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Assessing the expression of different biochemical indicators in scleractinian corals subjected to biotic and abiotic stresses.

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Citazione

Sii come le onde del mare, che pur infrangendosi contro gli scogli hanno la forza di ricominciare.

Jim Morrison

ABSTRACT

In the last few decades about the 27% of coral reefs have been destroyed worldwide caused by different environmental stressors, both abiotic and biotic. Moreover one of the main causes of coral reef destruction has been the dramatic increase in coral disease.

In reef building corals, as well as in other organisms, several components of the cellular stress response can be used as diagnostic indicators of stress, in order to assess their cellular physiological condition, expressing them with significant different patterns in relation to different stressors. In this study the diagnostic indicators used were: Heat shock protein 60-kDa (Hsp60), Heat shock protein 70-kDa (Hsp70), Heme-oxygenase (HO-1), and Manganese Superoxide Dismutase (MnSod), all involved in cellular response to stress. Two of the most studied diseases responsible for ongoing coral losses on Indo-Pacific reefs were chosen as the biotic stressors: the Brown Band Disease (BrB) and Black Band Disease (BBD). BrB is a virulent coral disease characterized by a dense concentration of ciliates ingesting coral tissue. In order to investigate the effect of the ciliate presence in the coral physiology, the level of the mitochondrial Hsp60 was analyzed in colonies of *Acropora muricata* affected by BrB in a Maldivian reef. Samples in the apparently healthy coral polyps located at different distances along the advancing front of the infection were analyzed. The BBD is characterized by a thick microbial mat, dominated by phototrophic cyanobacteria, which is responsible of the disease virulence and create the characteristic necrotic dark band. It is known to be persistent in reef, contributing to the long term mortality of the infected coral. In Maldives waters one of the highest prevalence was observed in *Goniopora columna*, which show a very slow progression rate. Due to its high persistence in infected corals, colonies of *G. columna* were analyzed in two time periods, space out by one year. Samples in the apparently healthy coral polyps were collected at three different fixed distances along the advancing front of the infection from the black band of necrotic tissue, in order to analyze how the disease's progression affect the expression of Hsp60 and Hsp70, HO-1, and MnSod. Finally Hsp60, Hsp70 and HO-1 were analyzed in relation to nictemeral and seasonal variations of temperature and light, in three different species of reef building corals living in the Maldivian waters: *Acropora tenuis*, *Echinopora lamellosa* and *Porites lobata*. These three species were chosen for their different growing morphology and susceptibility to stress. Samples were taken in November for the wet season and March for the dry season in order to made a comparison between the two seasons. During each selected month, corals were sampled in six time intervals while temperature and

light intensity were measured by data-loggers attached to the colonies. Results show the biochemical indicators used being modulate in different ways in relation to species and stressors.

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– Chapter 1 –

Background and General Introduction

1.1 Biology of the scleractinian corals

Scleractinian corals belong to the Cnidaria (class Anthozoa), a metazoan phylum, and represent the major building blocks of coral reefs. Coral reefs constitute some of the largest and most diverse ecological communities on earth, containing almost the 25% of the total marine species in only the 0.2% of the ocean total surface (Hoegh-Guldberg, 1999) and supporting an extraordinary biomass and diversity of life. Furthermore, coral reefs protect the shorelines from storms and wave action, making them suitable for human settlement and providing an invaluable array of human services (Pomerance, 1999; Hoegh-Guldberg et al., 2007). Scleractinian corals, known also as stony corals, live in relatively stable, often oligotrophic, environments (30° N and S of the equator) and are typically colonial animals composed by many genetically identical units called polyps. Their capacity to efficiently deposit an external calcium carbonate skeleton, modifying the sea substratum into the vast three-dimensional structures, plays an essential role in shallow tropical oceans. This extensive calcification results from the mutualistic symbiotic interactions between photosynthetic microscopic unicellular dinoflagellate algae, called zooxanthellae (*Symbiodinium* spp.), and the polyps. *Symbiodinium* inhabit coral tissues in concentration of millions of cells per square centimeter and use sunlight to get energy via photosynthesis, providing a metabolic benefit to their coral hosts. Zooxanthellae translocate carbohydrates, amino acids and lipids to the host (Trench, 1971) and represent the majority of daily carbon requirement for coral growth and skeletal deposition (Gates et al., 1995). In exchange, they receive, from the cnidarian host, inorganic nitrogen, phosphorus and carbon as well as a high light exposure environment and protection from predation (Venn et al., 2008; Yellowlees et al., 2008).

