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TECHNICAL ADVANCES

Gene expression normalization in a dual-compartment system: a real-time quantitative polymerase chain reaction protocol for symbiotic anthozoans

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Abstract

Traditional real-time quantitative polymerase chain reaction protocols cannot be used accurately with symbiotic organisms unless the relative contribution of each symbiotic compartment to the total nucleic acid pool is known. A modified 'universal reference gene' protocol was created for reef-building corals and sea anemones, anthozoans that harbour endosymbiotic dinoflagellates belonging to the genus *Symbiodinium*. Gene expression values are first normalized to an RNA spike and then to a symbiont molecular proxy that represents the number of *Symbiodinium* cells extracted and present in the RNA. The latter is quantified using the number of genome copies of heat shock protein-70 (HSP70) amplified in the real-time quantitative polymerase chain reaction. Gene expression values are then normalized to the total concentration of RNA to account for differences in the amount of live tissue extracted among experimental treatments and replicates. The molecular quantification of symbiont cells and effect of increasing symbiont contributions to the nucleic acid pool on gene expression were tested *in vivo* using differentially infected sea anemones *Aiptasia pulchella*. This protocol has broad application to researchers who seek to measure gene expression in mixed organism assemblages.

Keywords: anthozoan, coral reefs, real-time quantitative PCR, reference gene, symbiosis

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Introduction

Coral reefs are ecologically and economically important ecosystems that are threatened by increasing ocean temperatures associated with global climate change (Hoegh-Guldberg 1999; Hoegh-Guldberg *et al*. 2007). The relationship between anthozoans and endosymbiotic dinoflagellates belonging to the phylogenetically diverse genus *Symbiodinium* (LaJeunesse 2005; Stat *et al*. 2006) drives the productivity and structural integrity of the reefs (Muscatine & Porter 1977). Unfortunately, the functionality of coral–dinoflagellate symbioses is particularly sensitive to, and breaks down in the face of, temperature disturbances (Gates 1990), a scenario that manifests as paling of the external colouration of the anthozoan host. This loss of colour can reflect either the loss of dinoflagellate cells and/or a reduction in the amount of chlorophyll per dinoflagellate cell, and is the phenomenon known as bleaching (Brown 1997). Because incidents of mass bleaching are becoming more frequent and predictions for the future temperature environment are bleak, there has been a growing desire to better understand the biological capacity of the anthozoan–dinoflagellate symbiosis to accommodate altered temperature regimes (Hofmann *et al*. 2005; Edmunds & Gates 2008). Integral to this endeavour is the interest in monitoring expression of genes across a range of temperatures (van Oppen & Gates 2006). However, there is currently no appropriate means of applying real-time quantitative polymerase chain reaction (qPCR) techniques to accurately measure gene expression in dual-compartment (host and symbiont) symbiotic systems (Yellowlees *et al*. 2008).

In addition to addressing common qPCR pitfalls, such as differential tissue and RNA quality and reverse transcription efficiency, which is described below, anthozoan biologists also face the issue of varying RNA composition of their samples as a result of either natural ageing or environmental stress. For instance, during bleaching events, anthozoans

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lose their symbionts and the relative increase in host RNA in these extractions compared to those from nonbleached individuals will be reflected in an up-regulation of virtually all host genes. Thus, for messenger RNA (mRNA) expression results to be biologically meaningful, the symbiont contribution to the RNA extracts must be estimated and normalized across samples. With the goal of developing the capacity to quantify the endosymbiont contribution to a given extraction, the relationship between symbiont cell number and genome copy number of symbiont heat shock protein-70 (HSP70) was established by first qPCR-amplifying DNA templates extracted from serially diluted symbionts freshly isolated from corals or anemones. With this relationship established, the number of symbiont cells in mixed host and symbiont extractions can be estimated from the number of HSP70 genome copies amplified, a value referred to throughout this manuscript as 'symbiont molecular proxy (SMP)'. Gene expression values from real-time amplification of complementary DNA (cDNA) can then be normalized to the SMP to control for different symbiont densities in nucleic acid extractions. The empirically validated assumption is that there is a positive correlation between symbiont contribution to the DNA pool and contribution to the RNA pool.

In anthozoans exposed to temperature stress, few cellular processes remain undisturbed (Gates & Edmunds 1999; Mayfield & Gates 2007), and so housekeeping genes used in gene expression analyses in other systems, such as actin β (ACTB), are likely to be differentially regulated under the stress regimes of interest to most anthozoan biologists. For this reason, exogenous RNA spikes are potentially a better means of normalizing gene expression data and a RNA/DNA spiking approach (Bower *et al*. 2007, the 'universal reference gene' method) was taken in this study. This approach also controls for other technical issues such as pipetting errors and RNA quantity and reverse transcription efficiency differences which can also lead to inaccurate gene expression results (Huggett *et al*. 2005). Finally, even after normalizing to account for differential extraction of symbiotic dinoflagellates and using appropriate exogenous RNA and DNA spikes to accommodate variation in extraction and cDNA synthesis efficiencies, gene expression can still not be directly compared between scleractinian corals because of differing skeletal densities (Allemand *et al*. 2004). Two coral fragments that weigh 20 mg, for instance, may have very different amounts of tissue, as one may be of greater skeletal density or of different geometry or water content (Edmunds & Gates 2002). To control for these differences, total RNA and total protein were calculated for each sample, and an analysis was performed to evaluate which value better approximates the quantity of biological material used. This parameter was then used to normalize the gene expression data (Fig. 1a).

