The cellular stress response of the scleractinian coral *Goniopora columna* during the progression of the black band disease

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Abstract Black band disease (BBD) is a widespread coral pathology caused by a microbial consortium dominated by cyanobacteria, which is significantly contributing to the loss of coral cover and diversity worldwide. Since the effects of the BBD pathogens on the physiology and cellular stress response of coral polyps appear almost unknown, the expression of some molecular biomarkers, such as Hsp70, Hsp60, HO-1, and MnSOD, was analyzed in the apparently healthy tissues of *Goniopora columna* located at different distances from the infection and during two disease development stages. All the biomarkers displayed different levels of expression between healthy and diseased colonies. In the healthy corals, low basal levels were found stable over time in different parts of the same colony. On the contrary, in the diseased colonies, a strong up-regulation of all the biomarkers was observed in all the tissues surrounding the infection, which suffered an oxidative stress probably generated by the alternation, at the progression front of the disease, of conditions of oxygen supersaturation and hypoxia/anoxia, and by the production of the cyanotoxin microcystin by the BBD cyanobacteria. Furthermore, in the infected colonies, the expression of all the biomarkers appeared significantly affected by the development stage of the disease. In conclusion, our approach may constitute a useful diagnostic tool, since the cellular stress response of corals is activated before the pathogens colonize

the tissues, and expands the current knowledge of the mechanisms controlling the host responses to infection in corals.

Keywords Cellular stress response · Biomarkers · *Goniopora columna* · Black band disease · Maldives · Oxidative stress

Introduction

Coral diseases are strongly contributing to the rapid global decline in coral cover and diversity worldwide and their impact on reef ecosystems, which is expected to increase with global climate change, will play a crucial role in the structure and health condition of future coral reefs (Harvell et al. 1999; Rosenberg and Ben-Haim 2002; Rosenberg and Loya 2004).

Among the several coral diseases reported up to now, the black band disease (BBD) is one of the most destructive and virulent (Richardson et al. 2009; Gantar et al. 2011). Its numerous occurrences throughout the Caribbean, the Red Sea, the Indian Ocean, and the Indo-Pacific indicate that this disease has a global distribution (Edmunds 1991; Littler and Littler 1996; Barneah et al. 2007; Sato et al. 2009; Weil et al. 2012; Aeby et al. 2015; Johan et al. 2015). BBD affects more than 40 coral species belonging to many families (Sutherland et al. 2004; Kaczmarzsky 2006; Page and Willis 2006), although it preferentially infects large, massive, and slow growing scleractinian coral species (Frias-Lopez et al. 2004; Richardson 2004; Weil 2004). The BBD appears as a horizontally migrating dense and darkly pigmented microbial mat or line, between 1 mm and several centimeters wide and up to 1 mm thick. It rapidly moves across the surface of the coral colony causing tissue lysis and mortality and leaves behind the exposed bare skeleton (Carlton and Richardson 1995). Although BBD is generally characterized by low prevalence (Dinsdale 2002; Weil et al. 2002), it represents a serious

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threat to coral populations due to its persistence (Kuta and Richardson 1996; Page and Willis 2006; Zvuloni et al. 2009) and especially to its rapid progression rate, which is an average of 3 mm/day (Rützler and Santavy 1983) but in several cases has reached up to 2–3 cm/day (Kuta and Richardson 1997; Richardson 1998). However, in the Republic of Maldives, where the BBD presence was only recently reported (Montano et al. 2012, 2013, 2015a), a peculiar and extraordinary very slow progression rate was observed in large size colonies of the massive coral *Goniopora columna* (~0.5 mm/day). This occurrence may play an important role in determining the local persistence of the disease (Montano et al. 2015b).