Each individual coral colony contains only one or at most several individual clades of *Symbiodinium* at a time, and a large, diverse and specific population of microorganisms that have co-evolved with corals themselves (Rosenberg et al., 2007). This complex microbiota is called holobiont. Bacteria, Archea, Eucarya (*Symbiodinium* and several fungi) and Viruses

are hosted in the surface mucus layer, in the coral tissue and also in the coral skeleton. The species of Bacteria, such as Cyanobacteria, *Pseudomonas* nitrogen fixers and chitin decomposers, hosted in the holobiont are highly associated with particular coral species, and also the bacterial community differs from those found in the surrounding seawater. Symbiotic microorganisms benefit their host by various mechanisms such as photosynthesis, nitrogen fixation and production of antimicrobials in response to pathogen attacks. The relationship existing between microorganisms and polyps is considered to be dynamic and to respond to different environmental condition that selects the most advantageous coral holobiont (Rosenberg et al., 2007). By altering the structure of its resident microbial community the corals can adapt to changing environmental conditions rapidly and with great versatility (Mydlarz et al., 2009, Rosenberg et al., 2007)

1.2 Coral reef under threats

The reef health worldwide is seriously threatened by both biotic and abiotic factors. In the last few decades about the 27% of coral reefs have been destroyed worldwide (Frias-Lopez et al., 2004). Although corals have a remarkable ability to acclimatize to changes in solar irradiance (Anthony & Hoegh-Guldberg, 2003) abnormally elevated and low ocean temperatures, high UV radiations, severe changes in salinity, destructive storms, pollution, predation outbreaks (e.g. crown of thorn starfish), microbial attacks and imminent sea level rise (Dustan, 1999; Marshall & Schuttenberg, 2006) are contributing to weaken corals health. Furthermore, in the recent years an increase in the incidence of epizootic disease has been notice in many corals (Cervino et al., 2004, Sutherland et al., 2004, Montano et al., 2012). Furthermore, the increasing concentration of atmospheric and dissolved CO_2 resulted in a more acidic ocean chemistry leading to slowed deposition of calcium carbonate skeletons (Kleypas et al., 1999).

Also anthropogenic activities, such as destructive fishing practices, coastal development, agricultural land-use, increased nutrients and sediment load from run-off and eutrophication, contribute to threat the reef health. According to Wilkinson (2004, 2008) about 20% of the world's reefs have been already irreparably damaged, and a further 24% are threatened by anthropogenic pressures. Moreover, Frias-Lopez and colleagues (2004) stated that in the last few decades about the 27% of coral reefs have been destroyed worldwide.

In general the stressed condition of corals represent one of the major concerns of the last

decades for the marine environmental health as they posed a serious threat to its stability. "Stress" can be defined as *any external force or stimulus, or any environmental alteration, that causes a gradient between ideal conditions and the ultimate limits of survival* (Brown & Howard, 1985) and *extends homeostatic or protective processes into a compensatory state beyond the normal limits of an organism* (Moore, 2002).

Under adverse circumstances, the equilibrium between the partners of the holobiont may be compromised. This disequilibrium can culminate with the breakage of the symbiosis, generating the phenomenon known as coral bleaching, which represent one of the main cause of degradation of coral reefs worldwide (Hughes et al., 2003). During the coral bleaching events the *Symbiodinum* algae were released from the polyps and changes in the microbial composition occurred, which can lead to disease and also increase the susceptibility toward pathogens virulence.

The rapid spread of marine pathogens, the global warming and the increase of the anthropogenic disturbances enhanced the virulence and rate of transmission of pathogens, also compromising the mechanisms of host resistance (Mullen et al, 2004). In this context, more than twenty coral diseases have been described in the past 30 years and for only six of them the causative agent has been isolated and characterized. To date, among the most studied coral diseases there are the Black Band Disease and Brown Band Disease.

1.3 Coral Diseases

The first disease found in corals was identify in 1965 (Squires, 1965) despite the presence of bacterial microrganisms were found within the coral tissue since the beginning of '900 (Duerdon, 1902). The study of coral disease resulted to be complicated due to the polymicrobial nature of most diseases and the difficulty to fullify Koch's postulates without controversity (Mydlarz et al., 2009). Also the microbial agents which trigged the infection could not be the pathogenous itself (Sato et al., 2010). Actually the number of diseases recorded for the Carribean is 29 (Weil, 2004), 7 in the Indo-Pacifico Ocean (Willis et al., 2004), among which 4 were diffused globally (Sutherland et al. 2004). The most important coral diseases until now recognized are Skeletal anomalies (Squires, 1965), Black Band disease (Antonius, 1973), White Plague Type I, White Plague Type II (Richardson et al., 1998; Dustan, 1977), White Band Type I (Gladfelter, 1982), White Band Type II (Ritchie & Smith, 1998), White Pox disease (Holden, 1996), *Vibrio shiloi*-induced bleaching (Kushmaro et al. 1996), Yellow Blotch/Band (Santavy & Peters, 1997), Yellow Band

