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REPORT

Investigating the heat shock protein response involved in coral bleaching across scleractinian species in the central Red Sea

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Abstract Coral bleaching represents the most serious threat to contemporary coral reefs. In response, focus is being laid on understanding the cellular processes involved in the response of corals to the environmental stresses and the molecular mechanisms that determine the bleaching patterns. In the present study, a component of the cellular stress response such as the expression of the heat shock proteins (Hsps) was analyzed following the coral bleaching event which occurred in the central Red Sea (Saudi Arabia) in 2015. During this event, corals of different species, growth forms and sites showed variable bleaching susceptibility. In particular, we investigated the expression of Hsp70, Hsp60 and Hsp32 in both healthy and bleached colonies belonging to four different coral species (*Goniopora lobata*, *Porites lobata*, *Seriatopora hystrix* and *Stylophora pistillata*), in order to explore the intra- and inter-specific modulation of these biomarkers as well as the existence of spatial patterns of Hsp expression. In healthy colonies, the level of all the biomarkers was significantly different among the different species, although within each species it remained similar regardless of the distance from the shore. All the coral species showed a significant modulation of the Hsp expression in response to bleaching, whose typology and amplitude were species-specific. In all the species, Hsp70 and Hsp60 showed a coordinated dual expression, which, in response to bleaching resulted in an up-regulation in *G. lobata* and *P. lobata* and in a down-regulation in *S. hystrix* and *S. pistillata*. Hsp32 was up-regulated in all four species following bleaching, indicative of elevated oxidative stress. Overall, the protein expression profiles of each species contribute to assess the role of Hsps in regulating the susceptibility to thermal stresses of the various coral taxa of the Red Sea.

Keywords Heat shock proteins · Heme oxygenase-1 · Red Sea · Coral bleaching · Susceptibility

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Introduction

Ocean warming driven by climate change is resulting in increased occurrence of mass coral bleaching events. These events are becoming more frequent and devastating as evidenced by the recent episodes that affected coral reefs across the globe and led to a diffuse mortality (Hughes et al. 2017, 2018a; Oliver et al. 2018; Sully et al. 2019). In particular, the 2015–2016 coral bleaching has been considered the longest and most widespread global coral

bleaching event (Eakin et al. 2016; Hughes et al. 2018b; Lough et al. 2018). In this context, some reefs suffered severe bleaching twice and other areas experienced unprecedented mass bleaching (Couch et al. 2017; Rodgers et al. 2017; Le Nohaïc et al. 2017; Osman et al. 2018; Xie et al. 2017).

Coral reefs in the Red Sea are adapted to live in water characterized by high salinity and warm temperatures, which can also show seasonal variations of ~ 10 °C that overcome the tolerable limits of corals elsewhere (Kleypas et al. 1999; Davis et al. 2011). For this reason, bleaching thresholds for corals in the Red Sea are higher than those for most locations in the Great Barrier Reef, Indo-Pacific and the Caribbean (Berkelmans 2002; Osman et al. 2018). However, the surface temperature of the Red Sea has increased rapidly in the last 20 years, exceeding the global rate and making it one of the fastest warming areas in the world (Raitsos et al. 2011; Chaidez et al. 2017). These changes have generated serious impacts on coral communities, leading to an increase in coral bleaching cases recently recorded throughout the Red Sea (Kotb et al. 2008; Cantin et al. 2010; Riegl et al. 2012; Nir et al. 2014; Roik et al. 2015; Osman et al. 2018). In particular, in 2010 and 2015, two coral bleaching events hit the same area of the Saudi Arabian central Red Sea, near Thuwal (Furby et al. 2013; Monroe et al. 2018). In both events, the bleaching increased with proximity to shore and in shallow areas, where the majority of corals were affected. Interestingly, a different bleaching susceptibility among coral taxa and growth form was observed (Furby et al. 2013; Monroe et al. 2018).

Several factors, which operate in combination, can generate variable bleaching patterns over space and time and can determine the different sensitivity of coral taxa to thermal stresses. The extent of bleaching can depend on the duration and frequency of thermal anomalies and on site-specific environmental conditions (McClanahan and Maina 2003; Guest et al. 2012; Howells et al. 2013; Pratchett et al. 2013). However, several studies pointed out that intrinsic factors of the corals play a fundamental role in determining thermotolerance, including morphological and physiological characteristics (Loya et al. 2001; Van Woesik et al. 2011; Kenkel et al. 2013; Barshis et al. 2013; Wooldridge 2014; Dixon et al. 2015; Gardner et al. 2017). In this context, although it is known that several processes in the coral host and in the Symbiodiniaceae are affected by the onset of coral bleaching (Downs et al. 2002; Weis 2008; Baird et al. 2009; Vidal-Dupiol et al. 2009; Lesser 2011; Tolleter et al. 2013; Pinzón et al. 2015; Pogoreutz et al. 2017; Oakley and Davy 2018), a more thorough elucidation of the cellular mechanisms underlying bleaching can represent an area of critical importance in order to design mitigation strategies.

Changes in the expression of Heat shock proteins (Hsps) are emerging as ubiquitous and putative markers of temperature-induced cell stress in corals (Downs et al. 2000; Brown et al. 2002; Chow et al. 2012; Olsen et al. 2013; Seveso et al. 2016; Louis et al. 2017). As molecular chaperones, Hsps sustain protein homeostasis by facilitating proper protein folding and translocation, deterring and/or reducing the aggregation of other proteins damaged by heat or other environmental stress and assisting in refolding or degradation of stress-damaged proteins (Bozaykut et al. 2014; Balchin et al. 2016). Hsps are classified by molecular weight in major chaperone families (HSP40, HSP60, HSP70, HSP90, HSP100, and the small HSPs), which include several members with specific intracellular localization and function (Pockley 2003). In general, corals subjected to heat shock and other bleaching-causing stresses increase the level of Hsps as a defense mechanism (Barshis et al. 2010; DeSalvo et al. 2010; Kenkel et al. 2011, 2014; Rosic et al. 2011; Seveso et al. 2014), while in the absence of stress, a basal low level of Hsps is still required for normal protein folding, maintenance of signal transduction and/or normal development (Chen et al. 2018). However, the expression pattern of Hsps and the amplitude of their modulation may show species-specific characteristics, which may reflect different mechanisms/abilities of stress response (Robbart et al. 2004; Chow et al. 2009; Fitt et al. 2009; Seveso et al. 2014, 2018), as well as they may depend on the organism's natural environment and niche history (Feder and Hofmann 1999). Nevertheless, with regard to the corals of the Red Sea, this information is still scarce (Choresh et al. 2004; Chow et al. 2012; Maor-Landaw and Levy 2016) and to the best of our knowledge, no study has analyzed the expression of the Hsps during a bleaching event in the field.