The protocol developed was applied *in vivo* to differentially infected sea anemones *Aiptasia pulchella* to both test the molecular quantification of symbionts against traditional cell counting techniques and to demonstrate how *not* controlling for symbiont contribution to RNA extracts biases the interpretation of gene expression data.

Materials and methods

Collection of anemones and corals and isolation of dinoflagellate symbionts

Small branches of the Pacific coral *Montipora capitata* were collected from coral reefs adjacent to Coconut Island in Kaneohe Bay (21°26.2'N, 157°47.6'W) on the east side of the island of Oahu, Hawaii. *Aiptasia pulchella* specimens were gathered from seawater tables at the Hawaii Institute of Marine Biology (HIMB) on Coconut Island. Symbionts were freshly isolated from five *A. pulchella* specimens and five *M. capitata* fragments (Fig. 1b). Coral tissues containing symbionts were removed from the skeleton using a water pick and collected in a plastic bag. After decanting into 50 mL tubes, dinoflagellate cells were pelleted by centrifugation ($500 \times g$), repeatedly washed with 500 µL 0.2 µM filtered seawater (FSW) and spun at $500 \times g$ for 2 min (five repetitions). Intact symbiont cells readily form pellets during the centrifugation, while destroyed host cells fractionate in the supernatant and were discarded. The clean dinoflagellate cell pellets were finally re-suspended in 15 mL FSW. Anemones were diced with a razor blade, transferred to 1.5 mL microcentrifuge tubes, and ground with a motorized homogenizer for 20 s. Dinoflagellates were pelleted, washed and re-suspended as described above for coral. Haemacytometer counts were performed on isolated symbionts from each of the five anemones and corals and cells were diluted to 2.5, 2, 1, 0.5, 0.25 and 0.125×10^6/mL FSW before DNA extraction. A SYTOX (Invitrogen) stain was utilized to evaluate cell viability.

Aiptasia pulchella *infection*

In order to both demonstrate that the molecular method described below is effective at estimating the number of symbiont cells and show that increasing symbiont abundance *in vivo* leads to enhanced symbiont gene expression, aposymbiotic (free of symbionts) *A. pulchella* specimens were artificially infected with *Symbiodinium*. Each aposymbiotic anemone was infected with 4×10^6 *Symbiodinium* cells freshly isolated from symbiotic *A. pulchella* as described above. Eight individuals from each of five clonal lines of *A. pulchella* were used in the experiment. For each clonal line, six anemones were infected with *Symbiodinium* and two were left uninfected to serve as controls. Three infected and one uninfected anemones from each clonal line were sacrificed at 3- and 6-week post-infection. Sacrificed anemones were frozen in liquid nitrogen and stored at –80 °C until processed.

