MOLECULAR ECOLOGY
RESOURCES
Molecular Ecology Resources (2014) doi: 10.1111/1755-0998.12218

Diagnostic gene expression biomarkers of coral thermal stress

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Abstract

Gene expression biomarkers can enable rapid assessment of physiological conditions *in situ*, providing a valuable tool for reef managers interested in linking organism physiology with large-scale climatic conditions. Here, we assessed the ability of quantitative PCR (qPCR)-based gene expression biomarkers to evaluate (i) the immediate cellular stress response (CSR) of *Porites astreoides* to incremental thermal stress and (ii) the magnitude of CSR and cellular homeostasis response (CHR) during a natural bleaching event. Expression levels largely scaled with treatment temperature, with the strongest responses occurring in heat-shock proteins. This is the first demonstration of a 'tiered' CSR in a coral, where the magnitude of expression change is proportional to stress intensity. Analysis of a natural bleaching event revealed no signature of an acute CSR in normal or bleached corals, indicating that the bleaching stressor(s) had abated by the day of sampling. Another long-term stress CHR-based indicator assay was significantly elevated in bleached corals, although assay values overall were low, suggesting good prospects for recovery. This study represents the first step in linking variation in gene expression biomarkers to stress tolerance and bleaching thresholds *in situ* by quantifying the severity of ongoing thermal stress and its accumulated long-term impacts.

Keywords: cellular homeostasis response, cellular stress response, double-gene assay, *Porites astreoides*, qPCR

Received 1 October 2013; revision received 13 December 2013; accepted 14 December 2013

Introduction

The earliest steps of an organism's response to environmental stress occur at the molecular level, comprising the cellular stress response (CSR). Stress-induced damage to cellular components triggers the expression of response genes, including molecular chaperones, antioxidants and redox enzymes, and enzymes to mitigate damage to membranes, proteins and DNA (Kültz 2005). As the proximate interpreter between stressor impacts and the physiological response, transcriptomic analyses have been proposed as a standard metric for quantifying stress and evaluating an organism's condition *in situ* (Evans & Hofmann 2012). Gene expression analyses could therefore provide valuable information for conservation managers interested in linking organism physiology with large-scale climatic conditions.

Gene expression biomarkers could be particularly useful for monitoring the health of reef-building corals. Corals constitute the foundation of tropical reefs, the most diverse ecosystem in the ocean. As sessile marine organisms, corals cannot escape environmental conditions and must therefore mount a physiological stress response to cope with environmental perturbations. Reef-building corals are obligate symbiotic organisms, consisting of a cnidarian host harbouring intracellular populations of dinoflagellates of the genus

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Symbiodinium. Environmental stress can result in a disruption of this relationship, commonly known as coral bleaching, whereby symbiont cells are lost and/or their photosynthetic pigments are degraded, resulting in a coral that appears pale or white (Brown 1997). Climate change has resulted in an increase in the frequency, duration and severity of environmental perturbations (Glynn 1993; Brown 1997; Gardner *et al.* 2003; Hughes *et al.* 2003; Hoegh-Guldberg *et al.* 2007). Extreme temperature anomalies, in combination with additional stressors such as anthropogenic disturbance and eutrophication (Harvell *et al.* 1999; Lesser *et al.* 2007), impact corals worldwide with significant economic (Cesar 2000) and ecological consequences (Walther *et al.* 2002; Baker *et al.* 2008). Consequently, corals are the focus of major conservation efforts (Mumby & Steneck 2008). A biomarker system to rapidly and quantitatively evaluate coral stress condition *in situ* would facilitate prioritization of conservation efforts among reef locations and coral populations. Protein-based biomarker assays have previously been reported for reef-building corals (Downs *et al.* 2000, 2005). However, these assays rely on polyclonal antibodies that are difficult to standardize both between batches and between laboratories, while expression-based assays can be easily replicated in multiple laboratories, facilitating their broad practical application.

Early work on gene expression in a variety of coral species confirmed the presence of a largely conserved CSR. The most prominent responses to thermal stress in adult corals include immediate upregulation of heat-shock proteins (HSPs) and antioxidant enzymes (DeSalvo *et al.* 2008, 2010; Csaszar *et al.* 2009; Seneca *et al.* 2010; Kenkel *et al.* 2011). Differential regulation of antioxidant and calcification genes has also been shown in adult corals exposed to elevated salinity and pollutant levels (Edge *et al.* 2005; Morgan *et al.* 2005), suggesting that the coral CSR is a generalized stress response. Furthermore, the CSR response in corals is seen not only in adults, but in juvenile life history stages as well. Expression of HSPs, antioxidants and DNA and tissue repair enzymes have been reported in multiple species of coral larvae (Meyer *et al.* 2009, 2011; Rodriguez-Lanetty *et al.* 2009; Voolstra *et al.* 2009; Polato *et al.* 2013), supporting the idea of a conserved CSR. Similar generalized CSRs have been reported in yeast (Gasch *et al.* 2000), and components of this system occur in humans (Kültz 2003), indicating a conserved evolutionary strategy to deal with stress (Kültz 2005).

While the CSR is an immediate and transient response to stress, once damage control has been effected, a secondary response, called the cellular homeostasis response (CHR), is triggered to reestablish homeostasis under the new environmental conditions (Kültz 2005). Differential regulation enacted under the CHR is specific to the particular environmental variables that triggered the CSR and permanent until another change in environmental conditions occurs (Kültz 2003). Long-term consequences of corals exposed to thermal stress include downregulation of Ca^{2+} homeostasis and ribosomal proteins, and changes to cytoskeleton and extracellular matrix proteins (DeSalvo *et al.* 2008; Kenkel *et al.* 2013b), suggesting differential allocation to growth and regulation of symbiosis. Thus, biomarkers of the CHR could be particularly informative about specific stressors affecting different reefs. Additionally, as the CHR reflects long-term changes in organismal physiology, it may reflect remaining resilience potential and therefore better inform reef conservation efforts.

