INTRODUCTION

Coral diseases have been reported to be increasing in both the occurrence of known diseases and the incidence of newly reported infections, thereby threatening coral reefs worldwide (Sutherland et al. 2004, Cróquer et al. 2006, Weil et al. 2006, 2012, Montano et al. 2013, 2014). In this context, a coral syndrome called the Brown Band (BrB) disease occurring in scleractinians belonging to Acroporidae, Pocilloporidae and Faviidae families on the Great Barrier Reef was described for the first time by Willis et al. (2004). Thereafter, several reports of BrB highlighted its ongoing spread across the Indo-Pacific, especially affecting corals of the genus Acropora (Raymundo et al. 2009, Lamb & Willis 2011, Montano et al. 2012).

The macroscopic field sign of the syndrome is the presence of a distinctive brown zone of variable width located between healthy coral tissues and exposed white skeleton. This band is formed by a dense concentration of motile protozoan ciliates that feed on coral tissue and accumulate zooxanthellae (Symbiodinium sp.) intracellularly, resulting in the characteristic brown coloring and in rapid tissue loss (Willis et al. 2004, Ulstrup et al. 2007, Lobban et al. 2011).

ABSTRACT: Brown band disease (BrB), a virulent coral disease characterized by a dense concentration of ciliates ingesting coral tissue, is responsible for ongoing coral losses on Indo-Pacific reefs. Although several efforts have been made to identify the microbial communities associated with BrB and study the disease ecology, less attention has been given to the effect of ciliate presence on coral physiology. Levels of the mitochondrial heat shock protein 60-kDa (Hsp60, a biomarker indicative of cellular stress) were analyzed in apparently healthy coral polyps located at different distances along the advancing front of infection in Acropora muricata colonies affected by BrB in a Maldivian reef. Different Hsp60 levels were found in different parts of the same colony. Starting from a basal protein level in the healthy control colonies, a down-regulation of Hsp60 expression was detected near the ciliate band, indicating that the Hsp60 defense activity was probably already compromised due to the rapid progression rate of the BrB ciliate on the diseased branches and/or to the etiology of the disease. Moving away from the band, the Hsp60 levels gradually returned to a state comparable to that found in the control, showing that cellular damage was confined to areas near the infection. In conclusion, we propose the analysis of Hsp60 modulation as a useful tool for examining physiological variations that are not detected at the morphological level in corals subjected to epizootic diseases, while providing new insights into the immune response of corals.

KEY WORDS: Coral disease · Ciliates · Brown band disease · Acropora muricata · Hsp60 · Maldives · Coral immune system
Since different species of ciliates ingest *Symbiodinium* cells (Bourne et al. 2008, Sweet & Bythell 2012), the term BrB disease is generally used to describe coral infections characterized by a brown band associated with any ciliate containing *Symbiodinium* and actively consuming coral tissue (Nicolet et al. 2013, Sweet et al. 2013).

To date, although this disease is well documented and significant efforts have been made in the identification of the associated microbial communities, less attention has been given to the effects of ciliate presence on coral health and physiology, especially considering the high progression rate of the disease (Nicolet et al. 2013, Katz et al. 2014). In this context, the use of molecular biomarkers (e.g. heat shock proteins, Hsps, whose levels can be indicative of cellular stress due to pathogen activity) could clarify some aspects of the typology and severity of damage to coral tissues. It can also provide helpful information about coral stress responses and defense mechanisms before visible signs of disease become apparent. In fact, as ubiquitous molecular chaperones and fundamental cellular protective systems, Hsps are involved in preserving physiological protein homeostasis and maintaining regular cellular functions. They also play a crucial role in protein folding, refolding/unfolding, aggregation, degradation and transport (Sorensen et al. 2003, Mayer 2010, Hartl et al. 2011). In addition, it is well known that the expression of Hsps is up-regulated in all organisms, as well as in corals, in order to increase cellular repair and tolerance when environmental variations (both abiotic and biotic) perturb the organism’s physiological system (Rossi et al. 2006, Lanneau et al. 2008, Seveso et al. 2012, Ross 2014). Moreover, Hsps are also important factors both in the activation and modulation of the immune response linking cellular stress to immunophysiology (Pockley et al. 2008). In particular, Hsp60 and Hsp70 seem to play a dual role of stress biomarker and immune modulator, in addition to their primary function as molecular chaperones, providing the opportunity to use them as potential therapeutic agents (Pockley et al. 2008, Tsan & Gao 2009, Quintana & Cohen 2011). In corals, a recent paper suggests the involvement of the heat stress response gene, *hsp70*, in the immunological/defense response of *Acropora millepora* to microbial challenges (Brown et al. 2013). Furthermore, in the coral *A. muricata* infected by ciliates responsible for the coral disease Skeleton Eroding Band (SEB), an up-regulation of Hsp60 as a defensive mechanism against advancing infection was detected in the coral portions just above the SEB band; this suggests the involvement of Hsp60 in the coral immune response and implies its usefulness as a tool to evaluate physiological stress caused by coral diseases (Seveso et al. 2012).