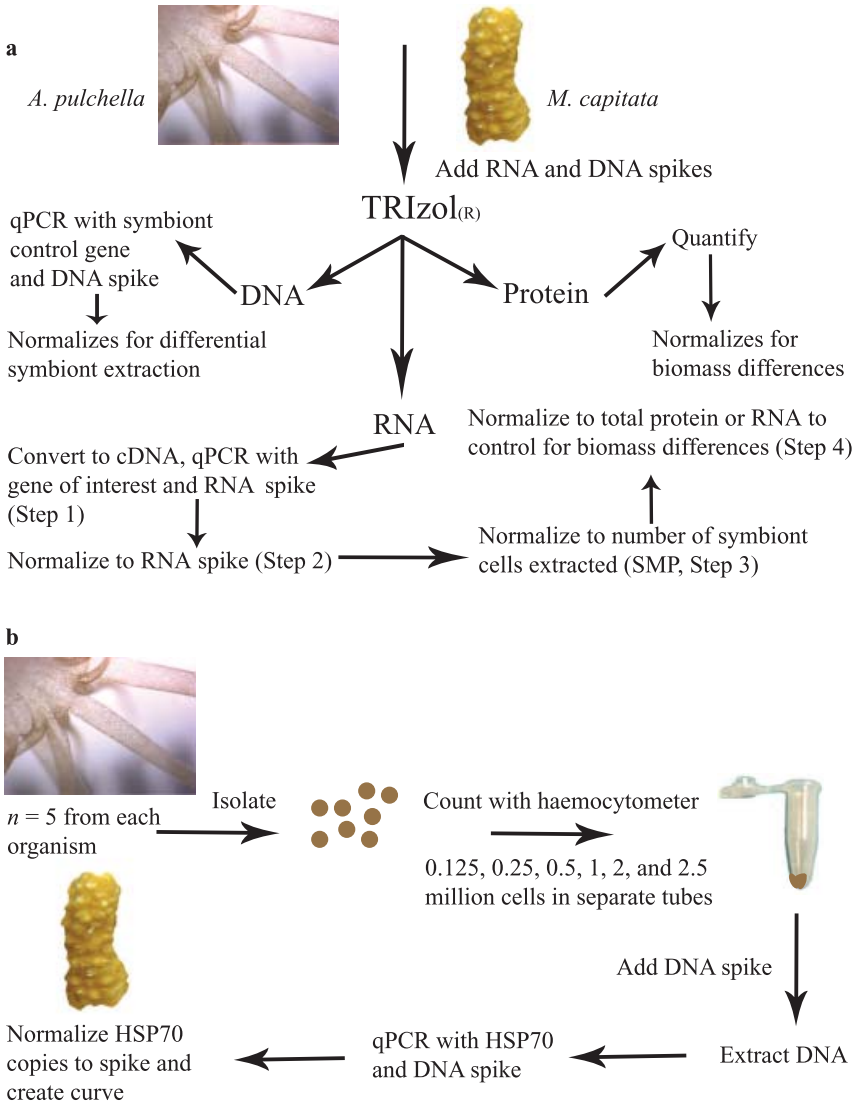

Fig. 1 A novel protocol for quantifying gene expression in an endosymbiosis. Messenger RNA expression is normalized to an RNA spike, an estimate of number of symbiont cells extracted based on genome copies of HSP70 measured in qPCR with the DNA phase (symbiont molecular proxy [SMP]), and protein or total RNA (panel a). *Symbiodinium* cells are isolated from anthozoans, diluted, and DNA is extracted and qPCR-amplified with HSP70 and DNA spike primers to correlate genome copies to number of symbiont cells to justify using HSP70 genome copies as a proxy for number of symbiont cells extracted (panel b).

Isolation of symbiont genes

DNA extraction. A cetyltrimethyl ammonium bromide protocol (Dempster *et al*. 1999) was used to extract DNA from *M. capitata* fragments (10–20 mg), collected as above. The resulting extract consisted of both host and symbiont DNA.

Primer design and PCR. For *Symbiodinium* HSP70 primer design, protein sequences from plants, algae, and alveolates were aligned with MacVector 8.1.1 software. Two regions that showed 100% conservation at the amino acid level were identified, and the nucleic acids representing these regions were aligned to design degenerate primers intended to amplify a 475-bp product. Primer sequences, annealing temperatures and concentrations are given in Table 1. Additional PCR reagents were used in the following concentrations in a 50-μL reaction: 1 μL DNA (~15 ng), 0.2 U Immolase (Bioline), 2.5 mM each dNTP, 2.5 mM $MgCl_2$, and 1× Immunobuffer (Bioline). PCR comprised an initial denaturation of 95 °C for 7 min, followed by 33 cycles of 94 °C for 30 s, 45 °C for 60 s, and 72 °C for 45 s, and a final extension at 72 °C for 5 min. Bands were excised from the gel and isolated from the agarose using the QIAEX II Gel Extraction Kit (Qiagen). The product was cloned into a pGEM-T Easy Vector (Promega) and sequenced. Nucleic acid sequences were assigned an identity using the National

Table 1 Primers, annealing temperatures, and concentration used in PCRs

Gene (length)	Type	Organism	Primer sequence (5′–3′)	Annealing temperature	Concentration (nM)
SL (88-bp)	Specific	*Tilapia* sp. (liver)	For: GGCTGGCTTTGCATGTATCA Rev: AGTGGAGCAACCATTTATCAGATATCT	57 °C	250
HSP70 (550-bp)	Degenerate	NA	For: GTNGGTGGTTCNACCCGTATT Rev: CCCTTNTCGTTGGTGATGGT	45 °C	500
HSP70 (86-bp)	Specific	*Symbiodinium*	For: CTGTCCATGGGCCTGGAGACT Rev: GTGAACGTCTGTGCCTTGTTGGTT	62.5 °C	500

Center for Biotechnology Information's (NCBI) BLAST database (TBLASTX). These sequences (accession: EU476880) were used to design *Symbiodinium* specific small amplicon qPCR primers (Table 1) that would not amplify host DNA/cDNA.