The black band consists of a microbial consortium, which makes the BBD the only polymicrobial disease affecting corals (Frias-Lopez et al. 2004). However, the disease etiology remains unclear due to its complex pathogenic nature (Sato et al. 2016; Arotsker et al. 2016). In fact, the specific members of the mat are highly variable in terms of species across geographic regions and host coral taxa (Cooney et al. 2002; Frias-Lopez et al. 2003; Myers et al. 2007; Voss et al. 2007; Sekar et al. 2008). Nevertheless, the mat is always volumetrically dominated by gliding, filamentous, nonheterocystous cyanobacteria from several genera (*Geitlerinema*, *Lyngbya*, *Leptolyngbya*, *Oscillatoria*, *Pseudoscillatoria*, *Phormidium*, and *Roseofilum*) and also includes sulfate-reducing *Desulfovibrio* spp. bacteria, sulfide-oxidizing *Beggiatoa* spp. bacteria, marine fungi, and other heterotrophic microbes (Ramos-Flores 1983; Richardson 1996; Frias-Lopez et al. 2002; Viehman et al. 2006; Casamatta et al. 2012; Buerger et al. 2016; Den Uyl et al. 2016; Séré et al. 2016). At the same time, it is known that these microorganisms always maintain a tightly organized synergistic community, showing vertical migration within the mat under changing diel light conditions. Host tissue necrosis and lysis are linked to the onset of an anoxic microenvironment, alternated with oxygen supersaturation conditions, and accumulation of sulfide at the base of the microbial mat (Carlton and Richardson 1995; Glas et al. 2012).

To date, studies on the microbial constituents of the BBD consortium and their virulence mechanisms are constantly increasing while, to our knowledge, the effects of the pathogen presence on coral tissue physiology have not been investigated. Since the earliest steps of an organism's response to any environmental stress occur at the cellular level (Kultz 2005), a useful diagnostic tool can be represented by the analysis of the expression of cellular stress molecular biomarkers that reflect changes in the cellular structural integrity and in the functional cellular pathways and performance (Downs 2005). Such an approach has been successfully applied in corals subjected to several abiotic stresses (Downs et al. 2002, 2009, 2012; Brown et al. 2002; Downs and Downs 2007; Rougee et al. 2006; Fitt et al. 2009; Kenkel et al. 2014).

For this reason, in the present study, the expression of a set of molecular biomarkers was analyzed in the massive coral *G. columna* during the slow BBD progression observed in Maldives (Montano et al. 2015b). The selected biomarkers included molecular chaperones, such as the 70 and 60-kDa heat shock proteins (Hsp70 and Hsp60) that preserve protein homeostasis (Balchin et al. 2016) and the oxidative stress-inducible protein heme-oxygenase-1 (HO-1) and the manganese superoxide dismutase (MnSOD) that are indicators of oxidative damage (Lesser 2006; Gozzelino et al. 2010). Each biomarker has a specific function performed in a specific cellular compartment (Table 1), which can provide useful information about its functionality under stress. The biomarker levels were analyzed in the apparently healthy coral polyps located at different distances from the advancing infection front and also in different development stages of the disease, in order to have new insights into the modulation over time of the cellular stress response triggered by the pathogen activity before signs of the disease become visible.

Materials and methods

Study site and selected colonies

Colonies of *G. columna* ($n = 7$) affected by BBD were located in the lagoon of Magoodhoo Island (3° 04' 42" N; 72° 57' 50" E) in the south east part of Faafu Atoll, Republic of Maldives (for a detailed description of the study site, see Montano et al. 2015b and Seveso et al. 2015). Here, they were tagged, photographed (Canon G11 with Canon housing), and monitored by snorkeling over a period of approximately 3 years (from the end of 2010 to 2013) to measure the slow disease progression rate as previously reported (Montano et al. 2015b). All the selected colonies had a similar large size (roughly >1 m) and were well isolated one from another (at least 5 m apart). However, they were located in the same reef flat zone of the lagoon, characterized by a similar shallow depth (around 2–3 m) and thus subjected to the same environmental and abiotic parameters. Above all, they showed the black band characteristic of the disease (Fig. 1a) and they had no other morphological anomalies except for BBD lesion. Furthermore, at the beginning of the sampling period, each selected colony showed a similar condition of disease progression and a similar percentage of living tissue not yet infected.

