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Strict thermal threshold identified by quantitative PCR in the sponge *Rhopaloeides odorabile*

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ABSTRACT: In light of increasing sea surface temperatures, quantifying the expression of stress-inducible genes in coastal organisms is imperative to identify early biomarkers of thermal stress. In the present study we developed a quantitative PCR (qPCR) assay to test the molecular response to heat stress in the Great Barrier Reef sponge *Rhopaloeides odorabile*. Suitable reference genes (coding for α-tubulin, 28S rRNA and ubiquitin) were identified among 5 candidates and then used to normalise expression of target genes (actin-related protein, calmodulin, ferritin, ubiquitin-conjugating enzyme, heat shock protein 90 [*Hsp90*] and heat shock protein 40 [*Hsp40*]) in samples exposed to high temperatures (31 and 32°C) for 1, 3, 14 and 15 d. A rapid down-regulation of most genes (actin-related protein, ferritin, calmodulin and *Hsp90*) was observed at both temperatures within 24 h, indicating an initial shut-down of the sponge's molecular systems in response to thermal stress. The increased expression of *Hsp40* and *Hsp90* in sponges at 32°C after 1 and 3 d respectively indicates an activation of the heat shock response system and is consistent with their role as chaperones for directing degraded proteins to proteolysis, this last process being sustained by an induction of the ubiquitin-conjugating enzyme gene at this temperature. While sponges kept at 32°C only survived for the first 3 d, none of the genes in sponges kept at 31°C were significantly different from those in the 27°C controls after 14 d. This indicates a very strict thermal threshold for *R. odorabile* between 31 and 32°C and is consistent with previous findings based on sponge necrosis and symbiotic disruptions in this species.

KEY WORDS: qPCR · Quantitative PCR · Thermal stress · Porifera · Great Barrier Reef

INTRODUCTION

Coral reefs are among the most biologically productive, diverse and economically important habitats, but they are threatened by an increasing number of sources, including climate change. It has been estimated that over the next 50 yr, surface ocean temperatures will exceed the temperature conditions under which coral reefs have flourished over the past 500 000 yr (Hughes et al. 2003) and drive corals to extinction in <100 yr (Hoegh-Guldberg 1999, Kleypas et al. 1999, Hoegh-Guldberg et al. 2007). Other more optimistic views predict that the long-term responses of coral reefs to climate change will be dictated by the capacity of reef organisms to adapt or acclimatise to warmer temperatures (Baker 2001, Baker et al. 2004, Rowan 2004). In order to predict the ability of reef organisms to adapt to changing environmental conditions, we need to enhance our understanding of their responses to stress, particularly at the molecular level. To date, research on thermal-stress responses in reef organisms has focused on corals and their symbiotic zooxanthellae, and only a few studies have assessed this response in other phyla (Kassahn et al. 2007, López-Legentil et al. 2008). This constitutes an important gap in coral reef biology, as other keystone reef organisms, especially invertebrates, remain largely under-studied (Przeslawski et al. 2008).

Porifera (sponges), are benthic filter-feeders and a critical component of coral reefs due to their abundance and diversity and their role in benthic-pelagic coupling (Bell 2008). Despite the ecological impor-

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tance of Porifera, the effect of thermal stress on sponges has rarely been investigated, particularly at the molecular level. The few studies published on this topic include a thermal-stress experiment in which Western blotting was utilised to show that the marine sponge *Suberites domuncula* expresses a 45 kDa polypeptide after heat treatment (Bachinski et al. 1997). Moreover, a drop in trehalose (a disaccharide that can protect yeast from temperature stress as shown by Hottiger et al. 1987 and Eleutherio et al. 1993), a reduction in the activity of the detoxifying and antioxidant enzyme glutathione-S-transferase (GST) and a concomitant decrease of its substrate glutathione (GSH) were recorded in the same specimens. In heat- and cold-stressed *Geodia cydonium*, an increased transcription of the heat shock protein 70 (*Hsp70*) gene and a reduction of the Rab GDP dissociation inhibitor (GDI) mRNA (a key element in the intracellular traffic system) were observed using Northern blotting (Krasko et al. 1997). These findings indicate the presence of active heat-stress protection mechanisms in sponges at the cellular level. More recently, 2 studies have utilised quantitative PCR (qPCR) to accurately quantify the stress response. The molecular response of the sponge *Xestospongia muta* undergoing fatal and cyclic bleaching in the field and during experimental thermal and salinity stress was assessed by measuring the abundance of the chaperone *Hsp70* gene transcript (López-Legentil et al. 2008) with qPCR. While *Hsp70* mRNA levels were significantly higher in the fatally bleached sponges than in normal tissues, expression of the *Hsp70* gene indicated that cyclic bleaching did not cause any additional stress. *Hsp70* gene expression varied over time in the salinity and temperature treatments, but while sponges were able to adjust to osmotic changes, higher-than-normal temperatures caused a significant increase in *Hsp70* transcript levels and a subsequent drop likely due to metabolic failure. The only other study to assess sponge stress responses using qPCR was an analysis of the immunological status of 2 populations of *S. domuncula* by comparing the allograft inflammatory factor-1 (AIF-1) transcript levels in specimens from different sites (Schröder et al. 2004).

