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Bleaching and stress in coral reef ecosystems: *hsp70* expression by the giant barrel sponge *Xestospongia muta*

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Abstract

Sponges are a prominent component of coral reef ecosystems. Like reef-building corals, some sponges have been reported to bleach and die. The giant barrel sponge *Xestospongia muta* is one of the largest and most important components of Caribbean coral reef communities. Tissues of *X. muta* contain cyanobacterial symbionts of the *Synechococcus* group. Two types of bleaching have been described: cyclic bleaching, from which sponges recover, and fatal bleaching, which usually results in sponge death. We quantified *hsp70* gene expression as an indicator of stress in *X. muta* undergoing cyclic and fatal bleaching and in response to thermal and salinity variability in both field and laboratory settings. Chlorophyll *a* content of sponge tissue was estimated to determine whether *hsp70* expression was related to cyanobacterial abundance. We found that fatally bleached sponge tissue presented significantly higher *hsp70* gene expression, but cyclically bleached tissue did not, yet both cyclic and fatally bleached tissues had lower chlorophyll *a* concentrations than nonbleached tissue. These results corroborate field observations suggesting that cyclic bleaching is a temporary, nonstressful state, while fatal bleaching causes significant levels of stress, leading to mortality. Our results support the hypothesis that *Synechococcus* symbionts are commensals that provide no clear advantage to their sponge host. In laboratory experiments, sponge pieces incubated at 30 °C exhibited significantly higher *hsp70* expression than control pieces after 1.5 h, with sponge mortality after less than 15 h. In contrast, sponges at different salinities were not significantly stressed after the same period of time. Stress associated with increasing seawater temperatures may result in declining sponge populations in coral reef ecosystems.

Keywords: chlorophyll *a*, gene expression, *hsp70*, salinity, temperature

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Introduction

Coral reefs and associated near-shore ecosystems are suffering dramatic changes in species abundance, diversity and habitat structure worldwide (Hughes 1994; Hughes *et al*. 2003). In the Caribbean, alterations in coral reef communities have been attributed to both natural and anthropogenic factors (Grigg 1994; Ostrander *et al*. 2000). These range from local anthropogenic modifications that affect the overall quality of marine habitats (Done 1999; Harvell *et al*. 1999) to increasing global temperature (Vicente 1989; Harvell *et al*. 1999).

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Both short- and long-term environmental perturbations place significant physiological stress on resident organisms (Hofmann *et al*. 2005). Adaptation of organisms to variable environmental parameters implies protection mechanisms including the heat shock protein (HSP) response (Bosch *et al*. 1988; Miller & McLennan 1988; Sanders 1993; Kültz 2005). During stress, HSPs are involved in proper folding or unfolding of nascent polypetides, proteins translocated through membranes or denatured proteins (Gelthing & Sambrook 1992; Parcell & Lindquist 1993; Morimoto *et al*. 1994). HSPs also participate in minimizing the aggregation of non-native proteins and targeting non-native or aggregated proteins for degradation and removal from the cell (Feder & Hofmann 1999). The proteins are also known as stress indicator proteins because their transcriptional and

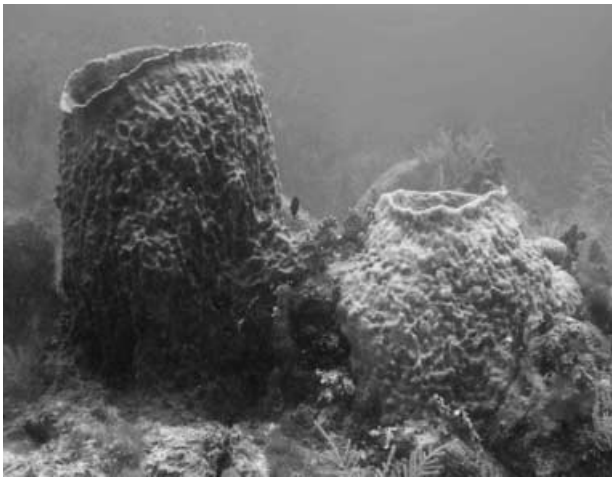

Fig. 1 Nonbleached and bleached *Xestospongia muta* on Conch Reef, Key Largo, Florida.

translational expression can be induced by a number of stressful conditions, such as extreme temperatures, ultraviolet radiation and presence of heavy metals (reviewed in Craig 1985; Feder & Hofmann 1999; Kültz 2005). The *hsp* genes are constitutively expressed in unstressed cells, but their expression increases when cells are under stress.