Production of RNA and DNA spikes

To control for differential RNA extraction and cDNA synthesis efficiencies between samples, equal amounts of an exogenous mRNA of the *Tilapia* gene somatolactin (*SL*, accession number AB120767) were added to whole *A. pulchella* and *M. capitata* samples as well as symbiont cells isolated from these organisms before RNA extraction. Likewise, an *SL* DNA spike was added to the same samples to control for differing DNA extraction efficiencies. For this method to prove effective, the mRNA and DNA spikes must be absent from the organism's transcriptome and genome, respectively (Johnson *et al*. 2005). *SL* has only been found in fish to date (Zhu *et al*. 2007) and the *Tilapia* specific *SL* primers (courtesy of Dr Andrew Pierce of HIMB) used in this study failed to amplify genomic DNA or cDNA from corals, anemones, or their endosymbionts.

DNA spike. An *SL* gene fragment was amplified from *Tilapia* sp. cDNA (provided by Dr Andrew Pierce of HIMB) with *SL* primers (Table 1), the same reagent cocktail used for the degenerate primer amplifications, and the following PCR cycling conditions: 95 °C for 7 min followed by 35 cycles of 94 °C for 30 s, 57 °C for 60 s, and 72 °C for 60 s and a final 72 °C extension for 5 min. The amplified 88-bp gene fragment was purified with a QIAquick PCR purification kit (Qiagen), analysed spectrophotometrically on a ND-1000 spectrophotometer (NanoDrop Technologies), and diluted to 5 pg/μL.

RNA spike. The 88-bp *SL* gene fragment was cloned into a pGEM-T Easy vector (Promega). The resulting plasmids were linearized by digestion with *Nsi*I (Fermentas) and used as template for *in vitro* transcription of *SL* RNA using T7 RNA polymerase (New England Biolabs). The residual template was removed from the RNA with an RQ1 RNase-free DNase (Promega), and the *SL* RNA was tailed using poly(A) polymerase (Ambion). Poly(A)-tailed RNA was precipitated with 1/10 vol 3YM sodium acetate and 2 vol isopropanol, washed twice with 75% ethanol, resuspended in 30 μL diethylpyrocarbonate(DEPC)-treated water, quantified, and diluted to 5 pg/μL.

Extraction of nucleic acids and protein

Anemone (from infection experiment) and freshly isolated dinoflagellate (from serial dilutions) RNA was extracted with a modified TRIzol (Invitrogen) protocol. After placing whole anemones or isolated symbiont cells in 500 μL TRIzol reagent, 25 pg (5 μL) of *SL* RNA and DNA spikes was added to each microcentrifuge tube and the tissues/cells were ground with a motorized homogenizer. Following homogenization, an additional 500 μL of TRIzol was added and the manufacturer's instructions were followed for total RNA isolation using a high-salt precipitation step involving the addition of 250 μL of a salt solution (0.8 M Na citrate, 1.2 M NaCl) before the addition of 250 μL of isopropanol. The RNA was eluted in 30 μL DEPC-treated water.

After the aqueous phase was removed from each sample and processed for RNA (as described above), 500 μL of back extraction buffer (4 M guanidine thiocyantate, 50 mM Na citrate, and 1 M Tris base) was added to the phase containing DNA/protein. The mixture was incubated for 10 min at room temperature (RT), spun at 12 000 × *g* for 10 min at 4 °C and the aqueous phase containing DNA was removed, transferred to a new tube, mixed with 2 μL Pellet Paint (Novagen) and precipitated at RT for 10 min with isopropanol and Na acetate. Samples were spun at 12 000 × *g* for 10 min at 4 °C, the supernatant was removed, the pellet washed twice with 1 mL of 75% ethanol and air dried briefly. The DNA pellet was re-suspended in 50 μL 1/10 TE buffer in water by heating for 10 min at 55 °C. A final spin was performed at 12 000 × *g* for 10 min at 4 °C, and the supernatant containing the DNA was transferred to a new tube. DNA and RNA quality were both assessed spectrophotometrically and visually on 1% agarose gels with ethidium bromide.

The protein phase remaining in TRIzol after the DNA extraction was only processed for the anemone samples.

Samples were sonicated and placed on a rotator for 10 min. Protein was sedimented by spinning at 12 000 × *g* for 10 min at 4 °C. The supernatant was removed and the pellets were washed with 1.8 mL of 0.3 M guanadinium hydrochloride in 95% ethanol and spun at 7500 × *g* for 5 min at 4 °C. The pellets were then disrupted with a pipette tip, and the mixture was placed on a rotator for 20 min. After three such washes, 1.8 mL of 96% ethanol was added and samples were placed on a rotator for 20 min. After drying the pellets, 200 µL of 1% SDS was added followed by incubation at 50 °C for 10 min. Samples were spun at 10 000 × *g* for 10 min at 4 °C, and the supernatant was transferred to a new microcentrifuge tube. Protein was quantified in triplicate using an RC:DC assay (Bio-Rad) read on a SpectraMax M2 spectrophotometer (Molecular Devices). [DNA] was regressed against total RNA or protein to determine which relationship demonstrated a stronger association and hence is more indicative of the amount of biological starting material.