qPCR is a highly sensitive method for accurately quantifying mRNA abundance levels in a variety of organisms (Perkins & Lotufo 2003, Bustin et al. 2009, Mieog et al. 2009, Jarosová & Kundu 2010). This technique is now routinely used in studies of marine ecology (Watanabe & Hamamura 2003, Hofmann & Place 2007, Smith & Osborn 2009), and guidelines have been developed to instruct researchers on how to optimally perform and interpret qPCR experiments (Bustin et al. 2009). An essential component of a reliable qPCR assay is the data normalisation: this process controls and corrects for experimental variations that may occur during the extraction, reverse transcription and amplification of RNA (Bustin 2000, 2002). The qPCR data can be normalised against sample size, total RNA concentration or genomic DNA. However, the use of reference genes as an internal control is highly advantageous as they have been exposed to the same treatment as the target genes and represent the cumulative error of the entire process (Huggett et al. 2005). The ideal reference gene is expected to maintain stable expression between experimental groups due to the fundamental function it plays in the cell (Ransbotyn & Reusch 2006). Unfortunately, a universal reference gene that is stably expressed in all tissues and across different treatments does not exist (Haller et al. 2004) and most importantly, recent studies have shown that many of the most commonly used reference genes, such as those coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), or ribosomal proteins (18S or 28S), do not actually maintain a constant level of expression across all tissues and under different experimental conditions. For this reason, it is necessary to validate the stability of candidate reference genes prior to their use in normalising the target genes in specific experimental designs (Suzuki et al. 2000, Vandesompele et al. 2002, Nicot et al. 2005, Gutierrez et al. 2008).

In a previous study, the effect of elevated seawater temperature on microbial symbiosis within the marine sponge *Rhopaloeides odorabile* was assessed (Webster et al. 2008). Sponges were exposed to temperatures ranging between 27 and 33°C. While no differences in bacterial community composition or sponge health were detected in treatments between 27 and 31°C, sponges exposed to 33°C exhibited a loss of microbial symbionts within 24 h and major cellular necrosis within 3 d. In order to determine the molecular-level sponge response to thermal stress, we have developed a qPCR assay to profile the expression levels of 6 genes that have previously been reported to be involved in the stress response (Feder & Hofmann 1999, Schroth et al. 2005, Schallreuter et al. 2007, Császár et al. 2009): the genes encoding for (1) ubiquitin-conjugating enzyme (UbC), which makes part of the ubiquitin-proteasome pathway responsible for the selective degradation of misfolded or damaged proteins produced during heat shock or oxidative stress and which cannot be repaired by the heat shock protein (HSP) molecular chaperones (Glickman & Ciechanover 2002, Goldberg 2003); (2) actin-related protein (arp2/3), which plays a central role in the control of actin polymerisation and cytoskeletal function (Machesky & Gould 1999); (3) ferritin (FER), a highly conserved and ubiquitous iron-sequestering protein that provides protection against the catalysis of deleterious oxidation of biomolecules by Fe^{2+}, playing a fundamental role in

protecting the cell from damage due to oxidative stress (Torti et al. 1988, Halliwell & Gutteridge 1989, Harrison & Arosio 1996); (4) calmodulin (CaM), a Ca^{2+}-binding protein that serves to maintain homeostatic balance and mediate the signal-transduction cascade by binding to and altering the activity of a variety of other proteins (Zielinski 1998); down-regulation of CaM has previously indicated oxidative and heat stress in the coral *Montastraea faveolata* (De Salvo et al. 2008); (5) heat shock protein 40 (*Hsp40*), which functions in parallel with *Hsp70* to promote protein folding, transport and degradation (Li et al. 2009); and (6) heat shock protein 90 (*Hsp90*), an abundant and highly conserved molecular chaperone that contributes to the folding, maintenance of structural integrity and proper regulation of many cytosolic proteins (Picard 2002). In addition, we rigorously assessed the suitability of 5 candidate reference genes that are commonly adopted as internal controls in gene-expression profiling studies.