Sponges are a prominent component of coral reef ecosystems (Diaz & Rutzler 2001), and play key functional roles (Diaz & Rutzler 2001). In terms of species richness, abundance and biomass, sponges are often rivaling both hard and soft corals (Targett & Schmahl 1984; Diaz & Rutzler 2001). Like reef-building corals, sponges have been reported to bleach and often die in both the Pacific and the Caribbean (Vicente 1990; Fromont & Garson 1999; Cowart *et al*. 2006). Although several studies have related bleaching in corals with stress (e.g. Black *et al*. 1995; Sharp *et al*. 1997; Downs *et al*. 2002), no study has targeted this same phenomenon in sponges. The giant barrel sponge *Xestospongia muta* (Demospongiae: Haplosclerida) is one of the largest and most common member of Caribbean coral reef communities (Armstrong *et al*. 2006). Tissues of *X. muta* contain cyanobacterial symbionts belonging to the *Synechococcus* group (Gómez *et al*. 2002; Steindler *et al*. 2005). This sponge is subject to occasional bleaching (loss of the reddish-brown coloration; Fig. 1). Reports of *X. muta* bleaching and other symbiont-containing sponges began to appear along with reports of coral bleaching events over a decade ago (Vicente 1990). Sponge bleaching and subsequent mortality have since been observed throughout the Caribbean (Nagelkerken *et al*. 2000; Gammill & Fenner 2005). Two types of bleaching have been observed in *X. muta*. The first, cyclic bleaching, is common and affects ~25% of the sponge population off Key Largo, Florida (Cowart *et al*. 2006). Sponges affected by cyclic bleaching recover over time. A second type, fatal bleaching, results in a complete whitening of sponge tissue and affects < 1% of the sponge population (Cowart *et al*. 2006). Fatal bleaching appears to be a different phenomenon and results in the complete disintegration of tissue, usually followed by mortality. Fatal bleaching has been associated with a distinctive sponge orange band (SOB) that migrates across the sponge, separating nonbleached and bleached portions of tissue (Cowart *et al*. 2006).

In this study, we measured the messenger RNA (mRNA) abundance of a main inducible *hsp* gene (*hsp70*) using quantitative real-time polymerase chain reaction (QRT–PCR). Because of its high sensitivity, QRT–PCR is currently the best method to accurately quantify mRNA abundance (Bustin 2000). We quantified *hsp70* levels in *X. muta* in the field undergoing cyclic and fatal bleaching and in the laboratory in response to different temperature and salinity regimes. In addition, because the abundance of cyanobacteria can be directly correlated with chlorophyll *a* concentration (Wilkinson 1983; Rai 1990), chlorophyll *a* content was estimated for all tissue samples. Our goal was to investigate whether there is a relationship between bleaching and *hsp70* gene expression in sponge tissue in the field and whether stress protein production could be induced under stressful conditions of altered temperature and salinity in both the field and laboratory. As dominant components of coral reef ecosystems (Diaz & Rutzler 2001), understanding the potential factors that cause sponge bleaching and stress is critical for ecosystem conservation and management.

Materials and methods

Samples

Samples from bleached tissue of *Xestospongia muta*, the sponge orange band, and normal coloured tissue (nonbleached) were collected from three sponges undergoing fatal bleaching on Conch Reef (24°57′13″N, 80°27′13″W) and Conch Wall (24°57′01″N, 80°27′25″W); Key Largo, Florida. For sponges undergoing cyclic bleaching, samples were taken from both bleached and normal tissue of three sponges. All samples were collected by SCUBA diving in June 2006.

Quantification and analysis of chlorophyll a

Three tissue cores were taken from each sponge using a 7-mm diameter cork borer. From each core, the top 5 mm of sponge tissue was sampled. Samples were transported to the laboratory on ice and in the dark. Chlorophyll *a* was extracted in a 90% acetone:water mixture held overnight at 4 °C. Each sample was then centrifuged and the supernatant

was transferred to a spectrophotometer cuvette. Absorbance was measured at 750, 664, 647, and 630 nm with an USB2000 Ocean Optics fibre optic spectrometer. Chlorophyll *a* content was calculated based on equations provided by Parsons *et al.* (1984), and standardized to sponge tissue volume.

Hsp70 *gene detection*

DNA extractions of frozen tissue from 10 different sponges were obtained using the PUREGENE kit (Gentra Systems) and nested PCRs were performed using the primers described in Borchiellini *et al.* (1998). The sequencing reaction was carried out in a Peltier PTC-200 with the BigDye terminator version 3.1 and using the same primers as in the amplification step. Sequences were obtained on an ABI PRISM 3100 automated sequencer. We obtained two haplotypes of 711 bp with 27.7% variable sites (GenBank Accession nos. EU302136 and EU302137).

QRT–PCR primer design

The QRT–PCR primer set 5′-TCAACGTCAAGCCACA-AAAG-3′ (forward) and 5′-TGTCGAGACCGTATGCGAT-3′ (reverse) was designed targeting a 101-bp region of the detected *hsp70* genes. The primers 5′-TGAGACTGCGAA-TGGCTCAT-3′ (forward) and 5′-GGCCTTCGGCATGTAT-TAGC-3′ (reverse) were designed against a 101-bp region of the 18S RNA gene of *X. muta* available at GenBank (Accession no. AY621510). All primers for QRT–PCR were designed using the PRIMER EXPRESS software (Applied Biosystems).