In our study, the expression of the host Hsp70, Hsp60 and heme oxygenase-1 (also known and herein referred to as Hsp32) was analyzed in four different coral species (*Goniopora lobata*, *Porites lobata*, *Seriatopora hystrix* and *Stylophora pistillata*), belonging to two coral families and showing two different growth forms, subjected to the 2015 coral bleaching event recorded in the central Red Sea (Thuwal, Saudi Arabia). For each species, the level of Hsps was compared in both healthy and bleached colonies, in order to investigate the intra-specific modulation of these biomarkers in response to heat stress-induced bleaching. Furthermore, the existence of expression patterns specific for each Hsp, coral species or growth form and site was also assessed. The results obtained from this comparative analysis could provide further insights into the molecular and cellular basis of coral bleaching of scleractinians of the central Red Sea, which is considered a key region for understanding future environmental conditions on reefs worldwide (Cantin et al. 2010).

Materials and methods

Sampling design

Samplings were performed in reefs of the central Red Sea near Thuwal, in Saudi Arabia, on November/December 2015, about 3 months after the bleaching event occurred in the central Saudi Arabian Red Sea (Monroe et al. 2018). Sites located at three different distances from the shore were surveyed by SCUBA diving: offshore sites placed ~ 15–20 km far from the coast, midshore sites (~ 8–9 km) and inshore sites (~ 2–4 km), (Fig. 1). In particular, three offshore sites (named OF1, OF2, OF3), four midshore sites (MI1, MI2, MI3, MI4) and four inshore sites (IN1, IN2, IN3, IN4) were explored.

Corals were classified as healthy (H) and bleached (B) using the color reference card (coral watch color card, Siebeck et al. 2006). This method has been used successfully for a wide range of coral species to monitor the organism condition over time or at a single time point (Siebeck et al. 2009; Montano et al. 2010; Cooper and Fabricius 2012; Coelho et al. 2017). In particular, in order to clearly distinguish two categories of coral pigmentation and, therefore, two opposite coral health conditions, colonies showing over their entire colony surface a normal dark coloration corresponding to color categories 5–6 of the reference card were defined as healthy (H). On the contrary, those showing tissue and polyps strikingly lightened (color categories 1–2 of the reference chart) were classified as bleached, as previously reported (Anthony and Kerswell 2007). It is important to emphasize that for branching corals, the color measurements were taken at a distance of about 3 cm from the tip, in order to avoid the variations in color that are frequently found in and around the axial polyp (Siebeck et al. 2006).

Hard coral species (Scleractinia) were selected based on growth morphologies and having a least six bleached colonies in the investigated area. Four species were sampled: *P. lobata* and *G. lobata* (Poritidae) showing a massive form and *S. pistillata* and *S. hystrix* (Pocilloporidae) having a branching morphology. For each species, from six to eight healthy colonies (totally 30 corals) were collected in the whole studied area. Of these colonies, nine were collected from inshore sites, ten from midshore sites and 11 from offshore sites. Six to eight bleached colonies per species (totally 30 corals) were sampled only from midshore and inshore sites, which represent the only sites where bleached colonies have been found. At each reef, colonies of the same species were chosen at least 5 m apart to avoid clone samples. Coral fragments were cut from the branching colonies using a plier, while massive colonies were excised using a hollow-point stainless steel spike (8 mm diameter) by applying constant rotational pressure to minimize the amount of sampling stress and limit excessive damage to the colonies (Bromage et al. 2009). Afterward, samples were immediately frozen in liquid nitrogen on the boat, brought to the Reef Ecology Laboratory (King Abdullah University of Science and Technology, Saudi Arabia), and maintained at − 80 °C until protein extraction.

To minimize any possible effect of abiotic variables on the Hsp expression and modulation, all the selected colonies were located at a similar shallow depth (around 5–8 m) and were sampled at the same morning time in non-cloudy days, in order to be subjected to the same seawater temperature (approximately 27 °C ± 0.5 °C), and light intensity. Moreover, only live colonies that showed no

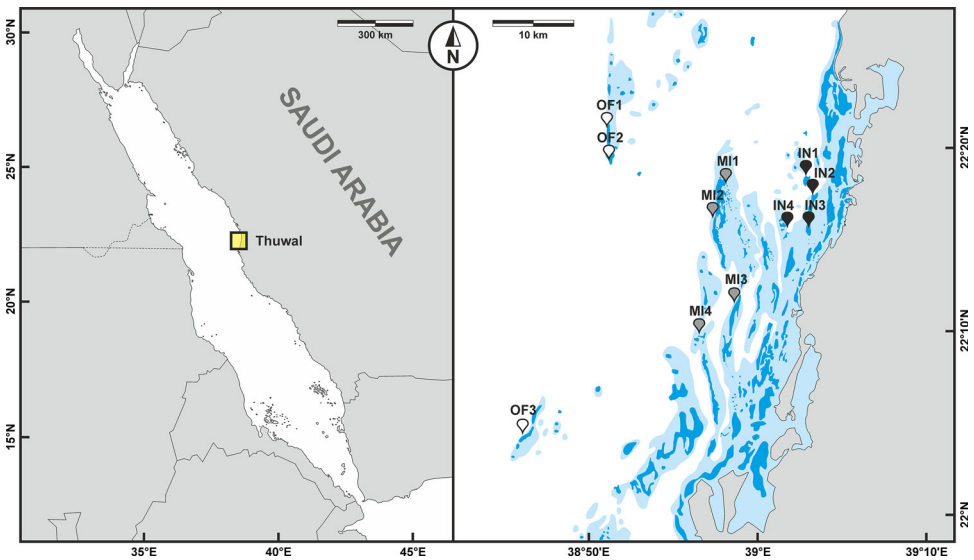


Fig. 1 Map of the 11 reefs located off the coast of Thuwal, Saudi Arabia, in the central Red Sea in which the samplings were performed. Abbreviations are as follows: OF (offshore), MI (midshore) and IN (inshore)

physical damage due to mechanical breakage or predation, partial mortality, disease and algal overgrowth were chosen.