MATERIALS AND METHODS

cDNA clone library construction and primer design. In April 2008, 9 specimens of *Rhopaloeides odorabile* were collected off Pelorus Island in the Great Barrier Reef (18° 32.710′ S, 146° 29.273′ E). Each sponge was placed in a separate sealed bag and quickly transported to the laboratory (~15 min). Three replicate specimens per treatment were each exposed to heat stress (33°C, salinity 35), salinity stress (27°C, salinity 25) or ambient (control) conditions (27°C, salinity 35) for 8 h in aquaria with static, but aerated water. The samples from the heat-stress and salinity-stress treatments were combined prior to preparation of cDNA clone libraries (1 of treated sponges and 1 of control sponges). Small pieces of the stressed sponges were ground together in liquid nitrogen using a mortar and pestle to make a single homogenate. The mRNA was extracted from control and treated homogenates using the DynaBeads mRNA Direct kit (Invitrogen) following the manufacturer's instructions. Equal amounts of mRNA (~27 ng) were reverse-transcribed with a Creator SMART cDNA Library Construction Kit (Clontech) and Prime Script Reverse Transcriptase (Takara) following the manufacturer's instructions. The cDNA (2 µl) was further amplified using the following protocol and primers: 95°C for 1 min, 24 cycles at 95°C for 15 s and 68°C for 6 min; CDS III/3′ PCR Primer 5′-ATT CTA GAG GCC GAG GCG GCC GAC ATG-d(T)30N–1N-3′ and 5′ PCR Primer 5′-AAG CAG TGG TAT CAA CGC AGA GT-3′. PCR products were digested with Proteinase K and *Sfi*I and successively size-fractionated following the Creator SMART cDNA Library Construction Kit instructions. The profile of the fractions was checked on a 1.1% agarose-ethidium bromide (EtBr) gel (3 µl of each fraction were run at 150 V for 10 min). For each library, 6 fractions containing cDNA were obtained and, according to manufacturer's instructions, the first 4 fractions containing cDNA within the correct size range were selected and pooled together, to be successively ligated into dephosphorylated pDNR-LIB vector using 3 different ratios of cDNA to the vector. A total of 6 ligation reactions (3 for the control-sponge library and 3 for the treated-sponge library) were sent to the Australian Genome Research Facility (AGRF, Brisbane, Australia) to be separately transformed into electrocompetent *Escherichia coli* cells and sequenced using M13 primers.

Sequences were edited with Sequencher v. 4.7 (Gene Codes): those lacking a $polyA^+$ tail and/or having poor-quality files were discarded and the vector was trimmed to isolate the actual genes. Putative gene sequences were then compared to GenBank sequences using the BLAST tool for a functional identification. A total of 116 unique genes were identified, from which 5 commonly used candidate reference genes (GenBank accession nos. GU951541, GU951542, GW667522, GW667523 and GW667524) and 6 target genes (GenBank accession nos. HS097565 to HS097570) were selected. The qPCR primers were designed using the open-access online software Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Rozen & Skaletsky 1999) according to guidelines given by Wang & Seed (2006). The presence of secondary structures such as primer-dimers, cross dimers and hairpins was checked using NetPrimer (www.premierbiosoft.com/netprimer/index.html). Primers were synthesised by Sigma-Aldrich. Primer sequences are given in Table 1.