RNA extraction and complementary DNA synthesis

Samples were collected in individual sealed plastic bags and immediately dropped in liquid nitrogen until transferred to a –70 °C freezer. From each sample, 90 mg of frozen tissue was homogenized in TRIzol reagent (Invitrogen) and purified RNA was obtained using the Micro-to-midi RNA purification kit (Invitrogen) according to manufacturer's instructions. RNA was re-suspended in 100 µL nuclease-free water. All samples were DNase treated using DNase Amplification Grade I (Invitrogen). For complementary DNA (cDNA) synthesis, ~400 ng of total RNA was reverse-transcribed using SuperScript Reverse Transcriptase II kit (Invitrogen) with specific primers targeting either *hsp70* gene or 18S ribosomal RNA (rRNA) gene. Initially, a total of 24 cDNA samples were obtained in duplicate and quantified by QRT–PCR (see protocol below) to test the efficiency of cDNA synthesis. No significant variation between duplicates was recorded in any case (signed-rank test, $P = 0.2$) and the variation between duplicate samples was less than 4.1%.

QRT–PCR of hsp70 *transcripts*

To quantify mRNA abundance of the *hsp70* gene, we used a 7500 Applied Biosystems quantitative real-time PCR and the standard curve method. Standards for 18S gene (reference gene) and *hsp70* gene (target gene) were obtained by cloning (TOPO TA Cloning Kit, Invitrogen). Positive colonies were analysed by PCR using specific primers targeting the plasmid. Colonies containing the correct insert were grown overnight in a Luria-Bertani liquid media containing kanamycin. Plasmid extraction was performed using the Perfectprep plasmid Mini kit (Eppendorf) and sequenced to re-verify that the correct fragment of 18S or *hsp70* gene was present. QRT–PCRs were performed with 2 µL of *hsp70* cDNA or 1 µL of 18S cDNA, in 10 µL SYBR GreenER SuperMix (Invitrogen), and nuclease free water to a total volume of 20 µL. The PCR was run for 40 cycles with the following cycle parameters: a single soak at 50 °C for 5 min, and 95 °C for 10 min, was followed by 40 amplification cycles (95 °C for 15 s; 58 °C for 15 s and 68 °C for 0.35 s). Each 96-well plate contained a negative control, samples in triplicates, and sevenfold serial dilutions of the corresponding standard. Samples from a same treatment set were analysed together. Melt curve analysis was performed following each PCR to confirm that only a single product was amplified. Fold change in the target gene and the reference gene were calculated by averaging the copy number in the experimental sample and dividing it by the average copy number of the control sample. To obtain the ratio of the target gene in the experimental sample relative to the control sample and corrected for the reference gene, we divided the fold change value of the target gene by the one of the reference gene. Ratios between experimental treatments and their controls were obtained to standardize the results and allow comparisons between experiments.

Laboratory experiments — salinity and temperature

Six sponges of similar size (~0.45 m high) were randomly selected and labelled on Conch Reef. For the salinity experiment, four pieces of approximately $14 \times 10 \times 5$ cm were cut from the upper margin of each of three sponges. For the temperature experiment, we cut five pieces of each of the remaining three sponges. All sponge pieces were left to heal inside the spongocoel from which the pieces were cut for at least 2 days. No bleaching or death of any of the pieces was observed, and cut edges healed rapidly (Walters & Pawlik 2005). Each piece was then placed in a separate sealed bag and quickly transported to the laboratory in a cooler filled with freshly collected sea water at ambient temperature. Before the start of the experiments, triplicate samples were taken from each piece to determine initial chlorophyll *a* concentration and *hsp70* expression (T_0). For

the salinity experiment, one piece from each of three sponge replicates was placed in a control tank with freshly collected reef water at 35.5‰ (control). The remaining three pieces per sponge were placed in tanks at 34‰, 39‰, and 41‰, respectively. The different salinities were achieved by adding freshwater or synthetic sea salt to control water. Salinity was measured with a refractometer. For the temperature experiment, one piece of each of three sponge replicates was placed in a control tank at 28 °C. The remaining four pieces per sponge were placed in tanks at 10 °C, 20 °C, 30 °C, and 40 °C, respectively. The different temperatures were achieved by heating the water with one to three 200 watt submersible heaters or using a cooling coil attached to a recirculating chiller, and using vigorous aeration of each tank to maintain a uniform temperature. Incubation times for both salinity and temperature experiments were T_1, 1.5 h; T_2, 5 h; T_3, 15 h. At the end of each incubation time, triplicate samples were taken from each sponge piece to determine levels of chlorophyll *a* and *hsp70* expression.

Field experiment

Three sponges from shallow Conch Reef (13 m) and three from a deeper location (26 m) of the same reef (24°56′59″N, 80°27′13″W) were randomly selected and labelled. Two pieces of approximately 14 × 10 × 5 cm were cut from each of the sponges and left to heal inside the spongocoel for at least 4 days. No bleaching or necrosis of any of the pieces was observed at the end of this time period. Vexar mesh cages of approximately 0.5 m^2 were fixed onto the limestone substratum of the nearby reef at 13 m and at 26 m. Triplicate samples were taken to determine the initial chlorophyll *a* content and *hsp70* gene expression level (T_0). Each piece was then carefully placed in a water-filled, sealed plastic bag and transported either to a cage located at the same depth (control) or to the reciprocal depth. Finally, we fixed each piece to a different corner of the vexar cage with a cable tie at ~20 cm from the bottom on June 2, 2006. Pieces were sampled again after 2.5 h (T_1), after 33 days (T_2), and after 163 days (T_3).