Analysis of the Hsp expression

In the laboratory, a pre-chilled mortar and pestle were used to pulverize the frozen coral samples (~ 1 g each) that were subsequently transferred into tubes and homogenized in 400 µl of SDS-buffer (0.0625 M Tris–HCl, pH 6.8, 5% SDS, 5% 2-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and complete EDTA-free cocktail of protease inhibitors (Roche Diagnostic) and processed according to the method previously described (Seveso et al. 2013, 2014). This method allows to remove, in addition to any calcium carbonate debris, any Symbiodiniaceae contamination, thus obtaining extracts containing only polyp proteins (Seveso et al. 2013). Polyp protein extracts were stored at − 80 °C until subsequent analyses. Aliquots of the extracts were used for protein concentration determinations using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Protein extracts were separated by SDS-PAGE on 8% polyacrylamide gels (Vai et al. 1986) using a Mini-Protean Tetra Cell (Bio-Rad Laboratories). The same amount of proteins (30 to 50 µg) was loaded on each lane of the gel, and duplicate gels were run in parallel. After electrophoresis, one gel was stained with Coomassie Brilliant Blue to visualize total proteins and the other electroblotted onto nitrocellulose membrane (Amersham Protran 0.45 mm) for Western analysis as described (Seveso et al. 2012). Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters.

Filters were processed as described (Seveso et al. 2017). Immunodetection was performed with anti-Hsp70 monoclonal antibody (IgG2a mouse clone BB70, SPA-822, Enzo Life Sciences), anti-Hsp60 monoclonal antibody (IgGI1 mouse clone LK-2, SPA-807, Enzo Life Sciences), anti-Hsp32 monoclonal antibody (IgG2b mouse clone HO-1-2, OSA-111, Enzo Life Sciences) and anti-β-Actin monoclonal antibody (IgG1 k mouse clone C4, MAB1501, Millipore). All the primary antibodies were diluted 1:1000 in TBS-0.1% Tween 20 and 5% skimmed milk with the exception of the β-actin (1:8000), and the filters were incubated overnight. After washing in TBS-0.1% Tween 20 (15 min, 3 times), membranes were incubated with the secondary antibody anti-mouse IgG polyclonal conjugated with horseradish peroxidase (ADI-SAB-100, Enzo Life Sciences) diluted 1:1000 for Hsp70, 1:10,000 for Hsp60, 1:7500 for Hsp32 and 1:15,000 for β-actin. All dilutions of secondary antibodies were in TBS-0.3% Tween 20 and 5% skimmed milk. Blots were developed using Pierce ECL Western Blotting Substrate followed by exposition of filters to Amersham Hyperfilm ECL.

Densitometric analyses

Densitometric analysis was performed by scanning films on a Bio-Rad GS-800 calibrated imaging densitometer and quantifying the pixel density of the scanned bands with the ImageJ free software (http://rsb.info.nih.gov/ij/) of the NIH Image software package (National Institutes of Health, Bethesda, Md.). For each blot, the scanned intensity of the bands of each biomarker was normalized against the intensity of the β-actin ones, which was used as internal loading control since in all our experiments the β-actin level did not display significant changes (ESM Fig. S1). The densitometric data were expressed as relative levels (arbitrary units). Due to the different availability of the colonies of the diverse coral species in the study sites, different numbers of samples were analyzed for each Hsp and species.

Statistical analyses

Data normality was verified using Shapiro–Wilk test and where assumptions were violated, the data were corrected by transformations. In both healthy and bleached colonies, differences in the expression of each biomarker were analyzed with a two-way ANOVA, using the species (*P. lobata*, *G. lobata*, *S. hystrix* and *S. pistillata*) and the sampling sites (offshore, midshore and inshore sites for healthy colonies; midshore and inshore sites for bleached colonies) as factors, and the normalized biomarker values obtained from the different groups of samples as the dependent variables. Following two-way ANOVA, Tukey's HSD post hoc tests for multiple pair-wise comparisons of means were performed to assess significant differences in the biomarker levels in the colonies belonging to the different species and located in the different sites.

For each biomarker, differences in its expression between healthy and bleached colonies belonging to each species were expressed as Log2 fold change (Log2FC) and were analyzed by one-way ANOVA followed by Tukey's HSD post hoc tests in order to assess significant differences between the coral species. Statistical significance was defined as $P < 0.05$. Analyses were performed using SPSS ver. 24 (IBM, New York). A multivariate analysis was performed using the statistical package PRIMER-E v.7 (Clarke and Gorley 2015) in order to analyze together the level of all biomarkers in the different situations. In particular, data were square root transformed, and a cluster analysis was carried out using Bray–Curtis similarity matrices and group-averaged hierarchical clustering.

Results

Hsp expression in healthy colonies

Reefs in the central Saudi Arabian Red Sea were not spared by the impacts of the 2015/2016 global coral bleaching event (Roik et al. 2015; Osman et al. 2018; Monroe et al. 2018), and we took advantage of this episode to explore the expression of the host Hsp70, Hsp60 and Hsp32 in *G. lobata*, *P. lobata*, *S. hystrix* and *S. pistillata*. Western analyses were carried out in healthy and bleached colonies living in different sites from the shore (Fig. 1). Each monoclonal antibody (anti-Hsp70, Hsp60 and Hsp32) recognized a single specific band in all the samples of all the species considered, whose molecular weight corresponds to that expected based on the amino acid sequences (ESM Fig. S2).

Regarding the healthy colonies, the average level of each biomarker did not change in sites located at the three different distances from the shore (inshore, midshore and offshore), both considering the species together (Fig. 2a and Table 1), as well as for each of the four investigated coral species (Table 1). This indicates that the site where the colonies live does not affect the level of the biomarkers. The two-way ANOVA also revealed that the level of all the biomarkers was significantly different among the healthy colonies belonging to the different species, suggesting that their expression in healthy corals is species-specific (Fig. 3 and Table 1). In particular, the Western analysis of the healthy colonies of *G. lobata* displayed a very weak signal intensity for the three Hsps (ESM Fig. S1) and the densitometric analysis indicated that their level was the lowest recorded among the species (Fig. 3). A very low basal level of Hsp70 and Hsp60 and a negligible expression of Hsp32 was already reported in healthy colonies of corals belonging to the same genus, such as *G. columna*, living in their natural reef habitat in Maldives (Seveso et al. 2017). *Porites lobata* also showed a low expression of all biomarkers, even if significantly higher than that of *G. lobata* (Fig. 3). Finally, the healthy colonies of the two branching species belonging to Pocilloporidae family, *S. hystrix* and *S. pistillata*, displayed a similar level of Hsp70 and Hsp60, which was the highest recorded among the species (Fig. 3). In addition, the expression of Hsp32 in their healthy colonies was also similar (Fig. 3).