Temperature-stress experiment. Sponges were collected at Pelorus Island on the Great Barrier Reef, Australia (18° 32.710′ S, 146° 29.273′ E) in July 2009. Briefly, 6 donor sponges were cut into a total of 100 clones (~15 cm^3) and randomly transferred to plastic racks without tagging for further identification. The sponge clones were allowed to heal on the reef for 12 wk before collection and transportation to the indoor temperature-controlled aquarium at the Australian Institute of Marine Science, Townsville (Queensland, Australia). The experimental design incorporated 3 temperatures (27, 31 and 32°C; range: ±0.2 °C) in 3 replicate 30 l flow-through aquaria per temperature, each holding 8 sponge clones. The experiment was conducted as described previously (Webster et al. 2008), randomly sampling 1 clone from each replicate tank per temperature at t = 0, 1, 3, 14 and 15 d. After 3 d, the sponges in the 32°C treatment displayed signs of cell discharge and necrosis and by the 4th day all sponges in this treatment were considered dead. In the

Table 1. *Rhopaloeides odorabile*. Forward (f) and reverse (r) primers used in the quantitative PCR assay to amplify target and reference genes in *R. odorabile*. e: reaction efficiency, rLSU: 28S rRNA, Ub: ubiquitin, a-tub: α-tubulin, act68: actin, mtLSU: mitochondrial rRNA, arp2/3: actin-related protein, CaM: calmodulin, FER: ferritin, *Hsp40*: heat shock protein 40, *Hsp90*: heat shock protein 90, UbC: ubiquitin-conjugating enzyme

Gene coding for	GenBank accession no.	Function	Primer sequence 5′-3′	Length (bp)	e
rLSU	GU951542	Structural component, large ribosomal subunit	f: TGTAGCCCGAAGCAGTTTT r: TTCATCCCGCATCGCCAGTT	184	1.06
Ub	GW667524	Protein degradation	f: TCAGCAACGATTGATTTTCG r: ACGCAGCCGTAGCACTAGAT	106	1.01
a-tub	GW667522	Microtubule constituent	f: ATTGGAGGGGGAGATGATT r: TGACCACGGGCATAGTTGTT	196	1.06
act68	GW667523	Cytoskeleton component	f: GCATGGAGAAGAGCTACGAA r: AAGAAGGATGGCTGGAACAA	97	1.09
mtLSU	GU951541	Structural component, large ribosomal subunit	f: CGAATGCCTCACTGTCTCAA r: CCCCAACTAAACTGTCTGTAT	139	0.9
arp2/3	HS097565	Control of actin polymerisation	f: ACCCGAGGTTGAAGTGAAGA r: CTATCTCATCCGCCTGTTT	142	1.04
CaM	HS097566	Ca^{2+} binding, signal transduction	f: GGGTGTTCGTAATCTTGTT r: ATCGTACCGTCTCCATCCTT	132	1.02
FER	HS097567	Iron sequestering	f: TCTTTGCTGTGCCAGTGAGTA r: CGCATTCAGGGTGGTAGTT	133	0.9
Hsp40	HS097568	Molecular chaperone	f: CGCATTCAGGGTGGTAGTT r: CTAAGTCCCGACCACGTTTC	200	1.02
Hsp90	HS097569	Molecular chaperone	f: TGGCGGTTCCTTTACAGTTC r: ACACCTCCTTGTCACGTTCC	200	0.97
UbC	HS097570	Protein degradation via the proteasome	f: AGCGACAATCATGGGACCT r: GGATAGTCCGTTGGAAAGT	89	1.06

remaining treatments, temperatures were maintained for the first 14 d and then all temperatures were returned to 27°C for the final 24 h of the experiment as a recovery period. Samples were immediately snap-frozen in liquid nitrogen after collection, and maintained at −80°C for no longer than a month before mRNA extraction and analysis.

RNA extraction and cDNA synthesis. RNA was extracted from all samples (1 cm^3) using the RNeasy Plant Mini kit (Qiagen) following the manufacturer's instructions. DNA contamination was eliminated with the DNA-free kit (Ambion). RNA quality, integrity and purity was confirmed by gel electrophoresis (the 18S and 28S bands were clearly visible) and by examining the 260:280 ratio (ranging from 1.92 and 2.06) using a Nanodrop spectrophotometer (Thermoscientific). The final RNA concentration was measured with a Nanodrop. First-strand cDNA synthesis was carried out using the Superscript Vilo cDNA synthesis kit (Invitrogen): the 20 µl reaction, containing 4 µl of 5× Vilo Reaction mix, 2 µl of 10× SuperScript Enzyme mix including random primers, 1 µg RNA and RNase-free water, was incubated at 25°C for 10 min, at 42°C for 80 min and terminated after 85°C for 5 min. All cDNAs were diluted to 1 ng μl^{-1} (RNA equivalent concentration) and aliquots were stored at −20°C.