Before gene expression can be applied as a universal biomarker, however, we must understand its limitations, particularly with respect to environmental sensitivity and how expression patterns relate to the physiological and ecological consequences of stress tolerance. To begin addressing these issues, we first ask whether CSR gene expression can be used as a quantitative measure. An 'all-or-nothing' response would provide little information regarding the intensity of stress experienced by the organism and would therefore be of little use as a quantitative biomarker. In contrast, a 'tiered' response where the magnitude of expression change is proportional to stress intensity would be directly applicable to conservation efforts as expression biomarkers could quantify the severity of stress and be used to establish thresholds for physiological function (Evans & Hofmann 2012). Similar quantifiable thresholds have been described for sediment-stressed corals using visual and histopathological descriptions (Vargas-Ángel *et al.* 2006, 2007). We address this question by profiling biomarker gene expression in coral fragments subjected to thermal stress of different intensity. We then apply these markers *in situ*, to profile an ongoing natural bleaching event in the Florida Keys, USA, to determine how well CSR- and CHR-based expression assays reflect the severity and progress of bleaching.

The expression assays used here were previously developed for use in the mustard hill coral, *Porites astreoides* (Kenkel *et al.* 2011, 2013b). This coral has two important advantages as a candidate bioindicator species: (i) it is ubiquitously distributed throughout coral reef habitats of the Caribbean Sea and (ii) it is not endangered, rendering sampling of coral colonies for biomarker analysis feasible from the reef conservation perspective. Furthermore, these gene expression assays are potentially transferrable to the Indo-Pacific congener, *P. lobata*, facilitating their cosmopolitan use as biomarkers (Kenkel *et al.* 2011).

Materials and methods

Tiered CSR experiment

Eleven colonies of *P. astreoides* were collected at a depth of 2–3 m from an offshore reef (N 24°31.303, W 81°34.605) near Sugarloaf Key, Florida Keys, Florida, on 21 July 2011 under Florida Keys National Marine Sanctuary (FKNMS) permit 2011-082. Colonies were immediately brought to Mote Marine Tropical Research Laboratory and quartered using a tile saw. The resulting four fragments were placed in a shaded (70% photosynthetically active radiation reducing) flow-through seawater trough (raceway) with an average water temperature of 27.5 ± 0.9 °C and allowed to acclimate for 4 days. Postacclimation, one fragment of each colony was randomly assigned to a temperature treatment (29, 31 or 33 °C), and a tank within that treatment (n = 3–4 fragments per tank).

Each temperature treatment consisted of three 40-L aquaria with clear plastic lids. Tanks were completely filled with seawater and equipped with a 2-W aquarium pump (HJ-311, Ningbo Hesen Arts and Crafts Co., Ltd., Zhejiang, China). These nine aquaria were placed haphazardly in one shaded (70% light reducing) raceway with flow-through serving as a water bath. HOBO Pendant Temperature/Light Data Loggers (UA-002-64, Onset Computer Corp., Bourne, MA, USA) programmed to record temperature and light intensity at 10-min intervals were placed in all three 33 °C tanks and two of the three 29 and 31 °C treatment tanks. Coral fragments were placed in their assigned treatment tanks at ambient temperature. No additional changes were made to the ambient (29 °C) temperature treatment tanks. In the elevated temperature treatments (31 and 33 °C), each individual tank was also equipped with a 200-W aquarium heater (ML90438-00, Marineland Aquarium Products, Blacksburg, VA, USA) set to the respective treatment temperature. The chosen target temperatures naturally occur at nearshore reefs of the Florida Keys during summer months (http://serc.fiu.edu/wqmnetwork/FKNMS-CD/). Post acclimation, the heaters were turned on, and water temperature gradually increased to the desired level over the course of 4 h (Fig. 1). Though rapid, this ramping rate is not unrealistic for natural coral populations. The phenomenon of fast thermal stress (Rosic *et al.* 2011) has been observed repeatedly on the Great Barrier Reef (Berkelmans 2002; Berkelmans & Willis 1999; Dove 2004), and more recently, Mayfield *et al.* (2013) reported that corals in Houbihu, Taiwan, experience 8–9 °C temperature changes over the course of a day during spring-tide upwelling events.

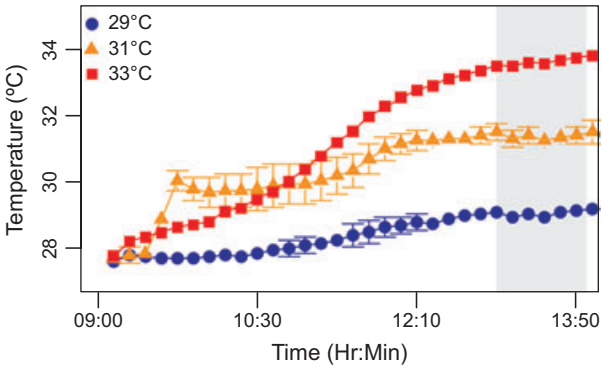

Fig. 1 Temperature profiles (average ± SEM) across treatment tanks (n = 3 tanks per treatment). Shaded area represents the time during which coral fragments were sampled for gene expression analyses.

At the time of sampling, mean temperatures were 29.1 ± 0.2 °C, 31.5 ± 0.3 °C and 33.5 ± 0.2 °C for the ambient, medium and high temperature treatments, respectively (Fig. 1). Levels of ambient light were sufficiently low in all tanks, marginalizing the effect of light on observed stress responses. The ambient and medium temperature treatment tanks received equivalent light levels, of 41.6 ± 1.5 lux and 43.8 ± 0.7 lux, respectively, whereas the high treatment tanks experienced lower light levels, of 21.5 ± 0.9 lux at the time of sampling (Fig. S1, Supporting information). Tissue samples for RNA isolation were removed from the skeleton using a razor blade, and RNA was extracted immediately.