(Korrûbel & Riegl, 1998), Dark Spots (Goreau et al., 1998), Skeleton Eroding Band (Antonius & Lipscomb, 2001), Fungal-Protozoan syndrome (Cerrano et al,. 2000), White Plague Type III (Richardson et al. 2001), Pink-line syndrome (Ravindran et al., 2001), *Vibrio coralliilyticus*-induced bleaching and disease (Ben-Haim & Rosenberg, 2002), Brown Band disease (Willis et al., 2004).

Diseases could cause a lot of very different effects on corals as a decline in fertility, and in growing efforts as well as the death. Also diseases could effect the reef comunity as a whole, with the decline of the infected species and the related alteration of the community structure and interactions. Often this situations lead to an habitat shift, as in Belize (Aronson et al., 2002) where death of the dominant species belonging to the familia Acroporidae pushed the predominance of Agaricidae. Often these disease spread due to the presence of predators such as *Drupella sp.*, which may play an important role as vectors and also create feeding scars from which the disease started (Nicolet et al., 2013). Also the fireworm *Hermodice caranculata* plays a role in diseases diffusion being a winter reservoir and summer vector of *Vibrio shiloi* (Sussman et al., 2003). The mollusk *Coralliophila abbreviata* is a vector and interepizootic reservoir of the White Pox pathogen (Sutherland et al., 2011) and the crown-of-thorns starfish, *Acanthaster planci* is considered a potential Brown Band vector (Nugues & Bak, 2009).

In the Indo-Pacific reefs at least 7 different diseases were recorded, as White syndrome, Ulcerative White Spot disease, Black Band disease, Brown Band disease, Skeletal Eroding band, Porites Dark Discoloration Response, (Raymundo et al., 2005; Willis et al., 2004). To date in the Republic of Maldives at least 5 coral diseases were found: Black Band Disease, Ulcerative White Spot Disease, White Syndrome, Brown Band Disease and Skeleton Eroding Band (Montano et al., 2012).

1.3.1 Black Band Disease

Black Band Disease (BBD) is one of the oldest and most studied coral disease. It is a widespread disease ranging from the Atlantic Ocean to the Indo-Pacific (Barneah et al., 2007), including the Red Sea and the Great Barrier Reef (Page & Willis, 2006), affecting a wide range of corals. To date BBD is described in relation with 9 coral families (Acroporidae, Faviidae, Poritidae, Siderastreidae, Agariciidae, Fungiidae, Pocilloporidae, Mussidae and Dendrophylliidae) (Page & Willis, 2006; Montano et al., 2013) and it is

characterized by a thick microbial mat, which can migrate up to 2 cm per day across apparently healthy coral colonies, actively killing tissue and leaving the skeleton exposed behind. At least 64 different species of bacteria were identified living into this consortium (Frias-Lopez, 2004). BBD infection is known to be persistent in the reefs, contributing to the long term mortality of the infected coral species (Bruckner & Bruckner 1997). The microbial community, dominated by cyanobacteria, is considered to be the main responsible of the disease virulence. This microbial mat include also sulfate-reducing *Desulfovibrio* species, sulfide-oxidizing *Beggiatoa* species and other heterotrophic microbes and marine fungi (Frias-Lopez et al., 2004). Nevertheless the identification of all this microbes is still uncertain as well as its complex developing and the nature of the primary causative agents. Even the presence of *Beggiatoa* species is in doubt as, in some cases, this species were not found in the mat (Frias-Lopez et al., 2004; Cooney et al, 2002), leaving the oxidation of sulfide happening in the BBD mat in need to be explained otherwise, probably by the activity of other organisms such as species of ε-proteobacterium which can oxidate sulfide into sultfate (Cooney et al., 2002). The first pathogen identified was a filamentous nonheterocystous cyanobacterium, *Phormidium corallyticum* (Rützler & Santavy, 1983), but a recent study found that the most common cyanobacterium was not *P.corallyticum* but a member of the genus *Geitlerinema,* closely associated to *Oscillatoria* species (Myers et al., 2007). However a lot of other bacteria have been proposed as possible primary causative agent of the disease in recent years. For example Cooney and colleagues (2002) found homology to *Cytophaga* species, to an α-proteobacteria already associated with Juvenile Oyster disease and to *Oscillatoria* coralline (which was, however, present also in non-BBD samples).