Data analysis

In order to standardize chlorophyll *a* and *hsp70* expression data, we calculated ratios between experimental treatments and their controls. For fatally bleached sponges, samples of normal tissue (nonbleached) were considered controls, whereas bleached and orange band tissues were considered experimental samples. For cyclically bleached sponges, normal tissue was considered control and bleached tissue the experimental sample. For the laboratory experiments, sponge pieces kept in tanks at ambient reef temperatures (28 °C) and salinities (35.5‰) were considered controls whereas those in manipulated water tanks were considered experimental samples. For the field experiment, we considered pieces kept at the same depth as control samples and pieces that were transplanted to the reciprocal depth as experimental samples. Differences between normal tissue and bleached and orange band tissues were assessed using a *t*-test.

To analyse changes in chlorophyll *a* and *hsp70* gene expression, we used a repeated-measures ANOVA design (ANOVAR), in which the between-subject factors were treatment and sponge (nested within treatment), and the within-subject factor was time of sampling. The assumptions for ANOVAR include the circularity of the variance–covariance matrix (Von Ende 2001), which is rarely met, so adjustments to the degrees of freedom of the *F*-statistics are necessary (Potvin *et al*. 1990; Quinn & Keough 2002). We tested circularity using Mauchly's sphericity test, and, as it failed in most cases, we have adjusted the degrees of freedom of ANOVAR and corrected the significance levels using the Huynh-Feldt and the Greenhouse-Geisser ε estimates (both correction methods yielded the same results). In addition, we confirmed the results with multivariate analyses that do not require the circularity assumption (MANOVAR, Von Ende 2001), and in all cases the results were coincident so only the univariate results will be presented. When interactions between treatment and time were significant, we performed ANOVA analyses for each time. A Tukey post-hoc test based on pairwise comparisons of treatments was run after significant ANOVA outcomes. Finally, a correlation analysis was used to assess relationships between chlorophyll *a* concentration and *hsp70* levels for salinity and temperature treatments. The statistical packages SYSTAT version 11, STATISTICA version 6, and SIGMASTAT version 2 were used for analyses.

Results and discussion

Bleached tissues of both fatal and cyclically bleached sponges had significantly lower chlorophyll *a* concentrations than normal tissues ($P < 0.001$ and $P = 0.004$, respectively; Fig. 2a). Orange band (SOB) tissues also had less chlorophyll *a* than the normal tissues ($P < 0.001$). Both bleached and orange band tissues of sponges undergoing fatal bleaching presented higher abundances of *hsp70* mRNA than normal tissues ($P = 0.014$ and $P = 0.049$, respectively; Fig. 2b), indicating higher levels of stress. In contrast, there were no significant differences in *hsp70* gene expression in tissues from sponges undergoing cyclic bleaching ($P = 0.930$, Fig. 2b). Fatal bleaching causes a gradual deterioration of tissue resulting in necrosis and sponge mortality within 2 months (Cowart *et al*. 2006). A similar phenomenon has been described for *Aplysina* species in the Caribbean (Olson *et al*. 2006). Affected specimens of *Aplysina* spp. presented a rust-coloured band (ARBS) due to the presence of a

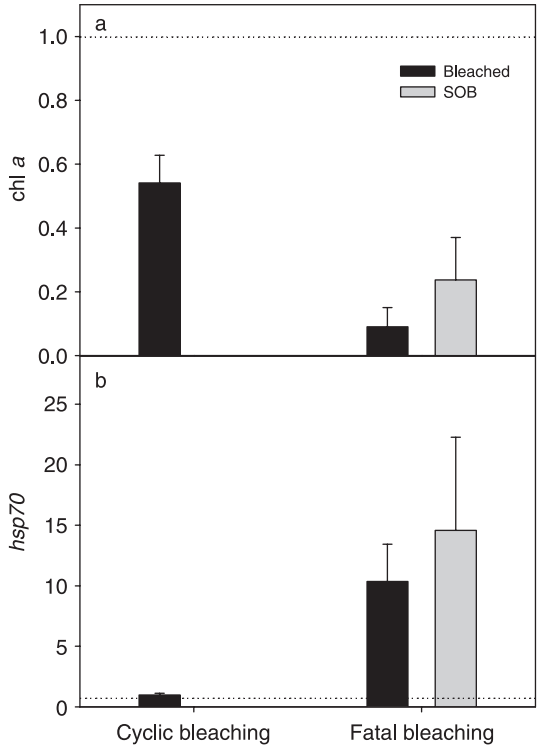

Fig. 2 Endogenous levels of (a) chlorophyll *a* and (b) *hsp70* expression in sponges undergoing cyclic and fatal bleaching in the field. Chlorophyll *a* and *hsp70* levels were determined for bleached tissue and tissue from the orange band (SOB) vs. normal tissue (nonbleached). Data are shown as a ratio of experimental: control values. Dotted lines indicate a ratio of one, where experimental samples results equal control results. Bars indicate standard errors.