Sampling of a natural bleaching event

A natural bleaching event occurred in late July/early August 2010 in the lower Florida Keys probably due to an extended period of water temperatures over 30 °C (Bartels & Walter 2010). On the morning of 11 August 2010, 20 colonies of *P. astreoides* were haphazardly selected for sampling at a depth of 2–3 m from an inshore patch reef near Sugarloaf Key (N 24°35.142, W 81°34.957) under FKNMS permit 2010-093. At the time of collection, visibility was 2–3 m and water temperature was 29–30 °C. Ten of these colonies displayed a bleaching phenotype, whereas the other ten appeared normal. The symbionts' photosynthetic efficiency at photosystem II was measured for each selected colony prior to sampling using pulse-amplitude-modulated fluorometer (Diving-PAM, Heinz Walz GmbH, Effeltrich, Germany) to confirm bleaching patterns. A tissue sample of approximately 3 cm^2 was then collected from each colony using a hammer and chisel. These samples were immediately brought to the surface and fixed in RNALater (AM7021, Ambion, Life Technologies, Grand Island, NY, USA) on

ice. Upon return to Mote Tropical Research Laboratory, samples were stored at −20 °C until processing.

Symbiodinium genotyping

Coral subsamples from the *in situ* bleaching event were stored in 95% ethanol for *Symbiodinium* genetic analysis. From each sample, total genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (A1120, Promega Corporation, Madison, WI, USA). The internal transcribed spacer 2 region (ITS2) of the *Symbiodinium* ribosomal DNA was amplified using the primers ITSinfor2 and ITS2CLAMP (LaJeunesse & Trench 2000) with the amplification conditions described by LaJeunesse (2002). The PCR-amplified ITS2 region was then electrophoresed on an 8% polyacrylamide denaturing gradient gel (45–80% urea–formamide gradient) at a constant temperature (60º C) for 13 h at 120 V (Thornhill *et al.* 2006). The denaturing gradient gel electrophoresis (DGGE) gels were stained for at least 20 min with SYBR Green I nucleic acid gel stain (S-7567, Invitrogen, Life Technologies, Grand Island, NY, USA). Informative bands were excised (LaJeunesse 2005) and sequenced on an Applied Biosystems 3730 capillary sequencer. The resulting sequences were analysed manually using GENEIOUS (version 5.3.6) and compared with GenBank submissions (Pruitt *et al.* 2012).

RNA extraction and cDNA synthesis

Total RNA was extracted using an RNAqeous kit (AM1912, Ambion, Life Technologies, Grand Island, NY, USA), and quality was assessed through gel electrophoresis and evaluated based on the presence of the ribosomal RNA bands. Of the 11 original paired samples in tiered CSR experiment, 9 fully paired (29, 31 and 33 °C treated fragments) were of sufficient quality for gene expression analysis. For the last two original paired samples, the 29 °C treated fragments were discarded due to insufficient quality of rRNA bands. Of the 20 field-collected samples, 12 were of sufficient quality for gene expression analysis (six bleached and six normal). RNA samples were then DNAse treated and reverse-transcribed into cDNA as in Kenkel *et al.* (2011).

Gene expression profiling

Expression of candidate stress response genes (Table 1) was measured using quantitative real-time PCR (qPCR) assays designed using Primer3 (Rozen & Skaletsky 2000). For the tiered CSR experiment, 10 target genes were chosen based on their use in a previous study aimed at identifying putative biomarkers of acute heat–light stress in coral (Kenkel *et al.* 2011). Five are associated with stress response: HSP16, Actin, HSP60, HSP90 and ubiquitin-like protein 3 (UBL3); two are putatively involved in the coral immune response: complement component C3 and spondin 2; two are metabolic genes: adenosine kinase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH); and one is of unknown function: a GFP-like chromoprotein (Table 1). For the *in situ* bleaching experiment, 13 targets were chosen based on short-term expression responses: the same 10 targets as for the tiered CSR with the addition of another immune candidate, a c-type lectin (Kenkel *et al.* 2011). Three additional genes were subsequently added to this analysis based on their expression responses to long-term thermal stress: an additional metabolic gene, phosphoenolpyruvate carboxykinase (PEPCK), and two genes putatively involved in cellular transport, exocyst complex component 4 (EXOC4) and a member of solute carrier family 26 (SLC26) (Kenkel *et al.* 2013b). qPCR reactions were performed in the Roche LightCycler 480 (Hoffmann-LaRoche Inc., Nutley, NJ, USA) in 15 μl volumes using 2× LightCycler 480 SYBR Green I Master Mix (04707516001, Hoffmann-LaRoche Inc., Nutley, NJ, USA), 0.1 μM forward and reverse primer and 1 ng of cDNA template. Preliminary analyses (CP calling and melting curve analysis) were performed using the GENESCAN software (Hoffmann-LaRoche Inc., Nutley, NJ, USA). Each cDNA sample was assayed in duplicate in independent qPCR runs, and these technical replicates were averaged for statistical analyses.