Sample collection

In October 2012 (Time 1), the percentage of living tissue in the selected large size colonies of *G. columna* was visually assessed and for all it was similar and comprised between 60 and 80%.

Table 1 Cellular biomarkers analyzed and their molecular weight (MW), cellular localization and main physiological functions

Biomarker	MW	Localization	Biological function
Hsp70	~72 kDa	Cytoplasm	Assembling of newly synthesized proteins; refolding of misfolded and aggregated proteins; degradation of unstable proteins; protein translocation; receptor regulation; cytoskeleton stabilization; regulation of stress-induced apoptosis; modulation of immune system. At basal level in normal condition, up-regulation in response to different stress factors.
Hsp60	~62 kDa	Mitochondria	Synthesis and transportation of mitochondrial proteins; folding of newly imported and stress-denatured proteins; anti-apoptotic ability; replication and transmission of mitochondrial DNA ; modulation of immune system. At basal level in normal condition, up-regulation in response to different stress factors.
HO-1 (Hsp32)	~32 kDa	Cytoplasm	Enzymatic activity; catalysis of oxidative degradation of heme into biliverdin, free iron and carbon monoxide using NADPH as a cofactor; cytoprotective activity in response to oxidative stress; antioxidant defense; therapeutic effects in immune-mediated inflammatory diseases. Only stress-inducible.
MnSOD	~25 kDa	Mitochondria	Catalysis of the dismutation of superoxide anion radical to molecular oxygen and hydrogen peroxide; first-line defense for protecting mitochondria and the cell against oxidative damage; important role in the prevention of various diseases and as therapeutic agent. Low level in physiological condition, up-regulation in response to oxidative stresses.

Ref: Hsp70 (Srivastava 2002; Mayer and Bukau 2005), Hsp60 (Martinus et al. 1995; Arya et al. 2007), HO-1 (Ryter et al. 2006), MnSOD (Fridovich 1995; Carillon et al. 2013)

In order to analyze the biomarker levels at different distance from the BBD mat along the disease progression direction, in each colony, three coral fragments were collected. The first at ~1 cm from the site of infection, just adjacent to the black band (these samples were marked as distance 1, D1). The second coral portion was collected at 5 cm (D2) and the third at 10 cm (D3) from the lesion. Since the band has always a rounded, semi-circular appearance, the coral fragments were sampled starting from its lowest part (Fig. 1b). In order to

investigate the modulation of the biomarkers during different development stages of the disease, the same sampling procedure was repeated 1 year later, in October 2013 (Time 2), in the same colonies, which, in the meantime, showed a significant reduction of their percentage of living tissue (comprised between 20 and 40%). Therefore, from each of the seven infected colonies, three other coral fragments were collected at the three predefined distances from the mat (D1, D2, and D3), which, despite the very slow progression rate of the

Fig. 1 **a** Detail of the infected area on a colony of *G. columna* showing the macroscopic sign of the BBD including (1) the exposed white coral skeleton without tissue and already covered by algae preceding the black zone, (2) the BBD circular mat composed by a microbial consortium migrating radially across the coral colony, and (3) the apparently healthy coral tissue with the extended polyps following the band. **b**

Representative picture showing the sampling design of a large size colony of *G. columna* affected by BBD at Time 1. The *white arrow* indicates the disease progression direction. Coral fragments were sampled just below and adjacent to the black band (D1), at 5 cm (D2), and 10 cm (D3) away from the site of infection. Distances are not in scale

BBD, has exceeded and killed the tissue portions sampled in October 2012.

In addition, to test whether the expression of the biomarkers could be influenced by the position of coral polyps within the colony and/or by the sampling time, seven entirely healthy large size colonies of *G. columna* were randomly selected as control within the same study site and at the same depth of the diseased ones. For each of them, coral fragments were collected in both the sampling periods at three different distances from the top of the massive colonies (C1, C2, C3) as previously reported (Seveso et al. 2015).