In line with the above studies, the present work investigated the effect of BrB ciliates on the physiology of coral tissue during disease progression through analysis of the modulation of mitochondrial Hsp60. This was performed by measuring Hsp60 levels in coral polyps situated at 3 different distances away from the disease lesion in colonies of the staghorn coral *A. muricata*, which is one of the most analyzed widespread species affected by the BrB syndrome worldwide (Ulstrup et al. 2007, Bourne et al. 2008, Lobban et al. 2011, Sweet & Bythell 2012, Nicolet et al. 2013).

**MATERIALS AND METHODS**

**Study area and sampling design**

In December 2013, after extensive surveys, a defined sampling area of ~200 m² located ~80 m from the shore and composed of several coral patches was chosen in the lagoon of Magoodhoo Island (3° 04’ 42” N, 72° 57’ 50” E), in the southeastern part of Faafu Atoll, Republic of Maldives (Fig. 1). This site was selected as it had been affected by BrB disease episodes in the past (Montano et al. 2012) and permitted easy and quick access suitable for rapid sampling activities. In addition, it extends along the same reef flat zone characterized by similar depths (~2–3 m) and may thus be subjected to the same environmental conditions. To confirm this, 3 HOBO pendant data loggers (Onset, UA-002-64) were placed in different locations within the whole area a few days before sampling, and were maintained until the end of the sampling in order to measure sea temperature and light intensity. In parallel, seawater samples were collected for salinity measurements with a refractometer (Milwaukee Instruments). Colonies of the branching coral *Acropora muricata* of similar size and exhibiting symptoms of BrB disease were randomly selected, tagged and photographed (Canon G11 with Canon housing) by snorkelers. To ensure that only colonies displaying active disease were sampled, colonies were monitored every 2 d prior to the sampling activity and only those showing lesion progression were subsequently sampled and analyzed to study Hsp60 modulation. Furthermore, we selected colonies in which the BrB band was located at approximately the same distance from the tips of the coral branches.
Coral collection

Among the selected colonies, 6 were randomly chosen and 3 intact and apparently healthy small coral fragments were collected from each of them. The fragments were obtained from the same coral branch at 3 different distances from the brown band, along the disease progression direction (Fig. 2A). The coral fragments sampled at ~1 cm from the site of infection just adjacent to the brown band, along the advancing front of the ciliate mass, were marked as distance 1 (D1). The coral fragments sampled approximately 5–10 cm away from the site of infection were marked as distance 2 (D2). Finally, the portions sampled far from the disease lesion (~15–20 cm away) were marked as distance 3 (D3) (Fig. 2A). In some fragments, a thin white zone of exposed skeleton was observed between the ciliate band and the healthy tissue that may comprise bleached tissue and/or denuded skeleton (Willis et al. 2004, Lobban et al. 2011) (Fig. 2B). In these fragments, the collection of coral fragments was started from the first portions of living tissue situated just above these thin white bands. Coral samples were excised from colonies of *A. muricata* using a hollow-point stainless steel spike (8 mm diameter) by applying constant rotational pressure to minimize the size of coral sampled; this also minimized the amount of sampling stress and limited excessive damage to the colonies, thereby allowing rapid coral recovery following the sampling (Bromage et al. 2009). Each sample was collected with a new hollow-point spike to avoid contamination. Samples were then immediately frozen at ~−80°C using an immersion cooler (FT902, JULABO, Labortechnik) in the MaRHE Centre laboratory at Maqhoodhoo Island, which is located a few meters away from the sampling area.