Real-time qPCR of co-extracted DNA from symbiont cell dilutions

Two microlitres of DNA extracted from six symbiont cell dilutions (2.5, 2, 1, 0.5, 0.25 and 0.125 × 10^6 cells) isolated from five *A. pulchella* specimens and five *M. capitata* fragments were qPCR-amplified with both exogenous DNA spike (*SL*) and symbiont-specific HSP70 primers using 2× SensiMix (Quantace) with SYBR green and 0.1 µL 50× BSA in triplicate 20-µL reactions. After 10 min at 95 °C, 40 cycles of 95 °C for 15 s and 57 °C/62.5 °C (for *SL* and HSP70, respectively) for 60 s were performed on an Applied Biosystems 7500 Fast real-time PCR system. A melt curve was performed to ensure primer specificity. Dilutions of *SL* and HSP70 PCR products (10^3–10^8 copies) were amplified to estimate PCR efficiency of each primer set.

The average threshold cycle (C_t) of symbiont HSP70 between the triplicate PCRs at each of the six cell dilutions was calculated and averaged across the five replicates for both coral and anemone. These non-normalized values were plotted against cell number. Then, HSP70 gene copies normalized to the DNA spike (as in Bower *et al*. 2007) for each cell dilution were averaged across the five organisms and plotted against the number of cells used in the extraction, corresponding to the SMP.

Real-time qPCR of nucleic acids from experimentally infected A. pulchella

Partially frozen anemones ($n = 39$) were homogenized for 10 s, and the number of symbiont cells in a 10-µL aliquot of the resulting homogenate quantified was using a haemocytometer and microscopy. *SL* RNA and DNA spikes (25 pg each) were added to the remaining homogenate and the RNA, DNA, and protein extracted as described above. RNA was DNase-treated as described above and converted to cDNA with a 'high capacity' cDNA synthesis kit (Applied Biosystems). The cDNA was diluted 2-fold before qPCR amplification with symbiont HSP70 and *SL* spike primers. Reaction conditions and reagent concentrations were as above with freshly isolated *Symbiodinium* cells, except that 2× Power SYBR green (Applied Biosystems) was used and the analysis was carried out on an MJ Chromo4 (Bio-Rad). Symbiont HSP70 expression was normalized to the RNA spike as in Bower *et al*. (2007).

Total holobiont DNA was amplified with symbiont HSP70 and *SL* primers as described above for amplification of freshly isolated symbiont DNA, and symbiont HSP70 genome copies were normalized to the *SL* DNA spike (Bower *et al*. 2007), corresponding to the SMP. Then, the mean normalized expression (MNE) was calculated by dividing the RNA spike-normalized HSP70 gene expression values by the SMP to account for differential extraction of symbionts. Finally, the MNE was divided by total RNA or protein to control for biomass differences between anemones.

Results

Symbiont dilution curves

Less than 0.5% of dinoflagellate cells showed signs of membrane damage in any of the isolations. Correlations between number of symbiont cells and HSP70 genome copies measured with qPCR were strong ($r^2 > 0.87$) regardless of whether or not data were normalized to amplification of the DNA spike (Fig. 2). For the coral-derived dilution series, the number of symbiont HSP70 gene copies normalized to amplification of the *SL* DNA spike (SMP) only conforms to the anticipated linear trend from 0.125–1 × 10^6 cells ($m = 1.73 \times 10^{-5}$, $r^2 = 0.97$, $P < 0.01$); at 2 and 2.5 × 10^6 cells, the SMP values were exponentially greater (Fig. 2a). Similarly, a strong linear correlation between cells and SMP is observed with freshly isolated symbionts from *A. pulchella* (Fig. 2l) over 0.125– 2 × 10^6 cells ($m = 5.90 \times 10^{-5}$, $r^2 = 0.88$, $P < 0.01$).

In vivo *demonstration of method*

The number of symbiont HSP70 genome copies increases with time post *Symbiodinium* infection (Fig. 3), regardless of whether or not the samples were non-normalized (Fig. 3a, student's *t*-test $P < 0.001$), normalized only to the exogenous DNA spike (SMP, Fig. 3b, $P < 0.05$), to the DNA spike and protein (SMP/protein, Fig. 3c, $P < 0.05$) or to the DNA spike and total RNA (SMP/total RNA, Fig. 3d, $P < 0.05$). Uninfected controls remained symbiont free over the 6-week period and no symbiont HSP70 genome copies were detected (data not shown). Non-normalized genome copies increased 2.06-fold over the last three weeks of infection (Fig. 3a)

