity (Kultz, 2001) and by reducing the expression of genes encoding spatially demanding proteins (Ovadi and Saks, 2004), such as tubulin (Minton, 2001) and actin. Actin, for example, can compose up to 15% of cell volume (Schevzov et al., 1992), and β-actin undergoes changes in expression in osmotically compromised cells in humans (Thirone et al., 2009) and sharks (Kultz et al., 2007). Likewise, α-tubulin exhibits fluctuations in expression in response to osmotic stress in human kidney (Dihazi et al., 2005) and liver cells (Haussinger et al., 1994). Accordingly, we hypothesized that the expression of β-actin, its polymerizing catalyst tropomyosin (Gunning et al., 2008), and α-tubulin would decrease at night, reflecting a temporary decrease in cell volume due to a drop in osmotic pressure stemming from the nocturnal cessation of *Symbiodinium* osmolyte translocation. As expected, we observed 4, 3.5, and 7.5-fold reductions in the mRNA levels of β-actin, tropomyosin, and α-tubulin, respectively, at 21:00 relative to 12:00 (Fig. 5).

A simultaneous decrease in expression of these three cytoskeleton genes at night provides support for the hypothesis that coral gastrodermal cell volume decreases temporally at night, perhaps reflecting the nocturnal cessation of *Symbiodinium* photosynthesis and the translocation of osmolytes into the host cell (Fig. 1). As *Symbiodinium* derived osmolytes are respired minutes to hours after
dusk, the coral host must ultimately re-establish intracellular osmolarity, leading to small changes in gastrodermal cell volume prior to RVI. As the Symbiodinium cells, which likely maintain similar size due to their ability to withstand turgor pressure, consume a relatively greater volume of the shrunken coral gastrodermal cell, host cytoskeleton gene expression may decrease to prevent crowding by spatially demanding actin and tubulin proteins (Papakonstani and Stournaras, 2008). Likewise, as the host gastrodermal cells enlarge upon increase in osmotic pressure after initiation of Symbiodinium photosynthesis in the morning, the cellular expansion will require an increase in actin filaments and microtubules via enhanced expression of β-actin and α-tubulin, respectively (Di Ciano et al., 2002). It should be noted here that, while we expect that only the gastrodermal cells housing Symbiodinium experience such diel variation in expression of these cytoskeleton genes, we extracted mRNA from whole coral polyps and so measured gene expression across both epithelial and gastrodermal tissue layers. Thus, it is likely that by averaging gene expression across both tissues, the extent of the temporal change in gene expression within the gastrodermal cells themselves may have been underestimated. In future studies, then, it may be worthwhile to physically separate the two tissue layers prior to biological extractions such that the reported gene expression trends better describe the cells within the tissue layer of interest. In such an experiment, tissue thickness could also be measured, thus shedding light on how tissue morphology influences the osmoregulatory behavior of cells.

4.5. Crowding and cytoskeleton reorganization in coral gastrodermal cells

The nocturnal increase in SMP observed in both P. damicornis and S. hystrix indicates that there may be a temporary increase in the density of Symbiodinium in the coral cells at night. As such, Symbiodinium division may also contribute to increased endosymbiotic gastrodermal cell crowding and a reorganization of the host cytoskeleton. Both the hypothesized nocturnal drop in osmotic pressure and the observed increase in intracellular Symbiodinium densities could, then, cause decreased cytoskeleton gene expression, or, in the case of P. damicornis, an increase in expression of bmk1. This cell volume compromised state (Fig. 1 in Venn et al., 2009) will lead to a RVI by the coral until either the cell volume of the holobiont is elevated or the “excess” Symbiodinium are released. Future work should address diel patterns in Symbiodinium DNA content and division (sensi Smith and Muscatine, 1999) in order to determine whether DNA replication ultimately results in cell division in all instances.

4.6. Conclusions

This study provides evidence that nocturnal cessation of Symbiodinium photosynthesis and/or cell division increases host gastrodermal cell crowding and promotes cytoskeleton reorganization. These data underscore the fact that there are still significant gaps in our knowledge of our coral–dinoflagellate symbioses that must be better understood and considered if scientists are to successfully interpret data from manipulative studies that seek to mimic future environmental conditions. Specifically, as temperature stress causes Symbiodinium photoinhibition (Jones et al., 2000) and hence a disruption to the metabolic dialogue that binds the holobiont, failure of the coral host to maintain the osmotic pressure of the holobiont may ultimately trigger apoptosis (Kultz, 2005) and necrosis pathways (Mayfield and Gates, 2007). Hence, cell volume regulation in coral–dinoflagellate symbiosis represents an important avenue for future research aimed at understanding the mechanisms by which corals will acclimatize to changing environmental conditions.

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