All the coral samples were collected approximately at the same morning time, at the same shallow depth, and during high tide regime (coral permanently submerged) to minimize any possible effects of abiotic variables on the biomarker expression and modulation. In this context, an HOBO pendant data logger (onset, UA-002-64) was used to measure continuously the seawater temperature during the entire sampling period and seawater samples were collected for salinity measurements with a refractometer (Milwaukee Instruments, USA). Sampling was performed by snorkeling and all the coral samples were excised from the colonies using a hollow-point stainless steel spike (8 mm diameter) by applying constant rotational pressure in order to reduce the size of coral sampled and reduce the amount of sampling stress limiting excessive damage to the colonies (Bromage et al. 2009). Afterwards, samples were immediately brought to the laboratories of the MaRHE Center located a few meters away from the study area, where they were instantly frozen at -80°C using an immersion cooler (FT902, JULABO, Labortechnik GmbH) and maintained at this temperature until protein extraction. In all the collected samples, the absence of BBD signs was carefully verified by microscopic examination of each frozen sample prior to their homogenization in order to avoid interference during the analysis of the biomarkers.

Protein extract preparation and Western analyses

All the frozen coral portions were powdered in a pre-chilled mortar and pestle, homogenized in SDS-buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol) containing 1 mM phenylmethylsulfonyl fluoride (Sigma) and complete EDTA-free cocktail of protease inhibitors (Roche Diagnostic) and processed according to a method previously described (Seveso et al. 2013, 2014). This method allows to remove any *Symbiodinium* contamination, obtaining extracts containing only polyp proteins (Seveso et al. 2013). Polyp protein extracts were stored at -80°C until the subsequent analyses. Aliquots were used for protein concentration determinations using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Equal amounts of proteins from each sample were separated by SDS-PAGE on 8% polyacrylamide gels (Vai et al. 1986) running duplicate gels 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) as described (Seveso et al. 2012, 2013). Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters. Following saturation with TBS and 5% skimmed milk, filters were probed with the following primary antibodies: anti-Hsp70 monoclonal antibody (IgG2a mouse clone BB70, SPA-822, Enzo Life Sciences), anti-Hsp60 monoclonal antibody (IgG11 mouse clone LK-2, SPA-807, Enzo Life Sciences), anti-HO-1 monoclonal antibody (Hsp32, IgG2b mouse clone HO-1-2, OSA-111, Enzo Life Sciences), anti-MnSOD polyclonal antibody (rabbit, SOD-110, Enzo Life Sciences), and anti- β -Actin monoclonal antibody (IgG1k 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 (1:3000). Filters were washed three times with fresh changes of TBS and then incubated with secondary antibody. Secondary antibodies used were anti-mouse IgG polyclonal conjugated with horseradish peroxidase (ADI-SAB-100, Enzo Life Sciences) diluted 1:1000 for Hsp70, 1:10,000 for Hsp60 and 1:7500 for HO-1 and anti-rabbit IgG polyclonal conjugated with horseradish peroxidase (ADI-SAB-300, Enzo Life Sciences) diluted 1:1000 for MnSOD. All dilutions were in TBS-0.1% Tween 20 and 5% skimmed milk. Western blots were developed using Pierce ECL Western Blotting Substrate followed by exposition of filters to Amersham Hyperfilm ECL.

Densitometric and data 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 (Fig. 2). The densitometric data were represented as means $\pm$ standard error of the mean (SEM) and expressed as relative levels (arbitrary units). Data normality was verified using Shapiro-Wilk test. For each biomarker, differences in its expression in both the healthy and diseased colonies were analyzed with a two-way ANOVA, using the distances from the top of the colonies (C1, C2, and C3) or from the black band (D1, D2, and D3) and the sampling time (Time 1 and Time 2) as factors, and the normalized biomarker values obtained from the different groups of samples as the dependent variables. Following two-way ANOVA, a Tukey's HSD post hoc test was performed to assess significant differences in the biomarker levels in response to the different

Fig. 2 Expression of the cellular stress biomarkers in both healthy and diseased colonies of *G. columnna* sampled in **a** October 2012 (Time 1) and **b** October 2013 (Time 2). In both periods, samples of coral fragments of the diseased colonies located at three different distances from the infection (D1, D2, and D3) were subjected to Western analyses. Immunodecorations were performed with anti-Hsp70, anti-Hsp60, anti-