Double-gene assays

A double-gene qPCR assay, as introduced by (Kenkel *et al.* 2011), takes advantage of the anticorrelated responses of two indicator genes to the factor being measured. First, raw expression values are corrected for amplification efficiency (Pfaffl 2001) using the formula $Ca = -Cp * \log_2(E)$ (Kenkel *et al.* 2011), where Ca is the relative abundance value proportional to $\log_2$ of the starting target amount, Cp is the raw expression measurement, and E is the amplification factor per PCR cycle measured using a dilution series as in (Pfaffl 2001). The difference in Ca values is then calculated by subtracting the Ca value of the downregulated gene from the Ca value of the upregulated gene. This assay does not require use of additional internal control genes as the template loading factor cancels out when computing the difference in expression of two genes assayed in the same sample (Livak & Schmittgen 2001). Furthermore, the anticorrelated responses of the two indicator genes increase the power and dynamic range of the assay, improving detection capability (Kenkel *et al.* 2011). In this study, three previously proposed double-gene assays were applied: the acute stress assay (Actin – HSP16, Kenkel *et al.* 2011), the bleaching-in-progress assay (EXOC4 –

Table 1 List of primer information for candidate genes used in expression analyses. Abbreviations are as listed in text. Oligonucleotide sequences and primer efficiencies used for qPCR analyses and efficiency correction

Gene name (abbreviation, if used)	Primer sequence (5′-3′)	Biological Process	Efficiency
Actin	F: CAGTGTTTCCCTCCATCGTT R: CAGTTGGTTACAATGCCGTG	Stress response	2.12
Adenosine kinase (ADK)	F: AAAGAACCCACTGGAACGTG R: CAAATGCCCAGTTTTCTGGT	Metabolism	1.99
Complement component C3	F: TGTGGCACTACAGGCTCTTG R: GACATCAATCGCTCTGCGTA	Immunity	1.99
C-type lectin	F: CCCGGTGATACTGTGTCAGA R: AAATGCCAACCCAAGTAACG	Immunity	2.00
Eukaryotic initiation factor 3, subunit H (EIF3H)	F: TTGATTGATACCAGCCCACA R: ACAAACTGCTTTGCTTTCCC	Control gene	1.88
Exocyst complex component 4 (EXOC4)	F: CTCCAGTCCATCCATCCAGT R: TCCACAAGAATTGCAGCATC	Transport	2.03
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	F: TCCATGGACTTCGTTCACAA R: CAGAAGATCCACCACCCTGT	Metabolism	1.85
GFP-like chromoprotein	F: AGGTGCCACCGTATCACTTC R: CACTATTGCCTTTTCGCCAT	Unknown	1.98
Alpha B crystallin (HSP16)	F: TCACAGGAAAACACAGAGCG R: GGGTCACGTGCCACTTCTAT	Stress response	1.88
60 kDa heat-shock protein (HSP60)	F: CCAGCAGCGGTTTTCTCTTA R: CGGCAACAGCATCAGTTAAA	Stress response	1.93
90 kDa heat-shock protein (HSP90)	F: GTTGGGTCGGTCAAACTCTC R: GAGCATCCGAAGAGTTGGAG	Stress response	1.91
Phosphoenolpyruvate carboxykinase (PEPCK)	F: CTTTCGCAGGGATTCACATT R: CAGGCACCATCAACACTGAC	Metabolism	1.93
60s ribosomal protein L11 (RPL11)	F: TTTCAAGCCCTTCTCCAAGA R: GACCCGTGCTGCTAAAGTTC	Control gene	1.95
Solute carrier family 26 member 6 (SLC26)	F: TCTAGTTTGGCTGCGTCCTT R: ATTTGTCTGATGGTGGCACA	Transport	2.06
Spondin 2	F: CACGAGCACAAAAATCATGG R: GCAGGTCCATTGTCACCTTT	Immunity	2.00
Ubiquitin-like protein 3 (UBL3)	F: ATGGACTTTTGACCCTCACG R: ATGGTCGGTTTCTACATGGC	Stress response	2.13

UBL3, Kenkel *et al.* 2013b) and the long-term stress assay (PEPCK – SLC26, Kenkel *et al.* 2013b).

Statistical analysis

The analysis followed the procedures outlined in Kenkel *et al.* 2011. All statistical analyses were carried out using R software (R Core Development Team 2013). Briefly, after initial correction of Cp values for amplification efficiency and normalization using two control genes, RPL11 and EIF3H, which were previously identified as the most stable control genes in *P. astreoides* under heat–light stress (Kenkel *et al.* 2011), the data were analysed using linear mixed models on a gene-by-gene basis. Double-gene assays were modelled like individual genes.

For the tiered CSR experiment, temperature was modelled as a fixed factor, with levels '29', '31', '33', and colony identity was added as a scalar random factor. Significance of fixed and random factors was evaluated using likelihood ratio tests (LRT). For the natural bleaching analysis, phenotype was modelled as a fixed factor, with levels 'normal' and 'bleached', and factor significance was evaluated using ANOVA. If the effect of temperature in the tiered CSR experiment was found to be significant at the $P < 0.05$ level following false discovery rate (FDR) correction using the method of Benjamini & Hochberg (1995), a post hoc Tukey's HSD test was used to evaluate the significance of each pairwise comparison using the function *ghlt* of the *multcomp* package (Bretz *et al.* 2010). A Welch's *t*-test was used to assess differences in symbiont's photosynthetic efficiency at PSII and ambient light levels between bleached and normal corals that were subsequently sampled for expression analyses *in situ*.

Results

Tiered gene expression response reflects thermal stress intensity

For the majority of genes tested, expression patterns increased or decreased incrementally with increasing temperature (Fig. 2). While all genes exhibited trends with respect to increasing temperature stress, only five genes in our panel were significantly differentially expressed following FDR correction: HSP16, HSP60, HSP90, actin and spondin 2 ($P_{LRT} < 0.05$, Fig. 2). These five genes included all the HSP genes in our panel, which were upregulated in response to temperature stress. HSP16 exhibited the greatest magnitude change, with a 4.5-fold upregulation between 29 and 31 °C