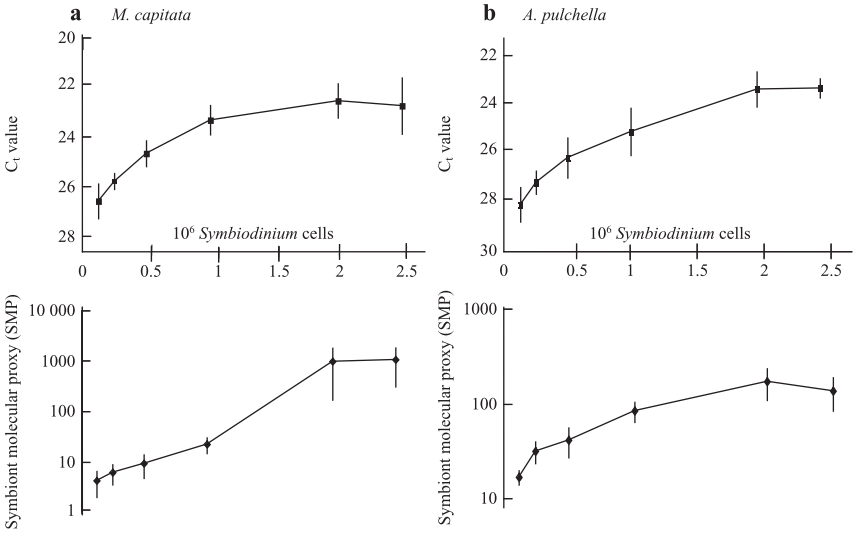

Fig. 2 Freshly isolated *Symbiodinium* cell dilutions and HSP70 genome copies. Symbiont cells were serially diluted to 0.125, 0.25, 0.50, 1, 2 and 2.5×10^6 cells per extraction. DNA was extracted from each dilution and qPCR-amplified with HSP70 and *SL* DNA spike primers. Symbiont cells were isolated from the coral *Montipora capitata* ($n = 5$, mean +/− SE, panel a) and the sea anemone *Aiptasia pulchella* ($n = 5$, mean +/− SE, panel b). C_t values (square) and DNA spike-normalized HSP70 genome copy numbers (SMP, diamond) both indicate a strong correlation between number of cells and HSP70 genome copies under 10^6 cells, justifying the latter's use as a symbiont molecular proxy in gene expression analyses. Note: As a C_t increase of 1 refers to a doubling of genome copies (or transcripts) the *y*-axes of the top graphs are on a $\log_2$ scale, as opposed to the $\log_{10}$ scale of the *y*-axes of the SMP data.

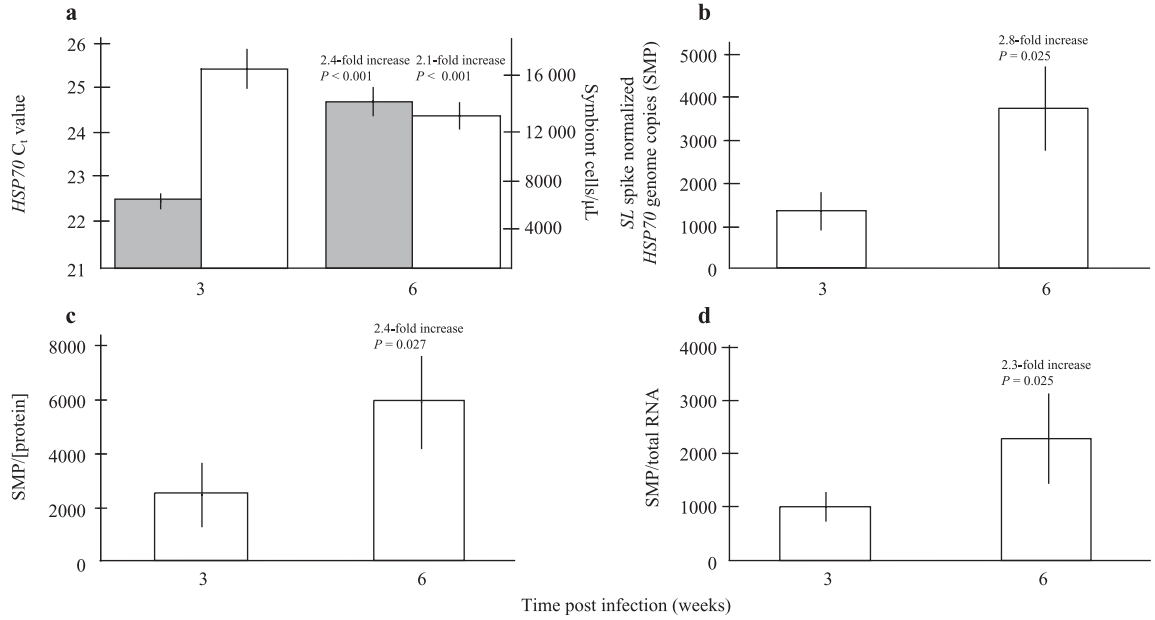

Fig. 3 *Symbiodinium* densities and HSP70 genome copies detected in *Aiptasia pulchella* evaluated at 3- and 6-week post infection with symbiont cells. Symbiont densities (cells/µL) within anemones at 3- and 6-week post infection are shown in panel a (grey columns). HSP70 genome copies are presented as non-normalized C_t values ($n = 5$, mean +/− SE, panel a, white columns); *SL* DNA spike-normalized genome copies (SMP, panel b); *SL* spike and protein-normalized genome copies (SMP/protein, panel c); and *SL* spike and total RNA-normalized genome copies (SMP/total RNA, panel d). Fold changes and student's *t*-test *P* values are also displayed. Fold changes in symbiont cell counts between the time points agree well with molecularly calculated values.