HO-1, anti-MnSOD, and anti- β -Actin antibodies. Equal amounts of total proteins were loaded in each lane. Samples prepared from healthy colonies were also analyzed; since in these colonies, no differences were detected in the signal intensity at the three different distances from the top, only a representative control sample (C1) is shown. Filter representative of seven experimental repeats ($n = 7$) is shown

distances for each sampling time. Moreover, paired t tests were used for comparison of the levels of each biomarker in samples collected at Time 1 and Time 2. Statistical significance was defined as $P < 0.05$. The statistical analyses were performed using SPSS ver. 22 (IBM, New York).

Results

Biomarker expression in the healthy colonies

In all the healthy colonies of *G. columnna* chosen as control, Western analyses detected weak signals relative to Hsp70 and Hsp60 in all samples collected at the three different distances from the top of the colonies and in both the two sampling periods (Fig. 2). Densitometric measurements indicated no significant effect of either distance (C1, C2, and C3) or sampling time period (Time 1 and Time 2) on the low basal constitutive level of these Hsps (Fig. 3a and Table 2). As far as HO-1 and MnSOD were concerned, their expression was negligible in all the coral samples collected from the healthy colonies (Figs. 2 and 3a and Table 2) in line with the characteristics displayed by these proteins under normal physiological conditions in other organisms (Stahnke et al. 2007; Mustafi et al. 2009).

Biomarker expression in the diseased colonies

As shown in Fig. 2a, in the colonies of *G. columnna* affected by BBD, strong signals were observed for the four biomarkers following Western analyses of the samples collected at the three distances from the black band (D1, D2, and D3) during the initial development stage of the disease (Time 1). Based on densitometric analyses, an evident and significant up-regulation in the expression of Hsp70, Hsp60, HO-1, and MnSOD was measured compared with that of the healthy colonies (Fig. 3). Their levels, although subjected to slight variations moving away from the infection, remained similar and comparable among D1, D2, and D3 and always higher than those observed in the healthy colonies. In particular, at Time 1, the average level of Hsp70 and Hsp60 determined in coral portions at D1 displayed approximately an 8 and 7.5-fold increase, respectively, compared with that in the healthy coral portions C1. In the same portions at D1, the up-regulation of HO-1 and MnSOD was even much higher, with a 30- and 65-fold increase, respectively (Fig. 3b).

After 1 year of disease progression (Time 2), with the coral colonies almost completely dead, the levels of all the four biomarkers always remained, at any distance from the lesion, significantly higher than those measured in the healthy colonies. However, differently to what observed at Time 1, they showed a modulation (Figs. 2 and 3b). In particular, a strong

Fig. 3 Bar charts of biomarker levels determined by densitometric analysis as described under the “Materials and Methods” section. The analyses were performed on samples of coral fragments located at the three different distances in healthy (a) and diseased (b) colonies at the two sampling periods (Time 1 and Time 2). Signals of seven different blots were analyzed. Data are expressed as arbitrary units and as

mean $\pm$ SEM (two-way ANOVA followed by Tukey’s HSD multiple pair-wise comparisons and paired *t* tests). For each biomarker, letters denote significant differences in the biomarker levels between the different distance groups ($P < 0.05$); thus, the same letter indicates no significant difference ($P \geq 0.05$)

down-regulation compared to Time 1 was observed for all the biomarkers in the coral portions closest to the infection (D1), while moving away of more than 1 cm from the infection, they displayed a clear and significant up-regulation compared to D1. In fact, their levels increased significantly returning

comparable to those measured at the same distance D2 during Time 1. Finally, at 10 cm from the band (D3), a general downward trend of the expression of Hsp70, Hsp60, HO-1, and MnSOD was observed, albeit this decrease resulted significant only for the Hsp70 (Figs. 2b and 3b).