Hsp expression in response to bleaching

Even regarding bleached colonies, no difference was observed in the average level of each biomarker in colonies sampled at different distances from the shore (see "Materials and methods" section), both considering the species together (Fig. 2b and Table 1) as well as for each coral species (Table 1). In addition, the levels of each biomarker were different among the different coral species (Fig. 4), pointing to a species-specific expression of the Hsps also in response to bleaching, as suggested in a previous study (Seveso et al. 2014). In particular, the bleached colonies of the two massive species belonging to Poritidae family, *G. lobata* and *P. lobata*, showed strong and similar Hsp70 and Hsp60 signals (ESM Fig. S1), which correspond to the higher level of Hsp70 and Hsp60 recorded among the species, while the level of these biomarkers was generally inferior in *S. pistillata* and significantly lower in *S. hystrix* (Fig. 4). Differently, the expression pattern of Hsp32 was roughly similar to that observed in healthy colonies, although less significant differences in its level were found between the species (ESM Fig. S1 and Fig. 4). Indeed, the densitometric analysis revealed that the only significant difference was found between *G. lobata* and *S. pistillata*, whose Hsp32 levels were the lowest and the highest, respectively, among the species (Fig. 4).

Finally, the comparison of the Hsp levels in healthy and bleached colonies showed that all the coral species had a significant modulation of their expression in response to bleaching (Table 2). An up-regulation of all the biomarkers

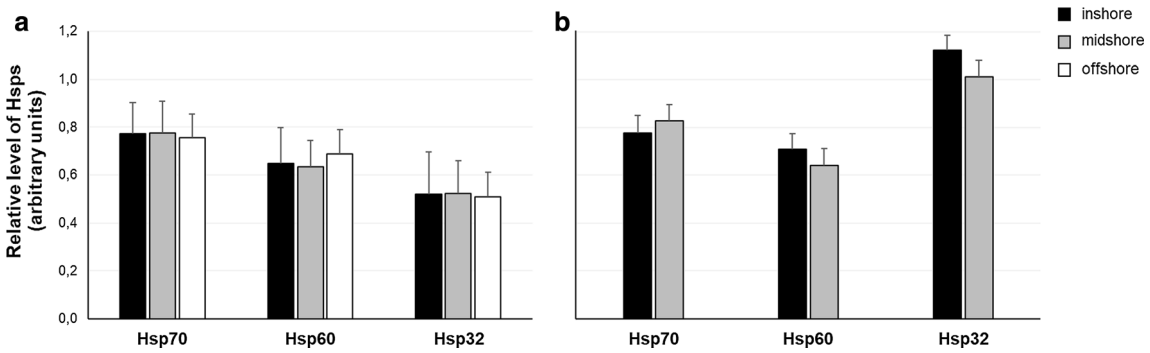


Fig. 2 Levels of Hsp70, Hsp60 and Hsp32 in healthy (**a**) and bleached (**b**) colonies detected in reefs located at different distance from the shore. The values were determined by densitometric analysis as described under "Materials and methods" section. Data are expressed as arbitrary units and as mean ± SEM considering all the coral species together

Table 1 Summary of the two-way analysis of variance (ANOVA) for all the normalized intensity values of Hsp70, Hsp60 and Hsp32 obtained from the healthy and bleached colonies of each coral species

Source of variation	Healthy colonies				Bleached colonies			
	Df	MS	*F*	*P*	Df	MS	*F*	*P*
Hsp70								
Site	2	0.21	0.543	ns	1	0.045	1.312	ns
Species	3	1.524	38.530	***P* < 0.001**	3	0.346	10.097	***P* < 0.001**
Site × Species	6	0.010	0.246	ns	3	0.030	0.877	ns
Residual	16	0.040			20	0.034		
Hsp60								
Site	2	0.008	0.224	ns	1	0.040	2.167	ns
Species	3	0.969	26.174	***P* < 0.001**	3	0.440	24.000	***P* < 0.001**
Site × Species	6	0.021	0.569	ns	3	0.006	0.304	ns
Residual	16	0.037			20	0.018		
Hsp32								
Site	2	0.000	0.20	ns	1	0.059	2.735	ns
Species	3	0.484	27.548	***P* < 0.001**	3	0.087	4.041	***P* < 0.05**
Site × Species	6	0.001	0.044	ns	3	0.011	0.504	ns
Residual	12	0.018			4	0.022		

The different sites depending on their distance from the shore (site) and the membership of the different coral species (species) were used as fixed factors. Significant values are in bold. Values not significant ($p \geq 0.05$) are indicated with ns

was observed in both the massive species (Fig. 5), although *G. lobata* displayed a strong induction of Hsp expression (more than two Log2FC for all the biomarkers) and *P. lobata* showed a lower up-regulation. Unlike the massive species, the branching Pocilloporidae species showed a significant down-regulation of Hsp70 and Hsp60 (Fig. 5). The magnitude of the down-regulation of Hsp70 was not significantly different in *S. hystrix* and *S. pistillata* (− 1.1 and − 0.6 Log2FC, respectively), while that of Hsp60 was significantly higher (− 1.8 and − 0.5 Log2FC respectively). Hsp32 displayed an up-regulation also in the bleached colonies of both the branching species, with a fold change similar to each other and to that of *P. lobata* (Fig. 5).

Discussion

In this study, we analyzed the expression of Hsp70, Hsp60 and Hsp32 in colonies of *G. lobata*, *P. lobata*, *S. hystrix* and *S. pistillata*. These colonies were on reefs located at different distance from the shore in an area of the Red Sea off Thuwal (Saudi Arabia) that had been hit by the coral bleaching event of 2015 (Monroe et al. 2018). Indeed, due to devastating impacts worldwide of coral bleaching, efforts have increased to understand the cellular processes involved in the response to the environmental stresses that cause bleaching. This is important especially considering that corals cannot migrate to new environmental optima and that the earliest steps of an organism's stress response occur at cellular level (Kültz 2005; Kenkel et al. 2014).