All coral samples were taken simultaneously at ~09:00 h at the same shallow depth and during high tide (coral permanently submerged) to minimize seasonal and/or daily variations in water temperature, UV intensity and salinity (Chow et al. 2009, 2012, Seveso et al. 2013). Furthermore, in order to confirm that ciliate infection was associated with the disease in all the analyzed colonies, coral fragments corresponding to the brown bands were also collected together with the samples D1, D2 and D3. In these fragments, the infection caused
by BrB ciliates was confirmed by microscopic analysis (Fig. 2B–D). Photomicrographs of the infected tissues containing ciliates were obtained using a stereomicroscope (Olympus SZ61) paired with a cold light source (Schott KL 300 LED), a camera (Olympus LC 20) and a microscope (Zeiss Axioskop, Carl Zeiss). To avoid contamination of the D1, D2 and D3 samples with other protozoans during the analysis of Hps60, the total absence of protozoans was carefully verified by microscopic examination of each frozen sample prior to their homogenization. As control, 6 isolated and entirely healthy colonies of *A. muricata* were randomly selected within the same sampling area, considering only those located at least 10 m away from diseased colonies. For each colony, coral fragments were collected at 3 different distances (C1, C2 and C3) from the tip of the coral branch corresponding approximately to the same fixed distances of samples in the diseased branches.

**Western analysis**

The frozen coral fragments were pulverized using a mortar and a pestle and polyp proteins were extracted as previously described by Seveso et al. (2013, 2014), removing any *Symbiodinium* contamination from the extracts. All protein samples were frozen at −20°C until used. Protein concentration was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Aliquots of proteins were separated by SDS-PAGE on 8% polyacrylamide gels (Vai et al. 1986) and duplicate gels were run in parallel. After electrophoresis, one gel was stained with Coomassie Brilliant Blue to visualize the total proteins and the other electroblotted onto nitrocellulose membrane for western blotting. Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters. For each blot, 75 ng of standard recombinant human Hsp60 (Enzo Life Sciences) was included as an internal control for signal differences.
across blots and also for quantification. Immunostaining was performed with anti-Hsp60 monoclonal antibody (IgG mouse clone LK-2, SPA-807, Enzo Life Sciences), anti-β-Actin monoclonal antibody (clone C4, MAB1501, Millipore) and secondary antibody anti-mouse IgG conjugated with horseradish peroxidase (Thermo Scientific) as previously reported (Seveso et al. 2013). Binding was visualized using Pierce ECL western blotting substrate followed by X-ray films.

Densitometric analysis was performed on a calibrated imaging densitometer (Bio-Rad GS-800) and the band intensities were quantified using the Image J free software (http://rsb.info.nih.gov/ij/). For each blot, the scanned intensity of the Hsp60 bands was normalized against the intensity of the β-Actin bands, which did not display a significant modulation at the different distances from the brown band, and were consequently used as internal loading control. To quantify the amount of Hsp60 expressed, the scanned intensity of the Hsp60 bands for each blot was normalized against the intensity of the Hsp60 protein standard. Data were expressed as means ± SEMs. Data normality was verified using a Shapiro−Wilk test. One-way ANOVA followed by Tukey’s HSD post hoc tests for pairwise comparisons (Tukey’s HSD multiple pairwise comparisons). (D) Hsp60 levels (mean ± SEM) were determined by densitometric analysis (described in ‘Materials and methods’). Signals of 6 different blots were analyzed. No significant differences were found (1-way ANOVA followed by Tukey’s HSD multiple pairwise comparisons). (C) Effect of BrB disease on Hsp60 modulation. Samples of coral fragments located at different distances from the brown band along the disease progression direction (D1, D2 and D3; see Fig. 2A) were subjected to Western blotting and immunostaining as in A. Samples prepared from healthy colonies (C1) are also shown. A filter representing 6 experimental repeats (n = 6) is shown. (B) Hsp60 levels (mean ± SEM) were determined by densitometric analysis (described in ‘Materials and methods’). Signals of 6 different blots were analyzed. No significant differences were found (1-way ANOVA followed by Tukey’s HSD multiple pairwise comparisons).