Table 2 Summary of the two-way analysis of variance (ANOVA) for all the normalized intensity values of Hsp70, Hsp60, HO-1, and MnSOD obtained from the healthy and the diseased colonies

	Healthy colonies				Diseased colonies		
Source of variation	Df	MS	<i>F</i>	P	MS	<i>F</i>	<i>P</i>
Hsp70							
Time	1	0.001	0.115	ns	4.727	27.276	<i>P</i> < 0.001
Distance	2	0.002	0.243	ns	0.581	3.351	<i>P</i> < 0.05
Time x Distance	2	0.003	0.473	ns	0.462	2.666	ns
Residual	36	0.007			0.173		
Hsp60							
Time	1	0.004	0.617	ns	2.352	9.740	<i>P</i> < 0.001
Distance	2	0.015	2.380	ns	0.774	3.212	ns
Time x Distance	2	0.003	0.386	ns	0.326	1.351	ns
Residual	36	0.006			0.242		
HO-1							
Time	1	0.000	0.001	ns	4.967	17.529	<i>P</i> < 0.001
Distance	2	0.001	0.401	ns	0.504	1.778	ns
Time x Distance	2	0.003	0.898	ns	1.106	3.903	<i>P</i> < 0.05
Residual	36	0.003			0.283		
MnSOD							
Time	1	0.001	0.426	ns	1.994	21.216	<i>P</i> < 0.001
Distance	2	0.001	0.693	ns	0.323	3.433	ns
Time x Distance	2	0.000	0.091	ns	0.653	6.951	<i>P</i> < 0.05
Residual	36	0.002			0.094		

The sampling time (time) and the distance from the top of the colony or from the BBD mat (distance) were used as fixed factors. Significant values are in italic. Values not significant ($P \geq 0.05$) are indicated with ns

Summing up, these data indicate that, as in the *G. columna* colonies affected by BBD, the average levels of all the cellular stress biomarkers, regardless of the distance from infection and sampling time, were significantly higher than those detected in the healthy colonies, regardless of the distance from the top of the colonies and the sampling time. Moreover, in the diseased colonies the levels measured were significantly more elevated in the initial development stage of the disease (Time 1) compared to the final one (Time 2). In this context, the two-way ANOVA showed as in the diseased colonies the factor that always affects most significantly the modulation of all the biomarkers is the sampling time, while the distance from infection and interaction of both factors differently affect the expression of each biomarker analyzed (Table 2). Indeed, in addition to the sampling period, Hsp70 levels also depend on the distances from the band while those of HO-1 and MnSOD by the interaction with the sampling time and the distance from the infection (Table 2).

Discussion and conclusion

Coral diseases have emerged as a significant threat to the world's coral reef ecosystems, causing unprecedented declines in many coral taxa (Bourne et al. 2009; Sweet et al. 2014). For this reason, novel approaches and diagnostic tools have recently substantially increased to assist in the early detection and characterization of such diseases (Pollock et al. 2011; Anderson et al. 2013, 2016; Morgan et al. 2015; Fuess et al. 2016). However, the knowledge of the cellular mechanisms used by corals to counteract infections remains still limited and the cellular stress response associated with coral diseases has up to now represented an under-studied area of the coral physiology. This especially applies to the BBD, although it is one of the most widespread and virulent coral diseases and probably one of the most extensively investigated (Sato et al. 2016).

Here, we analyzed the expression of some cellular biomarkers, such as Hsp70, Hsp60, HO-1, and MnSOD, during the progression and the development of the BBD infection in *G. columna*, with the objective to determine whether differences in their levels of expression may be associated with an altered physiological condition due to the disease infection. The same biomarkers have been already successfully used for detecting early signs of change in the coral's physiological state in relation to thermal anomalies causing bleaching (Fang et al. 1997; Barshis et al. 2010; Chow et al. 2012). At the same time, changes in other related molecular biomarkers (such as Cu/ZnSOD and Hsp90) have been employed to analyze the physiological response of corals to other bacterial infections (Couch et al. 2008; Fuess et al. 2016).