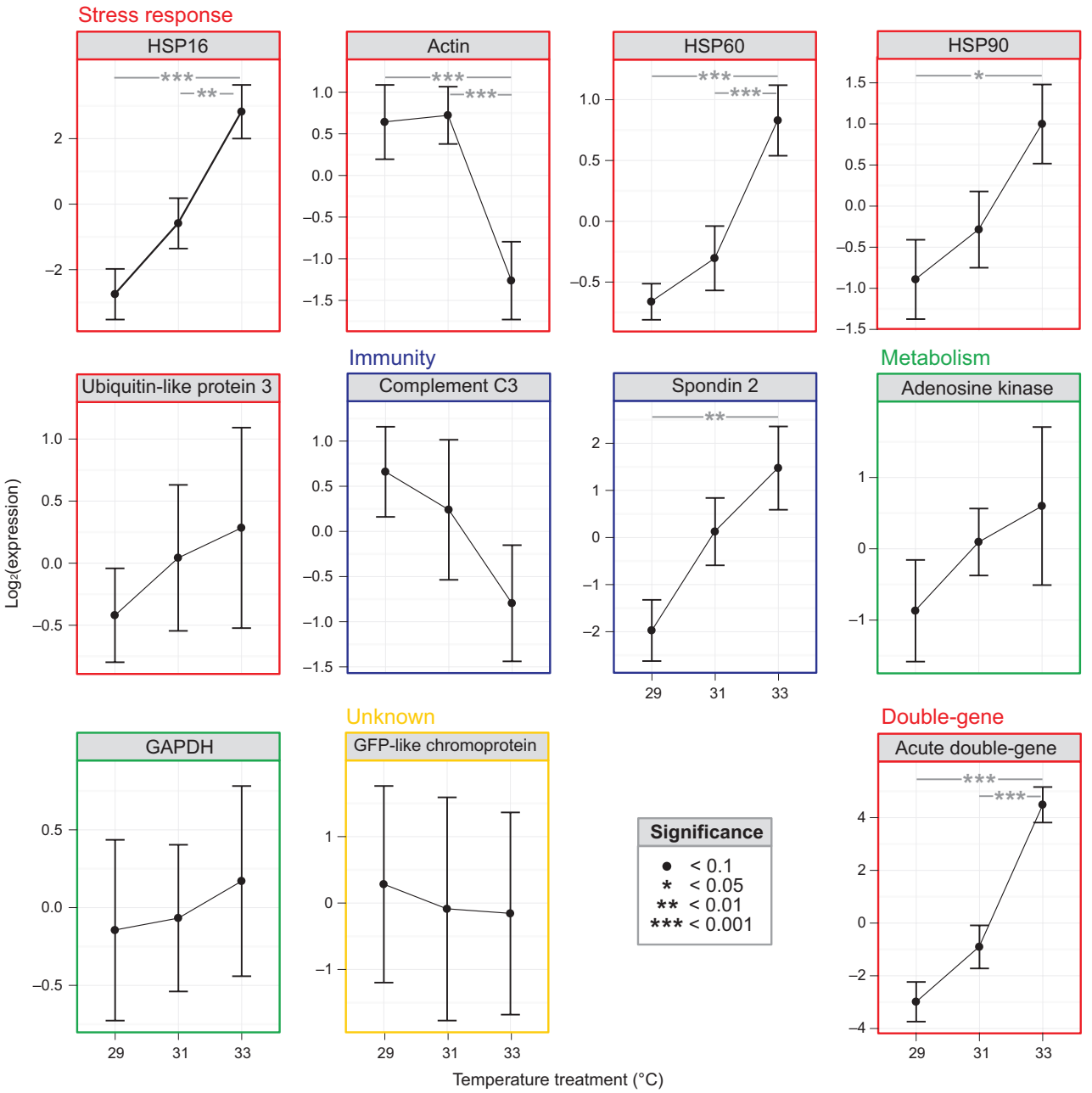

Fig. 2 Gene expression scales with the intensity of thermal stress experienced by a coral. Log_2-transformed relative expression values (± SEM) of candidate genes shown with respect to temperature treatment. Significance of post hoc Tukey's HSD comparisons between bleaching phenotypes is shown for genes with $P < 0.1$ after false discovery rate correction (Benjamini & Hochberg 1995). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSP, heat-shock protein.

(P_{HSD} = 0.14) and a 10.6-fold upregulation between 31 and 33 °C (P_{HSD} < 0.01) for a total change of 15.1-fold between 29 and 33 °C (P_{HSD} < 0.001, Fig. 2). HSP60 and HSP90 exhibited lesser upregulations of 1.3-fold and 1.5-fold between 29 and 31 °C (HSP60: P_{HSD} = 0.52; HSP90: P_{HSD} = 0.65) and 2.2-fold and 2.4-fold upregulation between 31 and 33 °C (HSP60:P_{HSD} < 0.001; HSP90: P_{HSD} = 0.12), yielding a total increase of 3.5-fold and 3.9-fold between 29 and 33 °C, respectively (HSP60: P_{HSD} < 0.001; HSP90:P_{HSD} < 0.05, Fig. 2). An immunity gene, spondin 2, also showed significant upregulation in response to temperature stress (P_{LRT} < 0.05), with 4.2-fold upregulation between 29 and 31 °C (P_{HSD} = 0.14) and 2.5-fold upregulation between 31 and 33 °C (P_{HSD} =0.41) for a total upregulation of 6.7-fold (P_{HSD} < 0.01, Fig. 2). Actin did not show a graded expression response between 29 and 31 °C, but was 4-fold downregulated between 31 and 33 °C (P_{HSD} < 0.001, Fig. 2).

We also tested for tiered expression using a previously described acute stress assay for *P. astreoides* (Kenkel *et al.* 2011), which is based on the upregulation of HSP16 and downregulation of *actin* under short-term heat stress. This assay revealed a 4-fold increase between 29 and 31 °C (P_{HSD}=0.14) and 42-fold increase between 31 and 33 °C (P_{HSD} < 0.001) for a total magnitude change of 46-fold (P_{HSD} < 0.001, Fig. 2).