RESULTS AND DISCUSSION

In all the Acropora muricata colonies affected by BrB disease, the microscopical analyses of the coral fragments collected at the level of the dark band revealed the presence of a dense mass of ciliates consuming the coral tissue and the total absence of living polyp tissue (Fig. 2B,C). As shown in Fig. 2D, the ciliates have an elongated, tube-like shape that is rounded at both posterior and apical ends, similar to other described ciliate taxa responsible for coral BrB disease (Bourne et al. 2008, Lobban et al. 2011, Sweet & Bythell 2012). Despite the extensive literature that highlights the opportunistic nature of BrB ciliates in rapidly invading corals through several vectors and their high rates of spreading, reproduction, virulence and tissue consumption (Ulstrup et al. 2007, Nugues & Bak 2009, Katz et al. 2014, Randall et al. 2015), little is currently known about the mechanisms employed by corals to fight and resist ciliate infection. For this reason the expression of Hsp60, which represents a useful molecular biomarker for detecting early signs of change in a coral’s physiological state caused by both abiotic and biotic stresses (Brown et al. 2002, Downs et al. 2005, Chow et al. 2012, Seveso et al. 2013, 2014), was analyzed in coral affected by the BrB disease at different distances along the advancing front of the infection.

In all the healthy colonies of A. muricata chosen as control, no significant difference in Hsp60 levels was detected among coral fragments collected at different distances from the tip of the coral branch (Fig. 3A). Based on densitometric analysis (Fig. 3B), a similar basal level of Hsp60 was found (2.24 ± 0.18,
2.09 ± 0.23 and 2.12 ± 0.23 ng Hsp60 µg⁻¹ proteins in coral fragments C1, C2 and C3, respectively (ANOVA, Tukey’s HSD post hoc tests for pairwise comparison of means; p ≥ 0.05 comparing C1, C2 and C3; Table 1). This also confirms the important role of this chaperonin even under normal physiological conditions (Choresh et al. 2001, Chow et al. 2009, Seveso et al. 2014). Consequently, only a representative control sample (C1) is shown in Fig. 3C. In contrast, a modulation of Hsp60 was observed in A. muricata infected by BrB disease, with different Hsp60 levels being found in the different parts of the same colony, suggesting that the distance from the diseased polyps can affect the Hsp60 trend (Fig. 3C). In fact, a strong down-regulation of Hsp60 expression was detected near the infected portions of the coral, with protein levels progressively increasing with distance from the dark band and returning to levels comparable to that found in the control only at the farthest distance from the band (D3). The densitometric analysis confirmed that Hsp60 levels approached values close to zero (0.48 ± 0.18 ng Hsp60 µg⁻¹ proteins) in the coral fragments sampled just above the dark band (D1) on the interface of ciliate progression (Fig. 3D), resulting in significantly lower values than those detected in the control (ANOVA, Tukey’s HSD post hoc tests for pairwise comparison of means; p < 0.05 when compared to C1; Table 1). Finally, in coral fragments sampled farthest from the dark band (D3), the Hsp60 level was almost twice compared to that in D2 (2.11 ± 0.14 ng Hsp60 µg⁻¹ proteins) and reverted to values close to those in the control samples (p ≥ 0.05 when compared to C1, p < 0.05 when compared to D1) (Fig. 3C,D, Table 1). In order to highlight the presence of the infection as the sole cause of the Hsp60 trend in the A. muricata colonies, it should be noted that all the coral tissue samples displayed normal tissue pigmentation, did not show morphological differences, were free of necrosis and were undamaged structurally. Furthermore, no significant changes in temperature and light intensity were observed within different locations of the sampling area during the sampling time and among different days (Table 2). Seawater temperatures were consistent with the normal mean seasonal trend (29.28 ± 0.26) recorded during the northeast monsoon season from December to April. In addition, no anomalies in the salinity values (~35.5‰) were detected.