while *SL* spike-normalized HSP70 genome copies (SMP) increased 2.80-fold (Fig. 3b). However, after accounting for different-sized anemones using protein (Fig. 3c) or total RNA (Fig. 3d), the fold changes of 2.37 and 2.27, respectively, are observed, which more closely correspond to the 2.41-fold increase in symbiont cells obtained from direct cell counts over this time period (5633 cells/µL at 3 weeks vs. 13603 cells/µL at 6 weeks).

Symbiont HSP70 expression was also measured and either treated as non-normalized or normalized to exogenous RNA spike only; exogenous spike, SMP, and protein; or exogenous spike, SMP, and total RNA (Fig. 4). Non-normalized HSP70 expression (Fig. 4a) showed an approximate 2.5-fold increase in expression between 3 and 6 weeks of infection, although the change is not significant (student's *t*-test, $P = 0.21$). When normalized to the SMP and either

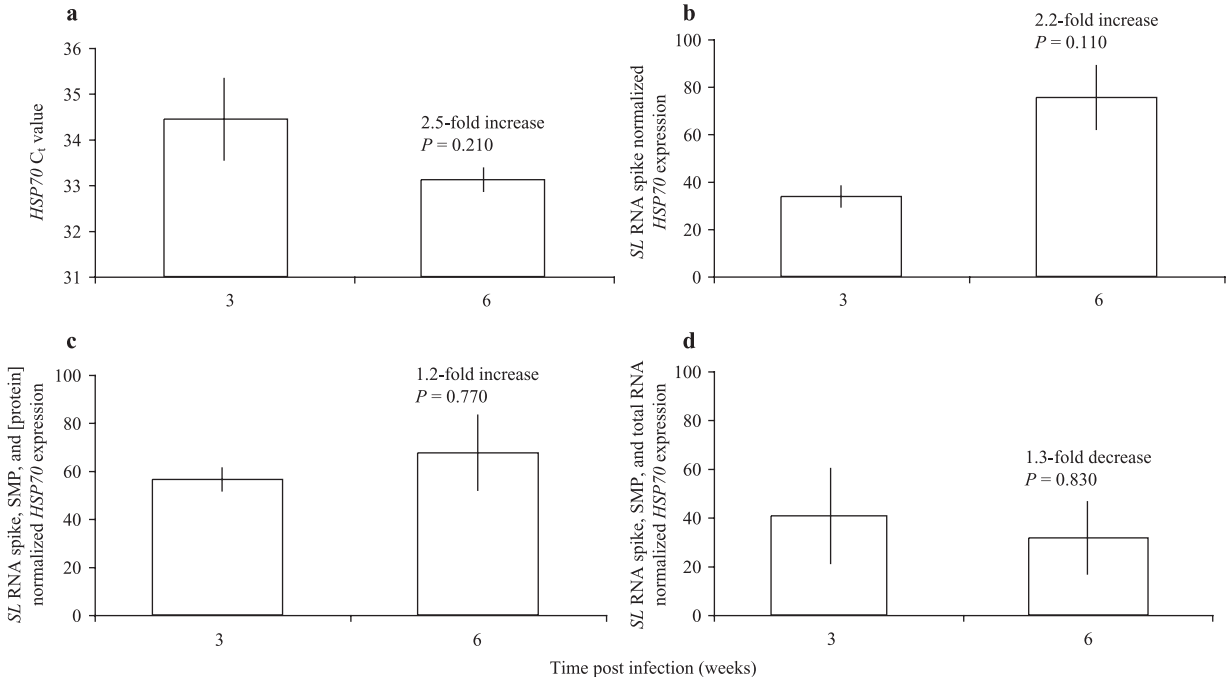

Fig. 4 *Symbiodinium* HSP70 expression in *Aiptasia pulchella* evaluated at 3- and 6-week post infection with symbiont cells. Mean symbiont HSP70 expression values (from cDNA, $n = 5$, +/− SE) presented as non-normalized C_t values (panel a); *SL* RNA spike-normalized transcripts (panel b); *SL* RNA spike, SMP and protein-normalized expression (panel c); and *SL* RNA spike, SMP, and total RNA-normalized expression (panel d). Fold changes and student's *t*-test *P* values are also displayed. Normalizing to the SMP effectively negates apparent changes in symbiont HSP70 expression.

protein or total RNA (Fig. 4c, d, respectively), there are no significant differences in symbiont HSP70 expression (student's *t*-test, $P = 0.77$, 0.83, respectively).