Analysis of a natural bleaching event

Chlorophyll *a* fluorescence, reflecting the effective quantum yield (Φ_{PSII}) of *in hospite Symbiodinium* photosynthetic function, was significantly reduced in bleached corals (P < 0.001, Fig. 3) confirming the visually bleached phenotype.

The *Symbiodinium* in both bleached and normal *P. astreoides* exhibited two dominant ITS2 DGGE profiles. Upon sequencing, samples either had only the A4 profile or a combination of the A4 profile with an A4a profile, as has been reported previously for this species in the Florida Keys (Thornhill *et al.* 2006; Kenkel *et al.* 2013a). Neither of these symbiont types were systematically associated with bleaching phenotype.

To evaluate stress level and its history in these naturally bleached corals, we used the acute-stress double-gene assay and two longer-term stress assays for

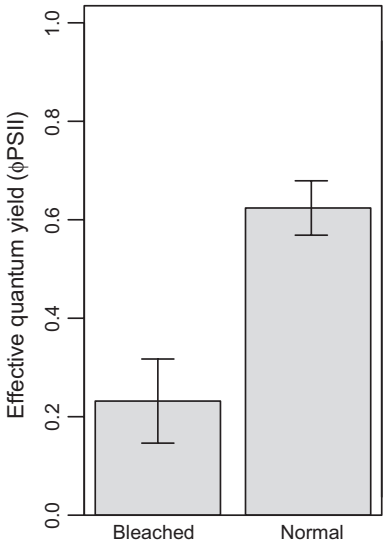

Fig. 3 Photochemical yield (± SD) of *in hospite Symbiodinium* at the time of sample collection for samples categorized as either bleached or normal.

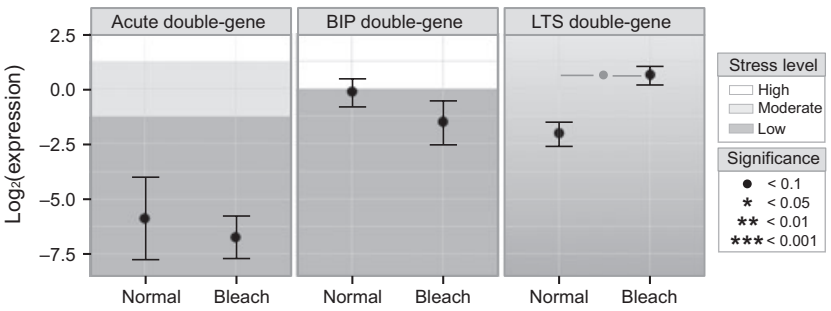

Fig. 4 Diagnostic double-gene assays to detect acute stress, bleaching-in-progress (BIP) and long-term stress (LTS). The assay value is the log2-transformed fold difference in expression of the two response genes relative to each other. Shading delineates assay values putatively indicative of coral stress level, from low stress (dark grey) to high stress (white). The stress thresholds for the acute double-gene assay are based on results presented in this article, and for the BIP double-gene assay on Kenkel *et al.* 2013b. Thresholds for the LTS double-gene assay remain to be established; therefore, putative stress level is indicated by a gradient. The acute double-gene assay values are well below zero for both phenotypes, indicating that none of these corals were experiencing acute stress on the day of sampling (Kenkel *et al.* 2011). The BIP assay also showed no significant difference between normal and bleached corals; however, BIP assay values for normal corals included positive values, suggesting that bleaching might be still ongoing in some of these individuals. LTS assay values were only slightly positive in bleached corals, suggesting that the severity of past stress was relatively minor.

P. astreoides based on expression patterns observed following a 6-week heat stress experiment (Kenkel *et al.* 2013b). The acute double-gene assay did not differentiate between normal and bleached corals (Fig. 4). In addition, assay values were well below zero for both phenotypes, indicating that none of these corals were experiencing acute stress on the day of sampling (Kenkel *et al.* 2011). The bleaching-in-progress assay (BIP), based on exocyst complex component 4 (EXOC4) and ubiquitin-like protein 3 (UBL3) (Kenkel *et al.* 2013b), also showed no significant difference between normal and bleached corals; however, BIP assay values for normal corals included positive values, suggesting that bleaching might be still ongoing in some of these individuals (Fig. 4). A long-term stress assay (LTS), based on the difference between a member of solute carrier family 26 (SLC26) and phosphoenolpyruvate carboxykinase (PEPCK), putatively reflecting the cellular homeostasis response (CHR), did distinguish a difference between normal and bleached corals, which remained marginally significant even after applying FDR correction ($P = 0.055$, Fig. 4). LTS assay values were only slightly positive in bleached corals, suggesting that the severity of past stress was relatively minor.

No other significant differences in gene expression were observed between normal and bleached corals for any of the individual candidate genes following FDR correction (Fig. 5), probably due in part to the number of corrections performed. Therefore, we explored trends in expression by looking for candidates with significant *P*-values prior to FDR correction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was downregulated in bleached corals by 11-fold (Fig. 5). SLC26, one of the genes comprising the LTS assay, exhibited substantial downregulation in bleached corals, by 16-fold on average (Fig. 5). Spondin 2 was the only candidate to show a strong trend of upregulation in bleached corals, with an increase in expression of twofold (Fig. 5).