The mitochondrial chaperonin Hsp60 is essential in mitochondrial biogenesis and in the synthesis and transport of essential proteins from the cytoplasm into the mitochondrial matrix, playing a central role in the folding of newly imported and stress-denatured proteins in the mitochondria (Hood et al. 2003). Generally, the up-regulation of the Hsp60 level implies a general shift in the protein chaperoning and degradation within the mitochondria, accompanied by

### Table 1. Tukey’s HSD multiple pairwise comparisons of means for all the normalized Hsp60 intensity values obtained from the different groups of samples (C1, C2, C3 and D1, D2, D3). N = 6 for each group. Statistically significant differences (p < 0.05) are indicated in bold

<table>
<thead>
<tr>
<th></th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
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<tr>
<td>C1</td>
<td>–</td>
<td>0.75</td>
<td>0.887</td>
<td>0.001</td>
<td>0.006</td>
<td>0.832</td>
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<tr>
<td>C2</td>
<td>0.75</td>
<td>–</td>
<td>0.999</td>
<td>0.004</td>
<td>0.008</td>
<td>0.999</td>
</tr>
<tr>
<td>C3</td>
<td>0.887</td>
<td>0.999</td>
<td>–</td>
<td>0.003</td>
<td>0.007</td>
<td>0.999</td>
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<tr>
<td>D1</td>
<td>0.001</td>
<td>0.004</td>
<td>0.003</td>
<td>–</td>
<td>0.016</td>
<td>0.004</td>
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<tr>
<td>D2</td>
<td>0.006</td>
<td>0.008</td>
<td>0.007</td>
<td>0.016</td>
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<td>0.009</td>
</tr>
<tr>
<td>D3</td>
<td>0.832</td>
<td>0.999</td>
<td>0.999</td>
<td>0.004</td>
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### Table 2. Sea temperature (°C) and light intensity (lux) recorded by the 3 data loggers (DL1, DL2, DL3) placed in different locations within the sampling area. Values measured during 5 representative days randomly selected during the sampling period (December 2013) are reported. The means (±SD) of both parameters recorded by the different data loggers in the different days are also shown.

<table>
<thead>
<tr>
<th>Day</th>
<th>DL1</th>
<th>DL2</th>
<th>DL3</th>
<th>Mean (±SD)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Temperature (°C)</td>
<td>Light intensity (lux)</td>
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<tr>
<td>Day 1</td>
<td>29.3</td>
<td>29.14</td>
<td>29.23</td>
<td>29.22 (±0.09)</td>
</tr>
<tr>
<td>Day 2</td>
<td>29.5</td>
<td>29.32</td>
<td>29.51</td>
<td>29.44 (±0.11)</td>
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<tr>
<td>Day 3</td>
<td>29.34</td>
<td>29.18</td>
<td>29.26</td>
<td>29.26 (±0.09)</td>
</tr>
<tr>
<td>Day 4</td>
<td>29.25</td>
<td>29.49</td>
<td>29.32</td>
<td>29.35 (±0.13)</td>
</tr>
<tr>
<td>Day 5</td>
<td>29.31</td>
<td>29.37</td>
<td>29.15</td>
<td>29.27 (±0.11)</td>
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<td>Mean (±SD)</td>
<td>29.34 (±0.10)</td>
<td>29.3 (±0.14)</td>
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changes in the equilibria of mitochondrial-associated metabolic pathways (Papp et al. 2003). In addition, this Hsp is also involved in the immune response, particularly interacting with the Toll-like receptor (TLR) signaling pathway (Pockley et al. 2008, Quintana & Cohen 2011). In corals, many immune mechanisms for resisting infections and maintaining tissue integrity have been described, including TLRs, the melanin-synthesis pathway, a component of the phenoloxidase pathway and antimicrobial and enzymatic activities (Geffen & Rosenberg 2005, Mydlarz & Harvell 2007, Palmer et al. 2008, 2011, 2012, Dunn 2009, Mydlarz et al. 2010, Palmer & Traylor-Knowles 2012, Libro et al. 2013); however, few reports have examined the involvement of a Hsp in coral immune response to any coral disease (Seveso et al. 2012, Brown et al. 2013). In this context, transcriptional up-regulation of the *hsp70* gene in the coral *A. millepora* infected by *Vibrio corallilyticus* has been proposed to be an element of the defense response of the coral, possibly by activating other components of the coral effector immune systems, such as the phenoloxidase cascade (Brown et al. 2013). Similarly, Baruah et al. (2011) presented evidence that Hsp70 up-regulation in the shrimp *Artemia* sp. increased resistance to pathogens by priming and enhancing the expression of the phenoloxidase system.