In the healthy control colonies of *G. columna*, both the cytosolic Hsp70 and the mitochondrial Hsp60 displayed a low basal level of expression that was always similar and comparable in the different parts of the coral colony (as also observed in another coral species, *Acropora muricata*, Seveso et al. 2015). Similarly, the expression of the cytosolic HO-1, as well as that of the mitochondrial MnSOD, was similar in the different parts of the colonies. These levels were negligible, indicating that the control colonies did not suffer oxidative stress. In addition, after 1 year from the former sampling and analysis, in the healthy control colonies, the expression levels of all the four biomarkers resulted unchanged, indicating that the cellular parameters herein investigated could represent a valuable tool to detect and define an unstressed and healthy coral status.

In fact, by contrast, in the diseased colonies of *G. columna*, the levels of Hsp70, Hsp60, HO-1, and MnSOD increased several fold compared to the control colonies. This up-regulation was observed in the coral tissues not yet directly infected but surrounding the infection, which were morphologically intact and apparently healthy. It is important to emphasize that all the coral tissue samples displayed normal

tissue pigmentation, were free of necrosis, and were structurally undamaged. Moreover, no thermal anomaly or significant difference of both the seawater temperature (Fig. 4) and the salinity values ($\sim 35.5\text{‰}$) in the sampling area of the lagoon were observed during the two sampling period. Consequently, the presence of the infection can represent the sole cause of the biomarker trend in the diseased colonies.

The high levels of all the biomarkers found up to 10 cm from the band along the direction of the disease progression indicate that the pathogens have generated, even in coral portions far from the infection, significant cellular damage. This damage affecting the metabolic and cellular equilibrium has triggered a cellular stress response by activating the main cellular defense mechanisms. These include, in addition to the Hsp70 and Hsp60, whose expression in pathogen-coral relationship has been recently explored (Seveso et al. 2012, 2015; Brown et al. 2013; Libro et al. 2013; Brown and Rodriguez-Lanetty 2015; Fuess et al. 2016; Garcia et al. 2016), an increased antioxidant response (HO-1 and MnSOD) that is indicative of oxidative stress. To date, cnidarian HO-1 and MnSOD have been investigated especially in relation to environmental abiotic stresses rather than to diseases (Fang et al. 1997; Richier et al. 2005, 2008; Downs et al. 2006; Lesser 2011).

A condition of oxidative stress reflects a serious imbalance between the production of reactive oxygen species (ROS) and the cellular antioxidant capacity. In the present study, we speculate that the oxidative stress produced in the coral tissues at any distance from the band could be principally caused by the alternation, at the progression front of the disease, of conditions of oxygen supersaturation and hypoxia/anoxia. Indeed, the BBD mat is characterized by steep microgradients of oxygen and sulfide along the vertical axis of the lesion causing a clear stratification in response to varying light levels (Carlton and Richardson 1995; Richardson 1997; Richardson et al. 2001; Glas et al. 2012). Due to oxygenic photosynthetic activities of the dominant cyanobacteria, under daylight

conditions, the BBD microbial mat appears at the surface more oxygenated than ambient seawater and the concentrations of hydrogen sulfide are minimal throughout the lesion. On the contrary, during the night, biogeochemical conditions at the base of the mat are typically hypoxic or anoxic with high levels of sulfide and low pH (reviewed in Sato et al. 2016). It is well known that usually both hyperoxia and hypoxia, as well as the exposition to sulfide, may lead to the up-regulation of the antioxidant systems HO-1 and MnSOD as well as that of Hsps (Lee et al. 1997; Yamashita et al. 1997; Carraway et al. 2000; Garrido et al. 2001; Richier et al. 2003; Calvert et al. 2009; Mohindra et al. 2015). Moreover, sulfide is toxic to most eukaryotes, inhibiting electron transport in aerobic respiration, leading to apoptosis and tissue necrosis, and increasing the cellular oxidative stress (Vismann 1991).