We found that the distance from the shore does not affect the level of each Hsp in both the healthy and bleached colonies of each coral species. Interestingly, the spatial homogeneity of the level of these biomarkers does not reflect the spatial pattern of the bleaching event, the prevalence of which had varied with the distance from the coast. Indeed, among the eleven sites surveyed in 2015 during previous studies in the same area, inshore reefs showed the highest levels of bleaching (from 54% to up to 67% of live coral cover exhibited bleaching), offshore reefs only experienced an average of ∼ 2% of bleaching, and midshore reefs displayed intermediate levels with approximately the 20% of hard coral cover bleached (Monroe et al. 2018). The same trend was also observed during the 2010 bleaching event (Furby et al. 2013). The distance from the shore had the most significant impact on bleaching susceptibility in the area, probably due to different sea surface temperatures, degree heating weeks and water circulation that characterized the three different reef systems (Furby et al. 2013; Monroe et al. 2018). Nevertheless, our findings show as in all the healthy colonies of each species, the level of each biomarker is not affected by the site where the colonies live and the intensity of the environmental factors that characterize it, thus excluding a direct involvement of Hsps in determining the spatial pattern of bleaching susceptibility observed. Indeed, as suggested by previous studies, other factors intrinsic to the

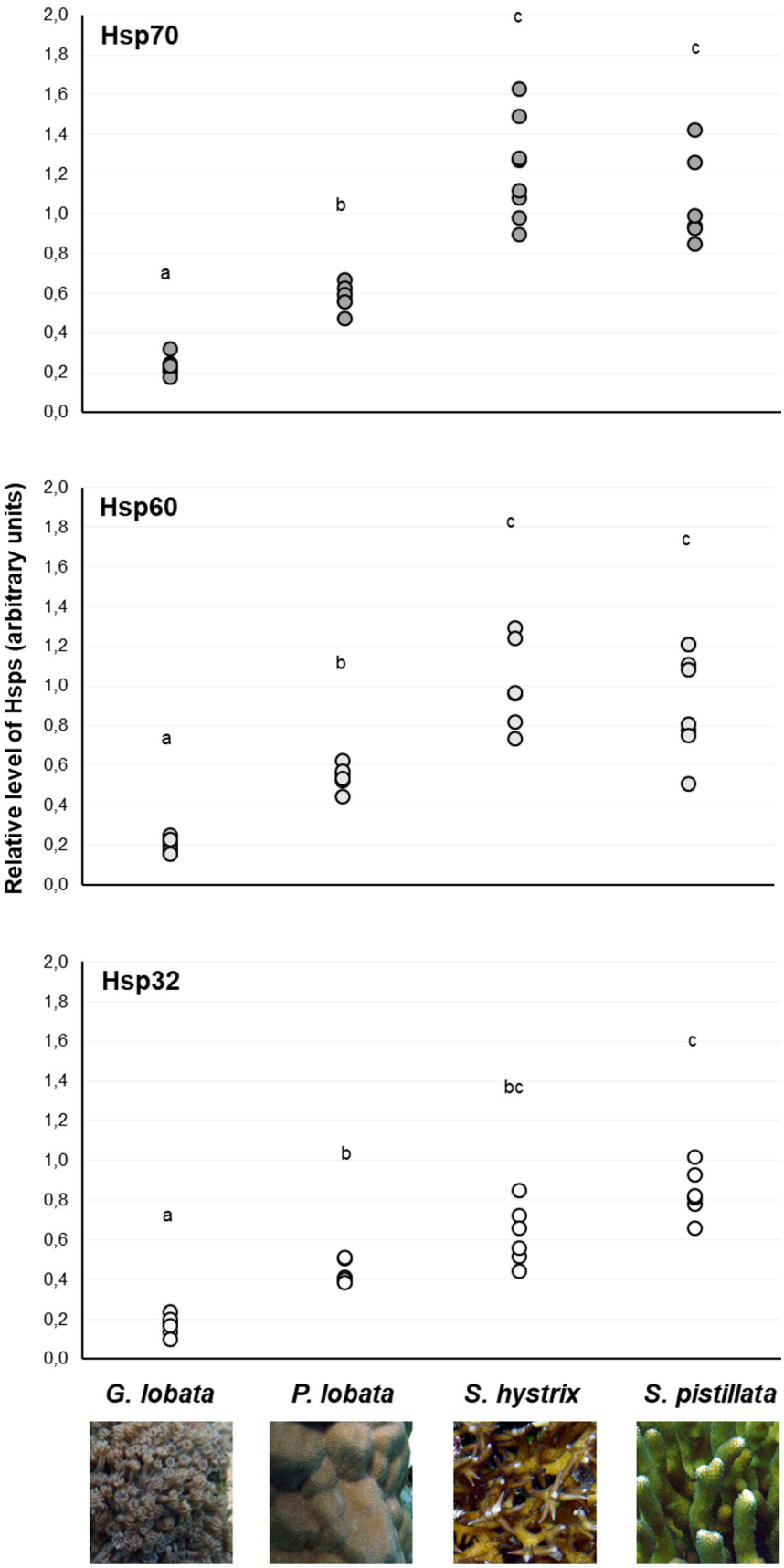


Fig. 3 Graph showing the general trend of the Hsp70, Hsp60 and Hsp32 levels measured in healthy colonies of *G. lobata*, *P. lobata*, *S. hystrix* and *S. pistillata*. For each species, the values of Hsps determined by densitometric analysis (see "Materials and methods" section) in each sample are displayed. Data are expressed as arbitrary units (two-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons). For each biomarker, letters denote Tukey's significant differences in the biomarker levels between the different species ($P < 0.05$). Same letter indicates no significant difference ($P \geq 0.05$). Number of colonies analyzed for each species: *G. lobata* ($n = 8$ for Hsp70 and 60, $n = 6$ for Hsp32), *P. lobata* ($n = 6$ for Hsp70 and 60, $n = 5$ for Hsp32), *S. hystrix* ($n = 8$ for Hsp70, $n = 6$ for Hsp60, $n = 6$ for Hsp32) and *S. pistillata* ($n = 6$ for Hsp70, $n = 8$ for Hsp60, $n = 5$ for Hsp32)