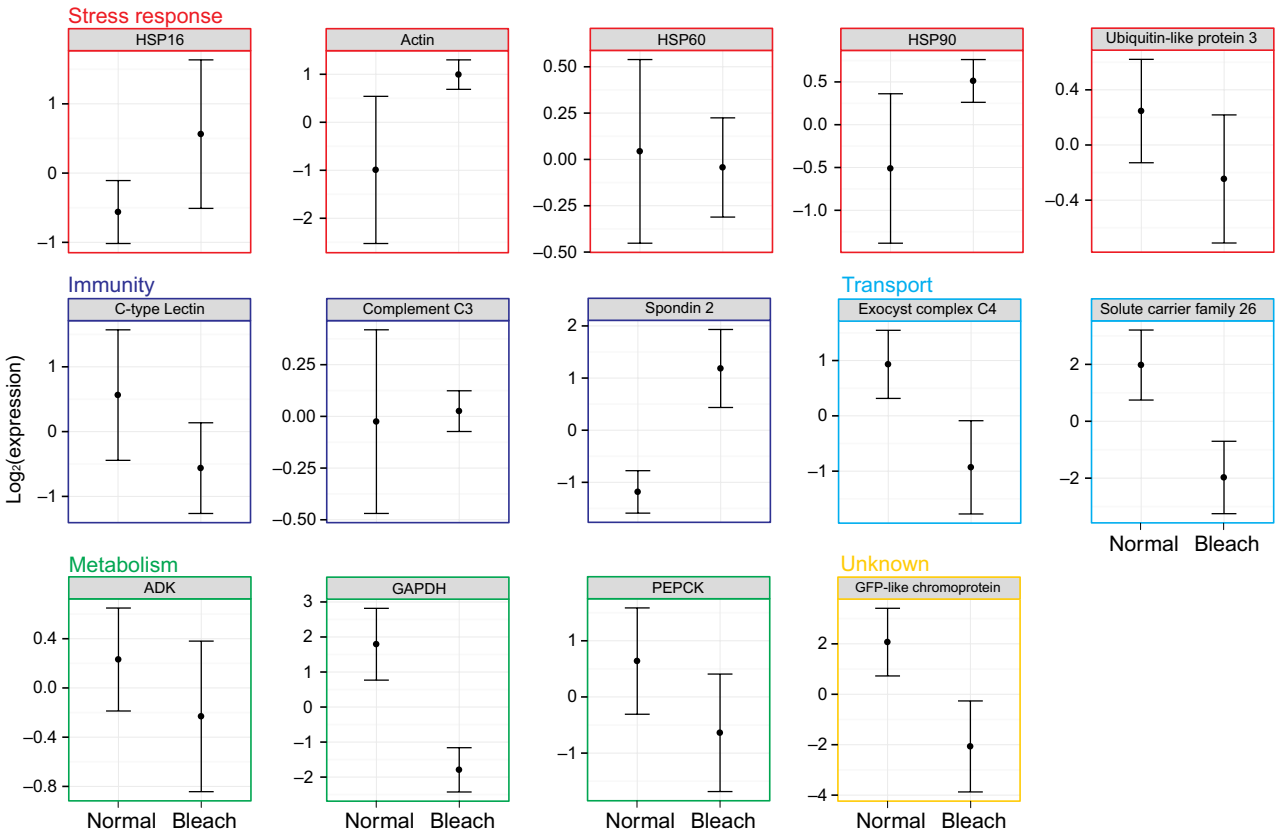

Fig. 5 Expression responses between phenotypically bleached and normal corals sampled during a natural bleaching event. Normalized $\log_2$-transformed expression values (± SEM) of candidate genes shown with respect to phenotype. Significance of post hoc Tukey's HSD comparisons between bleaching phenotypes is shown for genes with $P < 0.1$ after false discovery rate correction (Benjamini & Hochberg 1995). ADK, adenosine kinase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HSP, heat-shock protein; PEPCK, phosphoenolpyruvate carboxykinase.

Discussion

Expression of candidate stress response genes is tiered and may be regulated by thresholds

Empirically establishing the presence of a tiered cellular stress response (CSR) is an important step towards implementing gene expression biomarker monitoring into conservation management strategies (Evans & Hofmann 2012). Our study represents the first report of incrementally graded expression responses in a reef-building coral. While all genes showed trends in expression consistent with a tiered CSR, the molecular chaperones in our panel (HSP16, HSP16 and HSP90) all exhibited exponential upregulation of expression in response to linear increases in temperature. Fold changes were almost doubled for the two degree temperature increase from 31 °C to 33 °C as compared to the two degree increase from 29 °C to 31 °C (Fig. 2). Actin also showed a larger magnitude of change when the temperature increased from 31 °C to 33 °C than between 29 and 31 °C. This response may be due to the absolute difference in peak treatment temperatures or to the greater magnitude of change during the initial temperature ramping period. Although the interaction between stress intensity and duration remains to be tested in this system, the present data suggest that an upper threshold of coral tolerance could be reached between 31 and 33 °C, over which more extreme stress responses may be displayed. Data from several studies tend to support this hypothesis. For example, Leggat *et al.* (2011) reported that the PSII photosynthetic yields in dark adapted *Acropora aspera* symbionts subjected to heat stress (temperature increase of 1 °C/day) dropped significantly at 33 °C with a concomitant significant increase in HSP70 (8.2-fold) and HSP90 (10.5-fold) expression in the host coral. Similarly, *Acropora formosa* fragments exposed to either slow (0.5 °C/day) or fast (1 °C/day) heating from 23 °C to 33 °C showed significant reductions in *Symbiodinium* density (45–50% of controls), photosynthetic rates, lipid and protein contents when comparing these variables between 33 °C and either 30 or 31 °C (Middlebrook *et al.* 2010). However, as highlighted by Fitt *et al.* (2001), no temperature threshold can be mentioned without considering the duration of exposure and other confounding factors such as multiple stressors that vary considerably in natural reef environments. Furthermore, thermal history seems to play an important role in heat stress response (Bellantuono *et al.* 2012), with corals exposed to previous heat stress less likely to undergo significant damage (Middlebrook *et al.* 2008). CSR biomarkers could be a convenient method for integrating over these complexities as the CSR tends to be a nonspecific response to stress (Kültz 2005). Future work will aim to evaluate biomarker applicability under more complex environmental scenarios to validate this assertion.