Discussion

To date, real-time quantitative PCR has been utilized in cnidarian–dinoflagellate symbiosis to calculate the relative abundance of different symbiont types in mixed symbioses (Loram *et al*. 2007a; Mieog *et al*. 2007) and to explore whether different assemblages confer adaptive advantages to the host cnidarian (Loram *et al*. 2007b). However, qPCR has not yet been employed in gene expression studies where the chimeric nature of the anthozoan symbioses is taken into account. This is an important consideration when considering qPCR and microarray experiments comparing bleached vs. nonbleached anthozoans. In this scenario, it is likely that a significant up-regulation of many host anthozoan genes would be observed in the bleached treatment simply because the host anthozoan composes the majority, if not all, of the RNA extracted. As such, failure to consider that RNA extractions of symbiotic organisms produce RNAs with variable host/symbiont composition that reflect the specifics of the *in vivo* scenario will lead to a biased interpretation of host or symbiont gene expression data. We have developed a protocol that addresses this issue and incorporates controls for other template and methodological issues.

The strong correlation ($r^2 = 0.92$ for anemone-derived symbionts and $r^2 = 0.97$ for coral-derived symbionts) between cells used in extraction and symbiont HSP70 gene copies measured by qPCR (Fig. 2) suggests that this approach can be used in real-time qPCR to estimate the symbiont contribution to the total holobiont nucleic acid pool up to 10^6 cells, a value often used in studies of freshly isolated *Symbiodinium* (e.g. Leggat *et al*. 2007). In fact, symbiont cell numbers are generally far lower in coral and anemone samples suitable for nucleic acid extraction (Drew 1972), which rarely exceed a total tissue biomass of 100 mg. However, what is evident is that the standard errors for the high cell number extractions, 2 and 2.5×10^6, are so great as to prevent predictive value (Fig. 2b). These high standard errors could be due to copurification of PCR inhibitors, such as humic acids (Bustin 2002). Also, in addition to greater competition for reagents, pipetting errors become magnified with highly concentrated samples, potentially eliminating the ability to resolve even large differences in genome copies or transcripts.

Haemocytometer counts agree well with molecularly calculated HSP70 genome copies in estimating the increase in symbiont material from 3 to 6 weeks of infection: the SMP normalized to total RNA increased 2.27-fold, compared

to a 2.41-fold increase in cell counts (Fig. 3). Neither approach detected symbionts in uninfected anemones, confirming that these anemones were symbiont free. These findings justify normalizing gene expression data to the SMP, which is readily amplified from DNA co-extracted from the same TRIzol media used to isolate RNA. This justification is reinforced by the fact that dividing by the SMP effectively nullified the apparent 2.5-fold increase in symbiont HSP70 expression observed over the 3- to 6-week infection period because the number of symbionts increased approximately 2.3 to 2.4-fold over this same period (Fig. 4). Had no correction been made, an increase in gene expression would have been reported that was, in fact, an artefact of the increased symbiont densities found within the anemones. While data are expressed as normalized to either protein or total RNA, due to problems with re-suspension of the protein pellet, future normalization for tissue quantity appear more immediately tractable with total RNA. However, a recent work (Hummon *et al*. 2007) has addressed this technical issue, and so now it is likely that high-quality protein can be obtained from TRIzol. Thus, it is likely that protein or total RNA could be used to normalize for tissue biomass differences, granted that the appropriate technical modifications are made.

We have suggested that traditional housekeeping genes, especially those involved in cell scaffolding and cell volume alteration, could prove to be differentially expressed under heat stress given the hypothesis that thermal stress leads to cell volume changes before breakdown of the anthozoan-dinoflagellate symbiosis (Mayfield & Gates 2007). These genes, including ACTB and a-*tubulin* have shown to serve poorly as housekeeping genes in other systems (Tricarico *et al*. 2002). In addition, finding suitable housekeeping genes for nonmodel systems can be frustrating and time-consuming (Smith-Keune & Dove 2007). Nevertheless, recent research has focused on finding suitable housekeeping genes for studies with symbiotic anthozoans (Moya *et al*. 2008; Rodriguez-Lanetty *et al*. 2008), although neither study considered the issue of differential presence or extraction of symbionts and so putative stability of expression could be biased by altered host/symbiont nucleic acid ratios instead of actual biological consistency of mRNA transcription. As such, using an artificial molecule that represents a gene absent from an organism's genome or transcriptome represents a biologically sound and time-efficient alternative to the housekeeping gene approach (Bustin 2004). By normalizing to RNA/DNA spikes to account for extraction and reverse transcription efficiency, an SMP to account for differential extraction of *Symbiodinium*, and total RNA as a proxy for overall biological material, gene expression data can be reliably and robustly interpreted for anthozoan samples exposed to any variety of experimental manipulations and will be valuable for anthozoan and other symbiologists whose studies involve organisms in close association.

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