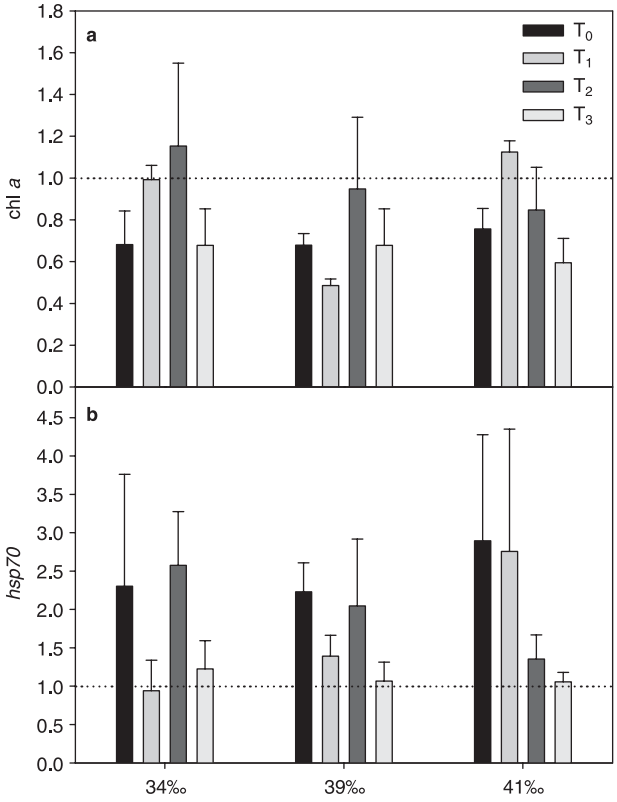

Fig. 3 Response of sponge pieces subjected to different salinities in the laboratory: (a) chlorophyll *a* content, and (b) *hsp70* expression level. Treatment tanks were maintained at 34‰, 39‰ and 41‰. Codes for incubation times: *in situ*, in the field, before cutting pieces from the sponge; T_0, before placing healed sponge pieces in treatment tanks; T_1, after 1.5 h; T_2, after 5 h; T_3, after 13 h (overnight). Data are shown as a ratio of treatment vs. control values. Dotted lines indicate a ratio of one. Control pieces were at 35.5‰. Bars indicate standard errors.

pathogenic cyanobacterium (Olson *et al*. 2006). The presence of SOB on the edge of the bleached tissue in *Xestospongia muta* appears to be equivalent to ARBS, and has been suggested to be pathogenic (Cowart *et al*. 2006).

There were no significant differences in chlorophyll *a* concentration over time for either the salinity or temperature experiments ($P = 0.175$ and $P = 0.257$, respectively; Tables 1a and 2a; Figs 3a and 4a). *Hsp70* gene expression varied over time in the salinity treatments (Fig. 3b), as revealed by a significant interaction time * salinity ($P = 0.02$; Table 1b). When ANOVA analyses were performed for each time separately, it was found that sponges kept at 41‰ had a higher level of *hsp70* gene expression after 1.5 h incubation than in the other treatments ($P = 0.001$). In general, *hsp70* transcript levels increased during the first observation times, but dropped to values close to the controls after less than 15 h of incubation (Fig. 3b), and no mortality was recorded in any case. Our results suggest that sponges are able to adjust and recover from salinity changes ranging from 34‰ to 41‰.

Significant differences were recorded in *hsp70* transcript levels of sponges incubated at different temperatures over time, as shown by a significant interaction time * treatment ($P < 0.001$; Table 2b). Separate ANOVA analyses at each time revealed significant differences among treatments at all times except the first one. The Tukey tests indicated that, after 1.5 h, the significant result was due to the fact that the sponges kept at 40 °C had significantly higher levels of *hsp70* gene expression than the other treatments, while after 5 h and 15 h the sponges kept at 30 °C displayed significantly higher levels of *hsp70* transcript than found in the other treatments (Fig. 4b). Therefore, the effect of increased temperature was evident earlier in the highest temperature treatment (40 °C) and later in the 30 °C treatment. After these time periods, *hsp70* gene expression decreased, which can be attributed to metabolic failure and sponge death. In fact, two out of the three sponge pieces incubated at 40 °C were dead (obvious tissue necrosis) after 5 h, whereas the pieces at 30 °C appeared healthy. Moreover, after 15 h incubation, two sponge pieces at 30 °C

Table 1 Salinity treatment results of ANOVAR for (a) chlorophyll *a* content and (b) *hsp70* expression. For within-subject effects, the corrected probabilities are given according to the Greenhouse–Geisser (G–G) and the Huynh–Feldt (H–F) epsilon values