qPCR assay optimisation. ***Amplification specificity and efficiency:*** PCR efficiency was assessed using the standard curve method: cDNA from control and treated samples were pooled together and 10-fold serial dilutions starting from 1 ng μl^{-1} to 10^{-4} ng μl^{-1} cDNA (RNA equivalent concentration) were run in duplicate in a Rotorgene 3000 (Corbett Research) using 250 nM primers (final concentration). All tubes, tips, qPCR tubes and nuclease-free water aliquots were UV-sterilised for 15 min before use. The PCR reaction consisted of 7.5 µl of 2× SensiMix *NoRef* (Quantace), 0.3 µl of 50× Sybr Green I solution (Quantace), 0.375 µl of each primer (forward and reverse, at 10 µM intial concentration), 2 µl of template cDNA and 4.45 µl nuclease-free water for a final volume of 15 µl. Master mix and cDNA template serial dilutions were prepared manually and dispensed in a robotic liquid-handling workstation CAS-1200 (Corbett Robotics). During each run, 2 no-template controls (NTC) were added to check for reagent contamination. Another reaction was run at the same time and with the same mix, with cDNA replaced by RNA in order to check for genomic DNA contamination. Cycling conditions were as follows: 10 min at 95°C, 35 cycles of 15 s at 95°C, 20 s at 55°C, 20 s at 72°C. The specificity of amplification was verified by melting curve analysis (50 to 99°C). In addi-

tion, agarose gel electrophoresis followed by ethidium bromide staining was performed to confirm correct size of the qPCR products. Reaction efficiency (e) was calculated from the slope (m) of the standard curve ($e = [10^{(-1/m)}] - 1$) obtained by plotting the quantification cycle (C_q) versus the log of the template concentration (after manually setting the threshold value with the Rotorgene 6.1 software).

Validation of reference genes: The expression stability of the 5 candidate reference genes was tested using biological triplicates and technical duplicates for sponge clones sampled after 0, 1, 3, 14 and 15 d at the 3 different temperatures (27, 31 and 32°C). Cycling conditions and reaction mixes were as described in 'Amplification specificity and efficiency' above. Data were collected as C_q values obtained after setting the threshold across the exponential phase of the amplification curve. The average C_q of the technical replicates was calculated and transformed to quantities (using standard curves) as input data required by the NormFinder algorithm. This software (available as a Microsoft Excel add-in at www.mdl.dk/publicationsnormfinder.htm) determines the stability of gene expression by evaluating the variation in expression within treatments compared to variation between treatments: low variation between treatments signifies stable expression. The software developers recommend evaluating the stability of at least 5 candidate reference genes and a minimum of 2 genes to assure reliable normalisation (Andersen et al. 2004).

qPCR assay. A total of 6 target genes (encoding for arp2/3, CaM, *Hsp40*, *Hsp90*, FER and UbC) covering a range of functions in the cell were chosen to assess the response of *Rhopaloeides odorabile* to thermal stress. The expression of the target and reference genes was tested with biological triplicates (3 clones, 1 from each tank per temperature) and technical duplicates for sponge clones sampled after 0, 1, 3, 14 and 15 d at the 3 different temperatures (27, 31 and 32°C). Cycling conditions and reaction mixes were as described in 'Amplification specificity and efficiency' above. Data were collected as C_q values obtained after setting the threshold across the exponential phase of the amplification curve. Amplification efficiency and C_q values of both target and reference genes were used as input data in the REST 2009 software (v. 2.0.13, Qiagen) developed for relative quantitation studies. The software applies a mathematical model that takes into account the PCR efficiencies of both the gene of interest and reference genes. The geometric mean of all reference gene concentrations is used to calculate the relative expression of target genes.

Genes of interest were separately normalised to the reference genes and then compared to the experimental control samples (maintained at 27°C) at each time point that were set as a calibrator. Results are expressed as gene expression fold changes relative to the calibrator. Significant differences between treated samples and controls were detected by the software through a hypothesis test for the difference between the samples (hypothesis H_1, significance for $p(H_1) < 0.05$) by performing up to 50 000 random reallocations of treated samples and controls between the groups.