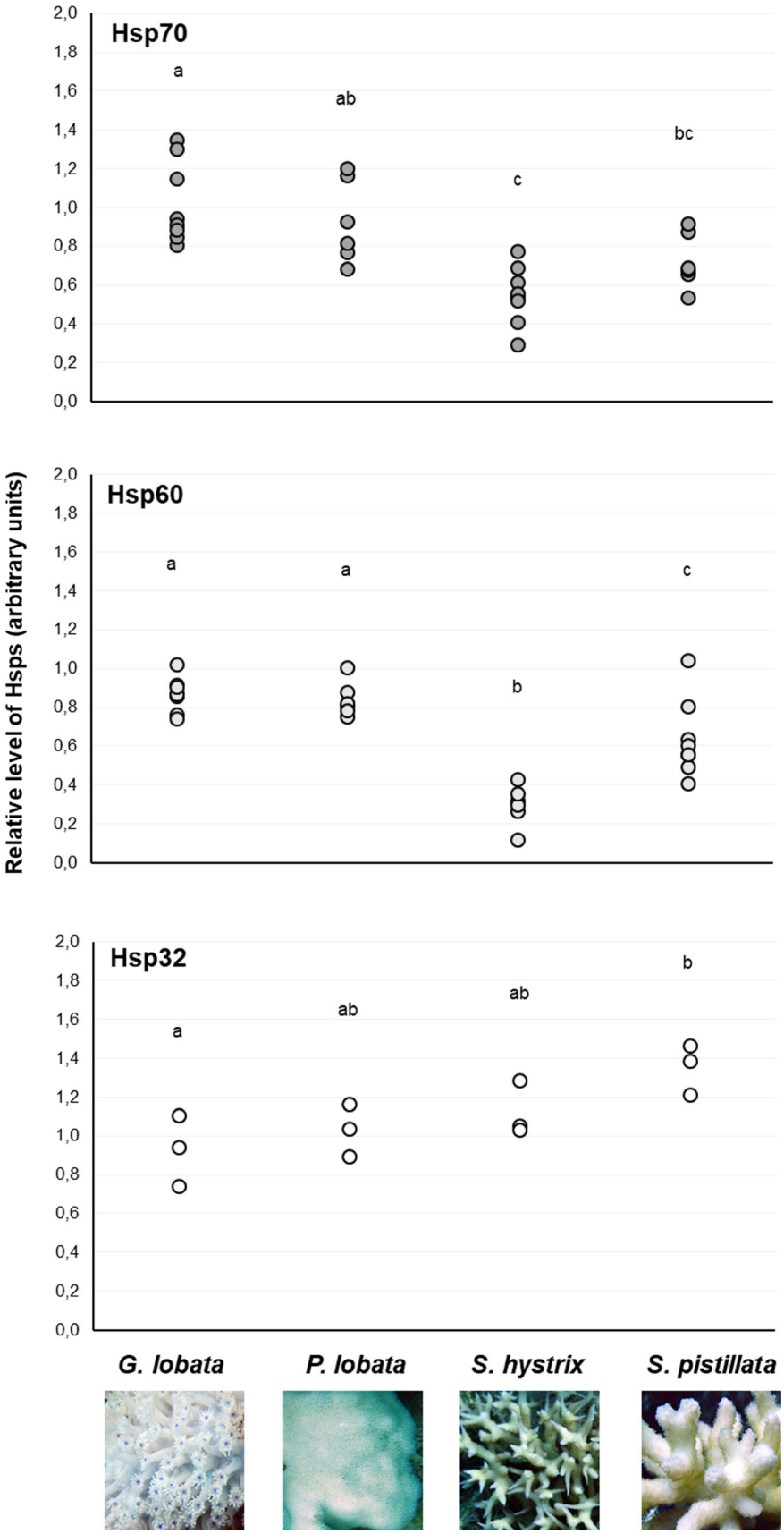


Fig. 4 Graph showing the general trend of the Hsp70, Hsp60 and Hsp32 levels measured in bleached colonies of *G. lobata*, *P. lobata*, *S. hystrix* and *S. pistillata*. For each species, the values of Hsps determined by densitometric analysis (see "Materials and methods" section) in each sample are displayed. Data are expressed as arbitrary units (two-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons). For each biomarker, letters denote Tukey's significant differences in the biomarker levels between the different species ($P < 0.05$). Same letter indicates no significant difference ($P \geq 0.05$). Number of colonies analyzed for each species as in Fig. 3, with the exception of those analyzed for Hsp32 ($n = 3$ per species)

Table 2 Summary of the one-way analysis of variance (ANOVA) for all the normalized intensity values of Hsp70, Hsp60 and Hsp32 in healthy versus bleached colonies of each coral species

	Healthy versus bleached											
	G. lobata			*P. lobata*			*S. hystrix*			*S. pistillata*		
	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*	MS	*F*	*P*
Hsp70	2.497	106.547	**0.001**	0.357	14.127	**0.004**	1.801	41.776	**0.000**	0.347	9.667	**0.011**
Hsp60	1.757	375.743	**0.000**	0.266	45.129	**0.000**	1.492	49.303	**0.000**	0.351	6.627	**0.022**
Hsp32	1.157	102.209	**0.001**	0.712	92.029	**0.000**	0.494	23.006	**0.002**	0.532	33.663	**0.001**

Significant values are in bold

coral could have contributed to produce the spatial bleaching heterogeneity, such as different stress-tolerant genetic variants of Symbiodiniaceae or bacterial communities associated with coral living in the different sites (Fisher et al. 2012; Hume et al. 2015, 2016; Ziegler et al. 2017) and a different gene expression plasticity of both coral hosts and their intracellular algae (Kenkel and Matz 2016).

Our results also suggest that the expression of all the Hsps in the healthy colonies is species-specific.

Furthermore, if we correlate our data of the Hsp level in the healthy colonies with the bleaching susceptibility recorded for the same coral genera in the Thuwal area during the same coral bleaching event (Monroe et al. 2018), a significant inverse relationship is found. As shown in Fig. 6, the genus *Goniopora* displayed the highest bleaching rate among all the coral genera, with almost the 80% of the colonies affected (Monroe et al. 2018), and the lowest level of Hsps in healthy colonies. The genus *Porites* showed a higher average percent of bleached corals than *Stylophora* ($\sim$ 30% and $\sim$ 20%, respectively, Monroe et al. 2018), and the level of Hsps in its healthy colonies was lower (Fig. 6). This can suggest that a low basal level of cellular defenses can make the coral more sensitive to temperature changes and more prone to bleaching. In line with this, previous studies suggested that healthy corals exhibiting in their natural environment higher Hsp levels may display enhanced tolerance toward environmental stressors (Poli et al. 2017), and as, under simulated bleaching stress, tolerant corals had higher constitutive level of Hsp genes (frontloading) under control conditions compared with sensitive corals (Barshis et al. 2013). Taken together, these findings point out the importance of investigating the level of Hsps in healthy corals in order to obtain information on the susceptibility of the species to stress.