	SS	d.f.	MS	*F*	*P*	G–G	H–F
(a)							
Between subjects							
Salinity	0.452	2	0.226	1.104	0.353		
Sponge	0.808	6	0.135	0.658	0.684		
Error	3.685	18	0.205				
Within subjects							
Time	1.625	3	0.542	2.055	0.117	0.147	0.118
Time * salinity	2.219	6	0.370	1.403	0.230	0.256	0.231
Time * sponge	6.772	18	0.376	1.427	0.157	0.206	0.158
Error	14.234	54	0.264				
Greenhouse–Geisser epsilon:		0.6163					
Huynh–Feldt epsilon:		0.9890					
(b)							
Between subjects							
Salinity	2.841	2	1.42	0.683	0.518		
Sponge	41.274	6	6.879	3.308	0.023		
Error	37.428	18	2.079				
Within subjects							
Time	23.471	3	7.824	11.154	0.000	0.000	0.000
Time * salinity	23.960	6	3.993	5.693	0.000	0.002	0.000
Time * sponge	113.085	18	6.282	8.957	0.000	0.000	0.000
Error	37.876	54	0.701				
Greenhouse–Geisser epsilon:		0.5756					
Huynh–Feldt epsilon:		0.9141					

SS, sum of squares; d.f., degrees of freedom; MS, mean of square; *F*, *F*-statistic; *P*, *P* value.

Table 2 Temperature treatments results of ANOVAR for (a) chlorophyll *a* content and (b) *hsp70* expression. For within-subject effects, the corrected probabilities are given according to the Greenhouse–Geisser (G–G) and the Huynh–Feldt (H–F) epsilon values

	SS	d.f.	MS	*F*	*P*	G–G	H–F
(a)							
Between subjects							
Temperature	1.007	3	0.336	0.746	0.535		
Sponge	5.965	8	0.746	1.657	0.161		
Error	10.800	24	0.450				
Within subjects							
Time	5.735	3	1.912	5.238	0.003	0.006	0.003
Time * temperature	4.140	9	0.460	1.261	0.273	0.287	0.273
Time * sponge	21.886	24	0.912	2.499	0.002	0.005	0.002
Error	26.274	72	0.365				
Greenhouse–Geisser epsilon:		0.7639					
Huynh–Feldt epsilon:		1.0000					
(b)							
Between subjects							
Temperature	1061.246	3	353.749	27.130	0.000		
Sponge	1954.081	8	244.260	18.738	0.000		
Error	312.846	24	13.035		0.000		
Within subjects							
Time	938.464	3	312.821	52.416	0.000	0.000	0.000
Time * temperature	3296.311	9	366.257	61.370	0.000	0.000	0.000
Time * sponge	6246.654	24	260.277	43.612	0.000	0.000	0.000
Error	429.696	72	5.968				
Greenhouse–Geisser epsilon:		0.7276					
Huynh–Feldt epsilon:		1.0000					

SS, sum of squares; d.f., degrees of freedom; MS, mean of square; *F*, *F*-statistic; *P*, *P* value.

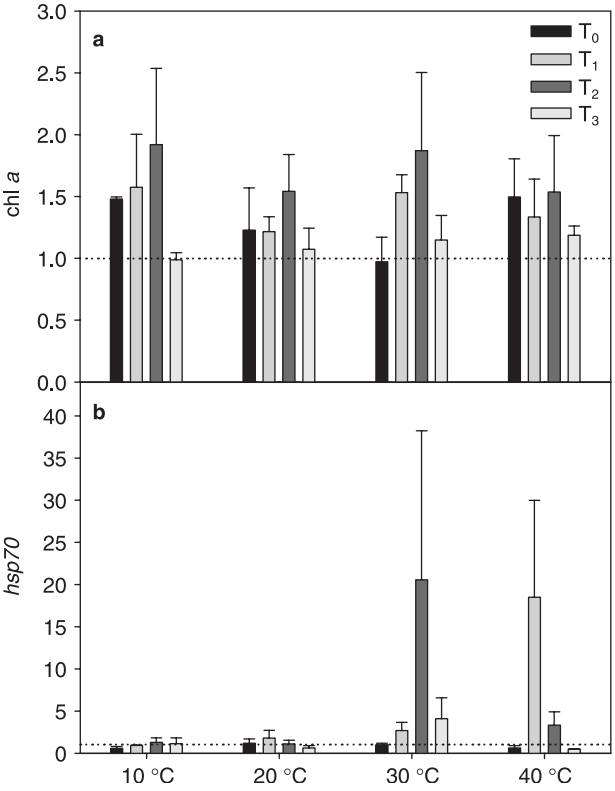

Fig. 4 Response of sponge pieces subjected to different water temperatures in the laboratory: (a) Chlorophyll *a* content, and (b) *hsp70* expression level. Treatment tanks were maintained at 10, 20, 30, and 40 °C. Codes for incubation times: *in situ*, in the field before cutting pieces from the sponge; T_0, before placing healed sponge pieces in treatment tanks; T_1, after 1.5 h; T_2, after 5 h; T_3, after 13 h (overnight). Data are shown as a ratio of treatment vs. control values. Dotted lines indicate a ratio of one. Control pieces were at 28 °C. Bars indicate standard errors.