RESULTS

Selection and validation of reference genes

A total of 5 candidate genes from different functional classes that are commonly used as reference genes were chosen for the present study (Table 1). The panel includes genes encoding for 2 different rRNAs (mitochondrial: mtLSU, and ribosomal: rLSU), ubiquitin, actin and α-tubulin. Primers were designed for all genes, and high specificity and reaction efficiency (Table 1) were confirmed through melting-point analysis and standard curves.

NormFinder analysis determined that the gene encoding for α-tubulin was the single best reference, with a stability value of 0.052. Also, α-tubulin and ubiquitin were the most stable 2-candidate gene combination. However, the use of the 3 most stable internal control genes is strongly recommended by Vandesompele et al. (2002); hence the next most suitable gene, rLSU, was also included (Table 2).

Gene expression profiles in *Rhopaloeides odorabile* exposed to temperature stress

We assessed the effects of thermal stress on the expression of 6 functionally diverse genes (candidate biomarkers) in the marine sponge *Rhopaloeides odorabile*. All genes could be amplified with an optimal efficiency (ranging from 0.9 to 1.06; Table 1). Expres-

Table 2. Expression stability values of the candidate reference genes calculated using NormFinder algorithms. The best combination was found to be α-tubulin and ubiquitin. The stability value for the best combination of 2 genes was found to be 0.033. mtLSU: mitochondrial rRNA, rLSU: 28S rRNA

Gene coding for	NormFinder stability value
mtLSU	0.162
Actin	0.096
rLSU	0.071
Ubiquitin	0.052
α-tubulin	0.052

sion data for each target gene were normalised to α-tubulin, ubiquitin and rLSU, and then compared to the calibrator of each sampling point (Fig. 1).

All sponge clones survived for the whole length of the experiment except for specimens kept at 32°C, which, after 3 d, showed signs of necrosis and died.

In sponges treated at 31 and 32°C, there was a significant reduction ($p(H_1) < 0.05$) in expression of the genes encoding for arp2/3, FER and *Hsp90* relative to the control after 1 d (54.6, 69.2 and 66.6% at 31°C; 73.7, 76 and 57.2% at 32°C, respectively) and a further reduction in sponges kept at 31°C for 3 d (48.1, 46.7 and 64.5%, respectively). In contrast, transcript levels of all 3 genes strongly increased after 3 d at 32°C (225.8, 117.4 and 48.8%, respectively). In sponges kept for 14 d at 31°C, a decline in arp2/3, FER and *Hsp90* transcript abundance was measured (55.7, 49.3 and 48.6%, respectively), and this down-regulation was maintained during the recovery period at 27°C (20.8, 33.9 and 32.8% for arp2/3, FER and *Hsp90*, respectively). The experimental treatment caused a reduction of CaM expression at both temperatures (ranging from 42.4 to 71.9%), with a significant (87.4 and 71.1%) down-regulation after 1 d at 32°C and 3 d at 31°C, respectively. CaM transcript levels rose (12.7%) during the 27°C recovery period. The molecular chaperone *Hsp40* was slightly induced after 1 d at 31°C (3.8%), only to be down-regulated for the rest of the experiment, although not significantly. A stronger induction was evident in specimens kept at 32°C: the *Hsp40* gene transcript showed an 82.8 and 167% increase after 1 and 3 d, respectively. *UbC* gene expression was significantly reduced after 3 d at 31°C (48.9%), and was slightly up-regulated (2.4%) during the recovery period. A significant increase in expression of UbC was detected in sponges maintained at 32°C for 3 d (Fig. 1).

It is worth noting that during the 24 h recovery period at 27°C, all target genes became up-regulated from the previous time point, with values still lower but closer to the expression levels in the controls. Only the *Hsp90* gene was significantly different from the control during the recovery period. *CaM* was the only gene that changed from a 40% down-regulation to 12% up-regulation during the last day of the experiment.

DISCUSSION

The present study is the first to employ a range of putative stress-response genes to investigate the molecular-level effects of thermal stress in sponges and identify potential genetic biomarkers. The qPCR analysis indicated that sponges go through a general shut-down of molecular systems immediately after thermal stress, with a subsequent induction of the heat shock response system. Overall, a very strict thermal threshold between 31 and 32°C was identified for the sponge *Rhopaloeides odorabile*.