Understanding natural stress regimes using gene expression biomarkers

The combined use of CSR and cellular homeostasis response (CHR) biomarkers represents one of the most promising routes to using gene expression as a predictor of coral health under stress. Our study of the natural bleaching event is the first attempt to investigate stress at a particular reef using a combination of CSR and CHR gene expression biomarkers. Corals did not appear to experience acute stress on the day of sampling, irrespective of bleaching phenotype, as the acute-stress double-gene assay values stayed well below zero (Fig. 4). This suggests that, despite some corals exhibiting a clear bleaching phenotype, the causative stress had already abated. Interestingly, the bleaching-in-progress (BIP) double-gene assay, presumably quantifying exocytosis relative to the rate of recycling of plasma membrane components (Kenkel *et al.* 2013b), showed higher values in normal-looking corals relative to bleached corals (Fig. 4), which might indicate ongoing bleaching. The long-term stress (LTS) double-gene assay proved most effective in discriminating between bleached and normal corals. The LTS assay capitalizes on previous observations in the response of two CHR components to long-term thermal stress: upregulation of gluconeogenesis as evidenced by increased expression of PEPCK, a key regulatory enzyme of this pathway (Pilkis & Granner 1992), and reduced skeletal deposition reflected in the downregulation of a putative bicarbonate–chloride exchanger SLC26 (Kenkel *et al.* 2013b). Notably, although PEPCK was not upregulated in corals undergoing a natural bleaching event, the LTS double-gene assay was still more powerful than the measurement of SLC26 relative to two control genes due to less residual variation. According to the LTS assay, the accumulated stress exposure was relatively mild: even in bleached corals, the LTS value was only slightly positive, whereas values exceeding 10 have been observed in severe stress cases measured in the laboratory (Kenkel *et al.* 2013b). We hesitate at present to name more specific stress thresholds for the LTS assay, as assay values obtained in our original experiment (Kenkel *et al.* 2013b) were universally higher than those reported here. One explanation for this discrepancy may be that all fragments in the earlier experiment were experiencing some form of stress. As they were kept in shaded tanks in filtered seawater for 6 weeks, nutritional deprivation seems likely, although further work is needed to test this conjecture.

Taken together, these double-gene assay results suggest that this reef site was affected by thermal stress and that bleaching might be still ongoing in some corals. However, the acute stress that initiated bleaching either abated prior to our sampling or was of mild intensity, indicating good prospects for recovery. Indeed, corals at this sampling site did not suffer any noticeable mortality as a result of the studied bleaching event (C. Walter, pers. comm).

Outlook for gene expression biomarkers of coral stress

An interdisciplinary approach, incorporating molecular technologies into traditional survey methods, can rapidly diagnose coral health across locations to help prioritize conservation efforts and facilitate resource management strategies aimed at mitigating causative stressors (Vasseur & Cossu-Leguille 2003; Downs *et al.* 2005). The results presented here show that expression biomarkers can quantify the intensity of stress experienced by a coral and can potentially provide insight into the past dynamics and future outcomes of the stress event at the sampled locality. These biomarkers involve minimal sampling, can be rapidly analysed and eliminate surveyor bias and therefore should be considered by managers in the development of reef conservation plans. Future analyses should aim to evaluate the ubiquitous *P. astreoides* as a bioindicator species, such that the biomarker measures obtained for samples of *P. astreoides* could be benchmarked to reflect responses on the scale of the whole coral community at the studied location. However, the diagnoses presented in the preceding section remain tentative without more extensive confirmation that must be obtained by linking field-collected data with gene expression variation, including historical environmental conditions, bleaching thresholds and risks of mortality. As such data can be accumulated through regular practical application of these biomarkers, we think that the most optimal way to proceed is to integrate gene expression biomarker sampling into current reef survey practices while establishing a central data storage and analysis platform to refine predictions based on accumulating observations.

Acknowledgements

This paper is the outcome of the second and third 'qPCR for Marine Biologists' workshops held at Mote Tropical Research Laboratory (Summerland Key, FL). The participation of A deVillers, L Hédouin, M Hellstrom, C Logan, E López, C Lowery, P Montoya-Maya, D Perez, Z Pratte, M Ramot, L Ruiz-Jones, JA Sánchez, T Schils, L Squiers and D. Wanless was instrumental in sample collection and processing. The efforts of E Bartels and the staff of Mote TRL greatly aided this work. Authors would like to acknowledge the following funding sources: Western Indian Ocean Association of Marine Science (WIOMSA) to RB; FNRS grant F3/5/5-A2/5-MCF/DM-A115 to CS; SFRH/BD/63783/2009 funded by the Fundação para a Ciência e Tecnologia (QREN-POPH-Type 4.1, subsidized by the European Social Fund and national funds MEC) to MCL; SPIRE program and UNC Chapel Hill Postdoctoral Scholars Award for Research Excellence to KDC; UM ORSP and the National Science Foundation (IOS-0747205) to TLG. These workshops are supported in part by Roche, Rainin, Nanodrop, Ambion and Clontech, who provided reagents and consumables and supplied loaner equipment. We are also very grateful to AC Baker (RSMAS) who loaned us the Diving-PAM.

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C.D.K. and M.V.M. conceived and designed experiments. C.S., M.C.L., K.D.C., N.K., C.D.K. and M.V.M. conducted tiered CSR experiment. R.B., E.M., T.L.G., C.D.K. and M.V.M. conducted *in situ* bleaching experiment. R.B. completed PAM analysis, and T.L.G. genotyped *Symbiodinium* types. C.D.K. conducted gene expression analyses. All authors contributed to writing.

Data Accessibility

All expression data, phenotypic data, sequence data and R scripts are available on DRYAD (doi:10.5061/dryad.1h0n1).

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Light profiles (average ± SEM) across treatment tanks (n = 3 tanks per treatment). Shaded area represents the time during which coral fragments were sampled for gene expression analyses.