As for healthy colonies, a species-specific expression of the Hsps was detected even in response to bleaching, as also observed in a previous study (Seveso et al. 2014). In detail, the typology and the amplitude of the Hsp modulation involved in the bleaching response were different among the species. Indeed, both the massive Poritidae species showed a significant up-regulation in the expression of the three Hsps, which in *G. lobata* was greater than in *P. lobata*, while both the branching Pocilloporidae species displayed a significant down-regulation of the expression of Hsp70 and Hsp60 in concert with an induction of the Hsp32. This indicates that bleaching triggered significant changes to normal cellular protein homeostasis in all the coral species, confirming that components of the cellular defense machinery, such as those investigated here, could play a role in the attempt of corals to adapt and counteract the environmental changes. In this context, the up-regulation of Hsps represents a common response of corals during heat stress-induced bleaching (Downs et al. 2002; DeSalvo et al. 2008, 2010; Seneca et al. 2010; Seveso et al. 2014, 2016; Ricaurte et al. 2016). Since many of the morphological and phenotypic effects of heat stress are associated with aggregation of proteins, protein misfolding and disassembly of multiprotein complexes in general, the marked induction of Hsps may reflect the increased requirement to protect preexisting proteins from denaturation and aggregation in order to restore protein homeostasis (Richter et al. 2010). On the contrary, the down-regulation of Hsp70 and Hsp60 may reflect a strong repression of the metabolic activity due to severe and intolerable stress that was leading the corals to death. In line with this, a similar Hsp modulation was also observed in branching corals subjected to extreme and prolonged thermal and osmotic perturbations (Seveso et al. 2013, 2014, 2016).

Finally, considering together the level of each biomarker in healthy and bleached corals, the Hsp expression profiles of the investigated coral species can be grouped in three main clusters (Fig. 7). *Goniopora lobata* clustered independently due to the lowest level of Hsps in healthy colonies and the strongest up-regulation in bleached colonies, reflecting the highest susceptibility to bleaching of

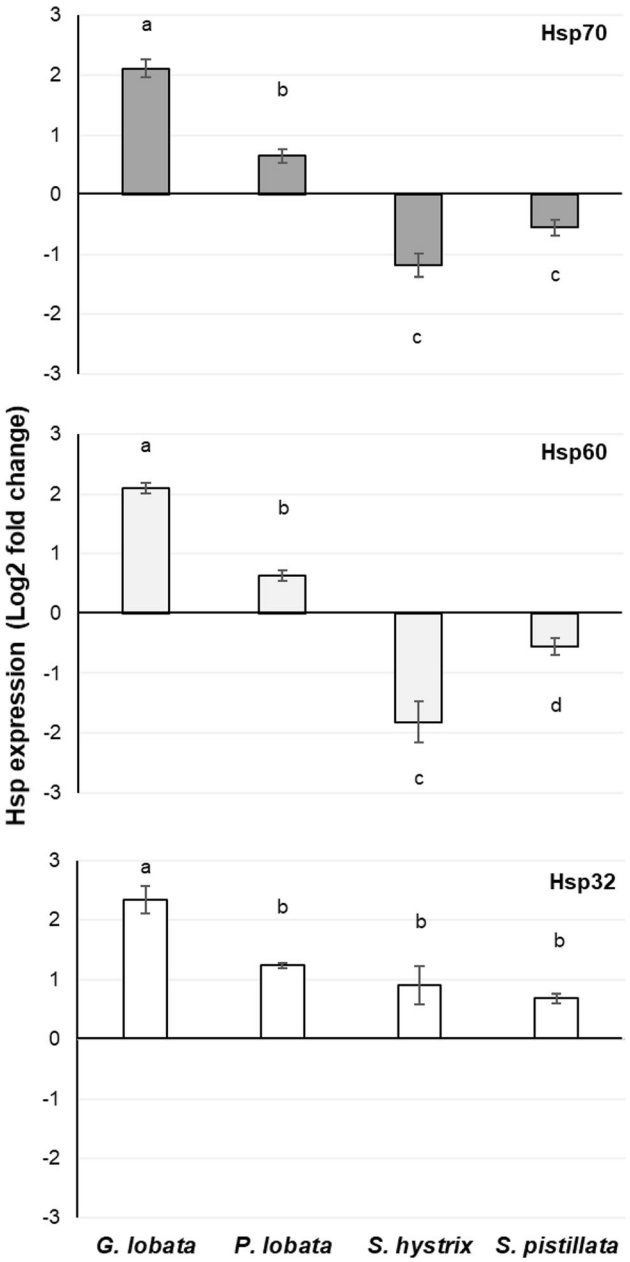
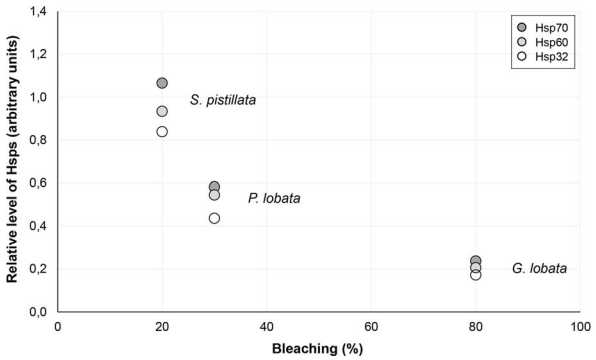


Fig. 5 Change in the expression of Hsp70, Hsp60 and Hsp32 in *G. lobata*, *P. lobata*, *S. hystrix* and *S. pistillata* in response to bleaching. Fold changes were calculated with respect to levels of Hsps detected in healthy colonies and were Log2-transformed. Data are expressed as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons). For each biomarker, letters denote Tukey's significant differences in the biomarker levels between the different species ($P < 0.05$). Same letter indicates no significant difference ($P \geq 0.05$)

this genus among all those analyzed (Monroe et al. 2018). The two branching Pocilloporidae species, *S. hystrix* and *S. pistillata*, are grouped together showing a similar pattern of expression of Hsps. This suggests that these corals that share comparable physical properties, such as growth form, tissue thickness and growth rate, similarly respond to environmental changes (Marshall and Baird 2000; Loya et al. 2001; Stimson et al. 2002). Differently, *P. lobata*, although it shares growth form and family with *G. lobata*, showed a protein expression profile with characteristics intermediate to those of *G. lobata* and the branching species (Fig. 7).