and all pieces at 40 °C were dead, and the *hsp70* transcript levels had dropped in both cases (Fig. 4b). *Hsp70* gene expression of sponge pieces at 10 °C and 20 °C were not different at any time (Tukey post-hoc tests, $P > 0.05$ in all cases), therefore lower than normal temperatures for less than 15 h did not significantly stress sponge tissue. We conclude that, as for corals, mortality or recovery is determined by the extent of damage to essential cellular structures (Halliwell & Gutteridge 1999; Downs *et al*. 2002), and minor damage can be repaired by an increase in chaperone (e.g. *hsp70*) turnover activity (Downs *et al*. 2002), while greater damage leads to a collapse of the metabolic defense systems in a relatively short time.

Statistical analyses revealed a lack of correlation between chlorophyll *a* content and *hsp70* gene expression for both salinity and temperature experiments (Fig. 5), and therefore, stress responses in *X. muta* could not be linked to the abundance of their symbiont populations. As for the transplantation experiment, the changes in chlorophyll *a*

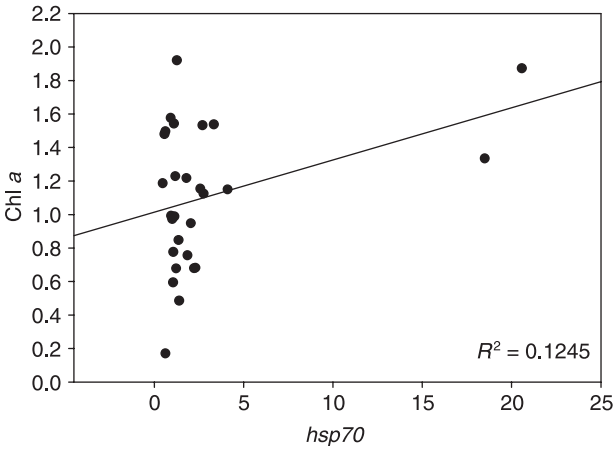

Fig. 5 Correlation analysis of the relationship between chlorophyll *a* concentration and *hsp70* expression during salinity and temperature treatments.

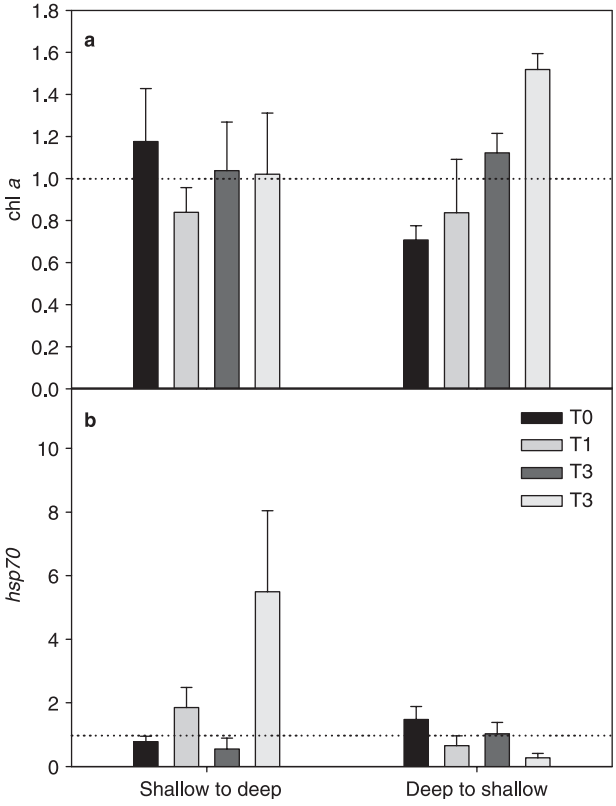

Fig. 6 Response of sponge pieces reciprocally transplanted to different depths: (a) chlorophyll *a* content, and (b) *hsp70* expression level. Codes for treatments: shallow to deep, sponge pieces moved from their original location at 13 to 26 m; deep to shallow, pieces moved from their original location at 26 to 13 m. T_0, before putting the pieces in cages; T_1, after 2.5 h; T_2, after 33 days; T_3, after 163 days. Data are shown as a ratio of treatment vs. control values. Dotted lines indicate a ratio of one. Control pieces were kept at the same depth over time. Bars indicate standard errors.