The strong oxidative stress observed could be also due to the production of the cyanotoxin microcystin by the cyanobacteria. Several variants of microcystin have been identified from both field samples of the BBD mat and from cultured BBD cyanobacteria, and the most common variant found associated with the disease is microcystin-LR, which is the most toxic one (Gantar et al. 2009; Richardson et al. 2007; Stanić et al. 2011). Microcystin uptake has been related to the ROS production and induction of oxidative stress in diverse aquatic species, generating lipid peroxidation, mitochondrial damage, and alteration of the antioxidant defense system (reviewed in Amado and Monserrat 2010). In corals, exposure to microcystin caused loss of tissue organization and structure, degradation of the coral epidermis and gastrodermis, and some degradation of the zooxanthellae even in the tissues surrounding the cyanobacterial lesions (Richardson et al. 2009; Miller and Richardson 2012; Kramarsky-Winter et al. 2014).

Interestingly, in the infected colonies, we found that the expression of all the biomarkers was analyzed, and consequently, the physiological condition of corals was significantly affected by the development stage of the disease. Indeed, at

Fig. 4 Graph showing the trend of sea surface mean temperature from September 2012 to December 2013 recorded in the sampling site, the lagoon of Magoodhoo Island (Faafu Atoll, Maldives), by a data logger. The monthly mean ($\pm$ SD) of daily records is shown. The arrows indicate the two sampling periods, October 2012 (Time 1) and October 2013 (Time 2)

the beginning of the infection, their levels resulted very high and comparable at any distance from the band. Therefore, this indicates that the microbial consortium has generated a strong cellular damage of a similar intensity in the whole diseased colony, within 10 cm from the infection, and that both the cellular compartments analyzed, mitochondria and cytoplasm, were equally affected by the pathogen action. Moreover, on the contrary to what observed in other coral species affected by other diseases (Seveso et al. 2012, 2015), the cellular stress and damage appeared not confined in a restricted area close to the infection but widespread in a more extended area. In this context, the up-regulation of all the defense mechanisms could represent a system that attempts to reduce the spreading of the infection throughout the whole colony, limiting the rate of the pathogen progression. This might also explain the exceptionally and peculiar slow progression rate of BBD observed in the investigated colonies (Montano et al. 2015b).

At the final stage of the disease, the levels of Hsps, MnSOD, and HO-1 showed an overall reduction compared to those observed at the beginning of the infection process, mainly in the coral portions closest to the infection. In these tissues, the continuous and prolonged pathogen action, coupled to a long time exposition to cyanotoxins, sulfide, and extreme conditions of hyperoxia and hypoxia, may have led to a depression of the defense mechanisms of the cellular stress response and immune system that was no longer able to counteract the strong cellular stress. Previous studies demonstrated that the BBD cyanobacteria can produce different toxins at different stages of the disease process (Stanić et al. 2011). In line with this, the compromised physiological status of the coral polyps nearby the infection could be related to the production, at the final stage of infection, of the most toxic and lethal microcystin variant, which has greatly increased the oxidative stress. Furthermore, during the progression of the infection, microcystin may promote the growth of some species of pathogenic bacteria in the coral tissue surrounding the lesion, which could indirectly and additionally affect the host, enhancing tissue lysis (Richardson et al. 2009).

In conclusion, our results suggest that the cellular stress response of corals is activated before visible disease lesions are evident and before the pathogens colonize the tissues. Furthermore, the approach used here highlights as a set of cellular stress biomarkers may constitute a useful diagnostic tool, capable to check specific variations in the coral health and physiology which cannot be detected morphologically and to differentiate between healthy and diseased colonies as well as between two different development stages of BBD within a diseased colony. Finally, the expression profiles of the cellular biomarkers analyzed, combined with other related and recent evidences (Seveso et al. 2012, 2015), clearly indicate as each coral disease, and thus each pathogen, triggers a specific cellular response in the host depending on the pathogen progression rate, their mechanisms of infection and on the

development stage of the disease. Moreover, since the pathogen virulence and host susceptibility are likely to increase with rising in sea temperatures (Bruno et al. 2007; Maynard et al. 2015), future studies could test the expression of a larger set of cellular biomarkers in different coral species subjected to different diseases and under different thermal regimes.

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