Interestingly, our results showed that Hsp60 expression was higher in the coral portions farther from the disease front that can potentially be infected than in those closer to the infection. This modulation of the Hsp60 contrasts with that in the same coral species infected by the ciliate *Halofolliculina corallasia* that causes SEB disease in the same geographic area (Seveso et al. 2012); a significant increase in Hsp60 compared to the healthy control was observed in colonies near the advancing front of the ciliate mass. The extremely low level of Hsp60 observed in front line colonies affected by BrB could indicate that the defense mechanisms were probably already overly stressed and were unable to counteract the strong cellular stress produced by the ciliates. Consequently, the physiological status and health of these coral polyps could probably be already compromised and ‘lost’ by the organism. Thus, even in neighboring cells that were not yet directly infected, the ciliate presence could have caused cellular damage. The BrB ciliates have been observed to migrate along the length of branching corals from base to tip at a rate much faster than SEB ciliates do. While SEB progression rate in *A. murioca* has been estimated to be at a maximum of 1 to 2 mm d⁻¹ (Antonius & Lipscomb 2001, Page & Willis 2008), the mean rate of BrB progression on diseased branches of *A. murioca* in the field varies from 5 to 20 mm d⁻¹ (Uistrup et al. 2007, Lobban et al. 2011, Nicolet et al. 2013, Katz et al. 2014, Randall et al. 2015). We speculate that this rapid migration of the BrB ciliate would not give the coral defense mechanism the time necessary to react in an attempt to block and confine the infection.

Alternatively, since the BrB ciliates are only feeding on coral tissue and may not produce harmful secretions that could inhibit Hsp expression, the Hsp60 down-regulation observed could be explained by referring to a study aimed at establishing the microbial diversity (bacteria and ciliates) associated with the BrB disease (Sweet & Bythell 2012). In this study, the authors suggested that bacteria such as *Arcobacter* sp. and *Aeromonas* sp., could be the primary disease causing agents in corals subjected to BrB; by invading healthy tissues and impairing physiological functions, bacteria allow ciliates to subsequently invade and consume the coral tissues (Sweet & Bythell 2012). In this context, one can hypothesize that the decrease in Hsp60 could be linked to toxic substances secreted by these pathogenic bacteria.

In conclusion, the present study provides new insights into the physiology of scleractinian corals subjected to epizootic disease. The results further support the notion that Hsp60 expression may constitute a useful tool for checking specific variations in coral physiological and cellular parameters, which cannot be detected simultaneously at the morphological level. Furthermore, the present data also suggest that different pathogens could trigger differences in Hsp modulation (cf. Seveso et al. 2012). Since ciliate feeding behavior and their role in coral tissue mortality remain unclear (Yarden et al. 2007), further investigation of other coral diseases and other molecular biomarkers is needed in order to elucidate major ecological and molecular aspects of pathogen–host relationships.

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