To assure reliable quantification of gene expression, we identified suitable reference genes among a panel of 5 commonly used 'housekeeping' genes. The gene coding for α-tubulin was the best reference gene and the one coding for mtLSU was the least stable gene. In addition, even with stable expression of α-tubulin and rLSU, NormFinder still determined that the addition of ubiquitin would be required for optimal normalisation. This is consistent with the findings of Vandesompele et al. (2002), who reported that the use of a single gene for normalisation resulted in relatively large errors and

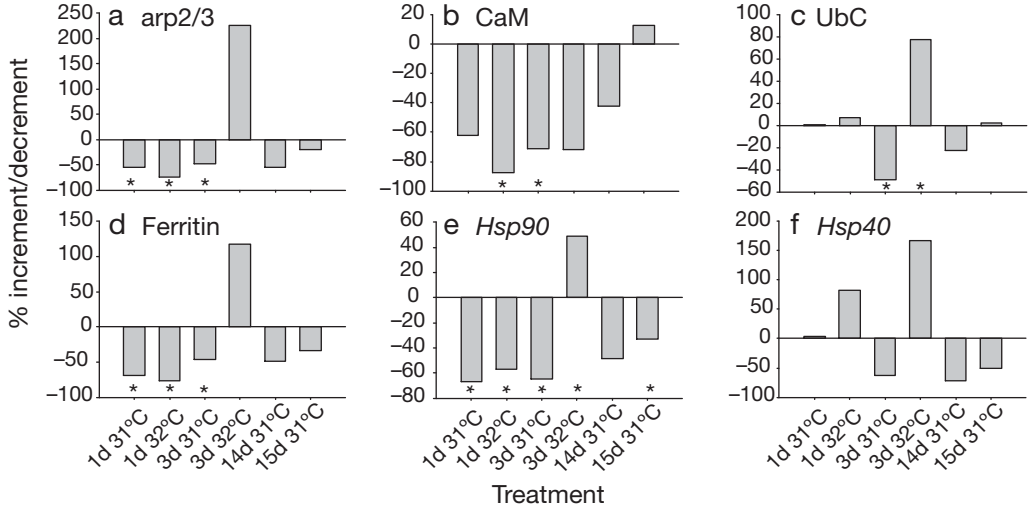

Fig. 1. *Rhopaloeides odorabile*. Gene expression variation in *R. odorabile* exposed to thermal stress for 1, 3, 14 or 15 d at 31 or 32°C. (a) arp2/3: actin-related protein, (b) CaM: calmodulin, (c) UbC: ubiquitin-conjugating enzyme, (e) *Hsp90*: heat shock protein 90, (f) *Hsp40*: heat shock protein 40. *Significant difference from the relevant control for each treatment

a minimum of 3 internal control genes was therefore recommended. Our results confirm that validation of multiple reference genes under the identical experimental conditions used for qPCR is essential to minimise error in data interpretation (Nicot et al. 2005, Ransbotyn & Reusch 2006, Bustin et al. 2009).

In a previous study that assessed the effects of elevated seawater temperature on *Rhopaloeides odorabile* and its symbiotic microbial community, no visible stress effects were detected in sponges kept in 31°C water, but at 33°C sponges exhibited minor surface necrosis (10% surface area) after 24 h and major necrosis (50 to 70% of surface area) after 3 d (Webster et al. 2008). In order to determine if sub-lethal stress was occurring in sponges exposed to elevated seawater temperatures prior to visual signs of necrosis, we assessed the expression of 6 functionally important genes previously reported to be involved in molecular stress response developing a multiple-gene profiling assay. A rapid decrease in the expression levels of the actin organiser arp2/3, the iron-storage protein FER, the signal-transducer CaM and the molecular chaperone *Hsp90* at 31 and 32°C within the first 24 h indicates that the sponge goes through an initial shut-down of its molecular systems in response to thermal stress to suppress cellular activity. These results are consistent with previous studies showing suppression of transcription, protein biosynthesis and cell growth in different taxa (Yost & Lindquist 1986, Duncan & Hershey 1989, Bell et al. 1988). A change in gene expression and the suppression of around 10% of assayed genes, many of which are involved in protein synthesis and cell growth, was reported in the yeast *Saccharomyces cerevisiae* in response to a range of environmental stressors (Gasch et al. 2000). A general disruption of Ca^{2+} homoeostasis, cytoskeletal dynamics, calcification, metabolism and protein synthesis was observed by De Salvo et al. (2008) in the reef coral *Montastraea faveolata* exposed to thermal stress and bleaching; Voolstra et al. (2009) observed similar negative effects on the overall metabolism of *M. faveolata* embryos exposed to high temperature. In the goby *Gillichthys mirabilis*, hypoxia caused down-regulation of many cytoskeletal and ribosomal proteins, likely reflecting the reorganisation of metabolism and the suppression of major energy-requiring processes shortly after the onset of stress (Gracey et al. 2001). The reef fish *Pomacentrus moluccensis* reacted to heat stress with a suppression of cell growth, repression of transcriptional activity and an increase of protein breakdown (Kassahn et al. 2007). The reported gene-expression profiles obtained in response to stressors, the present study included, are consistent with an interpretation of metabolic re-organisation following the onset of stress, which would precede the induction of genes and de novo synthesis of proteins, which may ultimately allow the organism to cope with prolonged exposure to stress (Kassahn et al. 2007).