Moreover, in each species, both the Hsp70 and the Hsp60 showed the same pattern of expression in response to bleaching, responding with a similar intensity and amplitude to thermal stress. The cytoplasmic Hsp70 is principally engaged in the assembly of newly synthesized proteins and the refolding of misfolded and aggregated proteins, contributing to the cytoskeleton stabilization and protein transfer to cellular compartments or the proteolytic machinery (Mayer 2013; Balchin et al. 2016). The mitochondrial chaperonin Hsp60 has a fundamental role in the synthesis and transportation of mitochondrial proteins and in the folding of newly imported and stress-denatured proteins, as well as being important for replication and transmission of mitochondrial DNA (Martinus et al. 1995; Arya et al. 2007). A parallel modulation and response of these Hsps have already been observed in corals subjected to different stressors, as well in bleached colonies (Downs et al. 2000, 2002, 2012; Seveso et al. 2017, 2018). This confirms that despite the different cellular localization, these molecular chaperones may work in tandem to facilitate the folding process, also due to the essential role of Hsp70 in the maturation of newly imported proteins into the mitochondria (Zhou et al. 1996; Papp et al. 2003). In contrast, Hsp32 was always up-regulated, even when the others Hsps were down-regulated. Hsp32 has an enzymatic activity, since it catalyzes the oxidative degradation of heme into biliverdin, free iron and carbon monoxide, performing a cytoprotective activity in response to oxidative stress. Therefore, it represents an important antioxidant defense (Gozzelino et al. 2010). It is widely known that thermal bleaching begins with photoinhibition and production of cytotoxic reactive oxygen species, which lead to severe oxidative stress in both polyp and symbiotic cells (Iglesias-Prieto et al. 1992; Lesser 1997; Smith et al. 2005; Baird et al. 2009). In corals, oxidative stress causes metabolic dysfunctions (Jones et al. 1998), due to lipid peroxidation, protein oxidation, inhibition of enzymes and damage to nucleic acids (Kültz 2005; DeSalvo et al. 2008; Voolstra et al. 2009), ultimately leading to coral death. The induction of Hsp32 in all the colonies of all the species indicates that this antioxidant response represents a common defense mechanism against heat stress-induced bleaching. In addition to thermal-type stress, in corals, the up-regulation of Hsp32 has been already observed in relation to osmotic stress, pollutants, pesticides and coral diseases (Rougée et al. 2006; Downs and Downs 2007; Downs et al. 2006, 2009; Seveso et al. 2017).



		Bleaching
Hsp70	Spearman coefficient	-0,595
	Sign. (two-tailed)	0,006
	N	20
Hsp60	Spearman coefficient	-0,580
	Sign. (two-tailed)	0,005
	N	22
Hsp32	Spearman coefficient	-0,047
	Sign. (two-tailed)	0,045
	N	18

Fig. 6 Relationship between the average level of Hsp70, Hsp60 and Hsp32 found in healthy colonies of *S. pistillata*, *P. lobata* and *G. lobata* and the approximate bleaching % of the genera *Stylophora*, *Porites* and *Goniopora* observed by Monroe et al. (2018) in the Thuwal area during the bleaching event of the 2015. Data about the Hsp levels are expressed as arbitrary units and as means (see Fig. 3 for the number of colonies analyzed for each species). Data about bleaching are expressed as average percent of bleached coral cover within each taxon (taken from Fig. 4b of Monroe et al. 2018). In the table, Spearman's rank order correlation values for Hsp are shown. The correlation between the level of Hsp70, Hsp60, Hsp32 and the bleaching % was assessed. Statistical significance was defined as $P < 0.05$

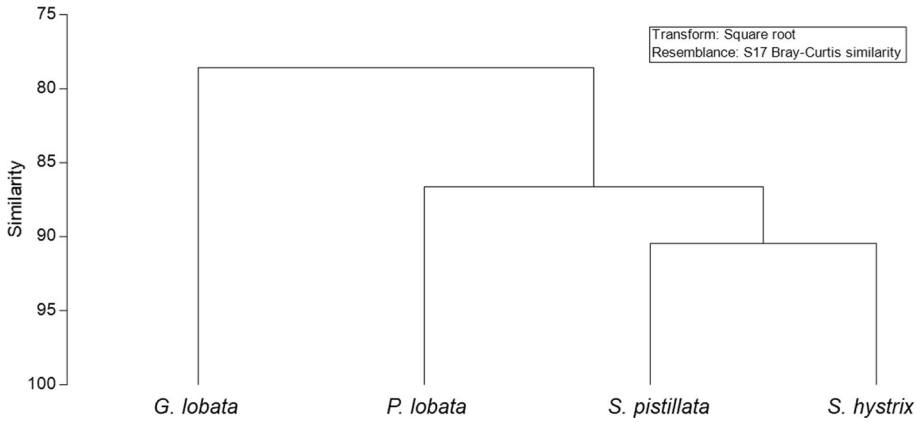


Fig. 7 Cluster analysis dendrogram of the coral species analyzed based on the level of Hsp70, Hsp60 and Hsp32 measured in healthy and bleached colonies

In conclusion, it is worth emphasizing that to the best of our knowledge, this is the first study on Hsps conducted on multiple species during a bleaching event in the Red Sea. Since the modulation of Hsps has shown patterns so specific to each taxa, the analysis of the cellular parameters herein investigated can be used as a valuable diagnostic tool to detect, define and distinguish a stressed coral from a healthy one and thus to understand if changes on a larger biological and ecological scale are in progress. Moreover, this approach could provide useful information on the role of Hsps in regulating the susceptibility to thermal stresses of the various coral taxa of the Red Sea and on the onset of future bleaching events, considering that coral reefs of the Red Sea will be increasingly threatened by global climate change.

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