Table 3 Depth treatment results of ANOVAR for (a) chlorophyll *a* content and (b) *hsp70* expression. For within subject effects, the corrected probabilities are given according to the Greenhouse–Geisser (G–G) and the Huynh–Feldt (H–F) epsilon values

	SS	d.f.	MS	*F*	*P*	G–G	H–F
(a)							
Between subjects							
Depth	0.014	1	0.014	0.175	0.683		
Sponge	0.952	4	0.238	2.959	0.065		
Error	0.965	12	0.080				
Within subjects							
Time	1.878	3	0.626	14.608	0.000	0.000	0.000
Time * depth	2.123	3	0.708	16.510	0.000	0.000	0.000
Time * sponge	4.417	12	0.368	8.589	0.000	0.000	0.000
Error	1.543	36	0.043				
Greenhouse–Geisser epsilon:		0.7903					
Huynh–Feldt epsilon:		1.0000					
(b)							
Between subjects							
Depth	31.021	1	31.021	201.534	0		
Sponge	32.407	4	8.102	52.635	0		
Error	1.847	12	0.154				
Within subjects							
Time	47.345	3	15.782	120.928	0.000	0.000	0.000
Time * depth	101.381	3	33.794	258.945	0.000	0.000	0.000
Time * sponge	101.492	12	8.458	64.807	0.000	0.000	0.000
Error	4.698	36	0.131				
Greenhouse–Geisser epsilon:		0.5360					
Huynh–Feldt epsilon:		0.8643					

SS, sum of squares; d.f., degrees of freedom; MS, mean of square; *F*, *F*-statistic; *P*, *P* value.

concentration varied with treatment (significant interaction treatment * time; Table 3a). Chlorophyll *a* contents in sponge pieces that were transplanted from 26 to 13 m increased over time and was significantly higher than in pieces transplanted from 13 to 26 m after 5 months (ANOVA, $P < 0.001$; Table 3a; Fig. 6a). For *hsp70* gene expression levels, the interaction treatment * time was significant ($P < 0.001$; Table 3b), and separate ANOVAs showed significant effects at all times. However, the increase of *hsp70* transcript levels over time did not show a clear pattern (Fig. 6b). During the first observation times (T_0, T_1, T_2), differences in *hsp70* transcript levels between shallow and deep transplants were inconsistent, but overall the mean values remained within 1 ± 0.8, so the variation was limited and may be explained by heterogeneity in sponge response. However, after 163 days, a significant increase of stress levels was observed in sponges that were transplanted from 13 to 26 m (Tukey test, $P < 0.001$; Fig. 6b). This increase coincided with a decrease of *hsp70* gene expression in shallow cages. We observed that four out of six sponge pieces at 26 m were developing new osculae. Therefore, the re-organization of tissues may be a stressful process that coincides with an increase in *hsp70* expression.

In most experiments, sponge pieces within a given treatment responded differently, as indicated by the large error bars in *hsp70* ratios (Figs 2–6), a significant sponge factor (nested within treatment), and a significant interaction for time * sponge (Tables 1, 2 and 3). This is evidence of heterogeneity in sponge response to different stressors. Intraspecific variability in stress response is common in marine invertebrates (e.g. Agell *et al*. 2001, 2004; Coles & Brown 2003; Osovitz & Hofmann 2005; Rossi *et al*. 2006), and ultimately depends on the genotype of each specimen.

Stress in *X. muta* cannot be directly related to the abundance of their cyanobacterial symbionts, in clear contrast to the relationship documented for reef-building corals (Hoegh-Guldberg & Smith 1989; Lesser *et al*. 1990; Coles & Fadlallah 1991; Muscatine *et al*. 1991; Glynn 1993; Fang *et al*. 1995; Brown 1997; Ben-Haim *et al*. 1991). Gómez *et al*. (2002) observed a loss of cyanobacteria from shaded *X. muta* after a 7-week experiment, but not a reduction in sponge mass. More recently, Thacker (2005) observed the same phenomenon in shaded specimens of *Xestospongia exigua*. Similarly, in our field experiment, sponge pieces that were transplanted to shallow depths steadily increased chlorophyll *a* content in their tissues, but no significant change was observed in control sponge pieces that remained at 26 m. Therefore, light availability may be the main factor influencing populations of cyanobacteria in sponges of the genus *Xestospongia*. Although chlorophyll *a* concentrations

in *X. muta* undergoing cyclic bleaching were significantly lower in bleached than normal tissue, there was no significant difference in *hsp70* gene expression. Our results corroborate field observations that indicate that cyclic bleaching is a nonstressful state characterized only by a temporary decrease in cyanobacterial density in sponge tissue, from which the sponges fully recover. Therefore, as suggested by Thacker (2005), our results support the hypothesis that *Synechococcus* symbionts are commensals that exploit the resources provided by their host without significantly affecting the sponge (Gómez *et al*. 2002; Thacker 2005), or influencing its physiological status. Further studies, including direct measurements of nutrient relationships between the sponge and its cyanobacterial symbionts, are needed to definitely settle this point.

While salinity changes did not produce significant variations in *hsp70* gene expression levels, increases in temperature of only a few degrees resulted in enhanced transcription of *hsp70* stress protein and detrimental effects leading to sponge death. Therefore, as amply demonstrated for corals (e.g. Glynn 1993; Hayes & King 1995; Brown 1997), sea water temperature increases may result in major declines of sponge populations in coral reef ecosystems.

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