The rapid increase in expression of *Hsp40* in sponges exposed to 32°C confirms the thermal stress effect, since the molecular chaperone system is activated in an attempt to preserve cellular functioning. The increased expression of *Hsp90* after 3 d at 32°C indicates a slower activation of this heat shock response system but is also consistent with its role as a chaperone for directing degraded proteins to proteolysis. The increase of FER transcript levels after 3 d at 32°C indicates the presence of and the attempt to neutralise reactive oxygen species. This is consistent with previous observations of experimentally bleached corals (Császár et al. 2009). The significant up-regulation of the UbC-encoding gene in the 32°C-treated sponges after 3 d indicates a full activation of the proteasome-mediated proteolytic system responsible for the degradation of damaged and unfolded proteins. A similar response was observed in the moon jellyfish *Aurelia* spp. upon exposure to tributyltin; in addition to the up-regulation of genes coding for HSPs and their associated co-proteins, genes responsible for degradative and cell-shaping processes were also induced (Schroth et al. 2005). In sponges and corals, the extent of damage to essential cellular structures determines the balance between mortality and recovery (Halliwell & Gutteridge 1989, Downs et al. 2002, López-Legentil et al. 2008). Even if minor damage can be repaired by an induction of the heat stress response (mediated by *Hsp70* and other chaperones), greater damage can cause a collapse of the metabolic defence systems and the rapid death of the animal (Downs et al. 2002). Despite the fact that only a limited number of genes were investigated, our study results suggest that this sponge is equipped with the molecular tools to accommodate even minor temperature changes, the only limit to the survival of the species being the extent of the seawater warming. Further studies should investigate the ability of these animals to cope with lethal temperatures in relation to the rate of temperature increase.

The general pattern of down-regulation for most genes over the course of the temperature exposure indicates that both 31 and 32°C can cause physiological impairments in *Rhopaloeides odorabile*. The 32°C treatment likely represents an acute stress condition that activates the primary heat stress response together with secondary processes (such as degradative and cell-shaping processes and elimination of oxygen reactive species) (Blackstone 2001, Schroth et al. 2005). A similar phenomenon has also been reported in *Aurelia* spp. (Schroth et al. 2005) and in sponges exposed to cadmium (Schröder et al. 1999). The 31°C treatment is much less acute but appears to cause some

sub-lethal molecular responses that are able to return to normal once the thermal stress has been removed.

The ability of marine organisms to adapt to and cope with temperature changes can be identified with laboratory experiments, so that responses to thermal changes in the natural environment can be explained or predicted. In the present study we assessed the ability of the Great Barrier Reef sponge *Rhopaloeides odorabile* to withstand seawater temperatures only 2 to 4°C above the *in situ* mean maximum annual temperature. Whereas sponges at 32°C only survived for the first 3 d, specimens kept at 31°C for the whole length of the experiment did not show any signs of necrosis. Moreover, none of the genes in sponges kept at 31°C were significantly different to those in the 27°C controls after 14 d. This indicates a very strict thermal threshold for *R. odorabile* between 31 and 32°C and is consistent with previous findings based on sponge necrosis and symbiotic disruptions in this species. These findings indicate that sponges may be as vulnerable as corals to changes in sea surface temperatures, and further research is required to determine the level of inter-species sensitivity to thermal stress and potential synergistic interactions with other water-quality stressors.

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