Sato and colleagues (2010) found successional changes occurring during the development of the disease in *Montiopora hispida,* and suggested that bacterial pathogens which trigged the BBD infection may be very different from which caused the BBD virulence. Moreover, in the 19% of the studied cases some cyanobacterium-infected lesions were found to evolved into BBD with a change in cyanobacterium species involved. It is still unclear which factors govern, whether a cyanobacterium-infected lesion develops in BBD or just loses the active microbial front. It has been suggested that the pathogens that trigger the BBD may become less abundant during the subsequent successional changes and this may be why it is so difficult to identify the primary causative agents of BBD.

It is also known that cyanobacterial toxins can have a role in the pathogenicity of BBD (Richarson et al., 2007) and that the presence of high concentrate of sulfide at the base of the

BBD mat, in anoxic condition, cause the necrosis of coral tissue (Richardson, 1997). The high concentration of cyanobacteria, the presence of bacterial sulfur cycle with nitrogen, iron, phosphorus and carbon cycles tied to it, may promote further anoxic sulfide rich micro-environment and hence the BBD progression (Sato et al., 2010). Also environmental factors as light, nutrients and temperature have been correlated with the prevalence and progression of this disease (Sato et al., 2011, Boyett et al., 2007: Voss & Richardson, 2007, Muller & Van Woesik, 2011). Since to date any of the proposed primary pathogens have fulfilled Koch's postulates (Richardson, 2004), it can be probably that the entire BBD community is the whole pathogenic consortium working as primary pathogen rather than an individual one.

1.3.2 Brown Band Disease

Brown Band (BrB) disease is widespread in Great Barrier Reef and is considered an ongoing disease in the whole Indo-Pacific reefs. It is known to affect at least three major coral families, including members of the *Acroporidae, Pocilloporidae* and *Faviidae* (Willis et al., 2004).

The most distinctive and macroscopic sign of BrB is a brown band formed by a dense concentration of motile protozoan ciliates that feed on coral tissue and accumulate zooxanthellae (*Symbiodinium*) intracellularly, resulting in the characteristic brown coloring and in rapid tissue loss (Willis et al., 2004; Ulstrup et al., 2007; Lobban et al., 2011; Nicolet et al., 2013; Sweet et al., 2013). Molecular studies showed that these ciliates belong to the class Oligohymenophorea, subclass Scuticoliatia (Bourne et al., 2008).

At least two distinctive morphs of ciliates have been found at the lesion interface, Sweet and Bythell (2012) found that one was closely related to *Philaster digitiformis*, while the second was related to *Philaster guamensis*. Since different species of ciliates ingest *Symbiodinium* cells 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. BrB has an high progression rate (up to 2.16 cm $days^{-1}$) (Nicolet et al., 2013). Also ciliate of the BrB do not seem to increase in virulence with increasing temperature but some variation in biological characteristics of colonies, for example coral bleaching presence, can influence the prevalence and progression of BrB (Nicolet et al., 2013). It is also hypnotized that the presence of ciliates might attract micro-invertebrates, as copepods

(Ajiboye et al., 2011), to which the band might represent an easy source of nutrients (Nicolet et al., 2013).

The transmission patterns and potential pathogen vectors are still unknown, although this disease is well documented and significant efforts have been performed in the identification of the microbial communities associated with it. To date, few vectors of the BrB were hypothesized: the crown-of-thorns starfish (*Acanthaster planci*) (Nugues & Bak, 2009), the coral-eating snail (*Drupella* sp.) (Nicolet et al., 2013) which produces large scars while feeding on the corals, that have been observed to be the origin of BrB infection.

1.4 Expression of Heat shock proteins in relation to stresses

Marine environment is a complex habitat and fluctuations in amplitude and frequency of both biotic and abiotic conditions (Brown, 1997) and the physical characteristics of seawater itself, can make the aquatic environment extremely stressful to its inhabitants (Feder & Hofmann, 1999). Scleractinian corals in particular, being sessile organisms inhabiting several niches in the intertidal zone, are subject to a wide range of these stressors, undergoing constant direct exposure to the surrounding environmental conditions.

In general extreme or unexpected, short or long-term environmental fluctuations could be very stressful for all marine organisms and can cause cell damages. To avoid these dangerous situations, organisms have developed several mechanisms, such as behavioral adaptations, morphological changes, physiological regulations and biochemical and cellular specializations (Feder & Hofmann, 1999).

Corals, instead, seem to lack any developed physiological regulatory system and for this reason they are expected to possess well-developed cellular adaptation abilities (Brown, 1997). A recent research showed that corals could develop resistance to specific pathogens and higher temperature (Rosenberg et al., 2007). In this context, scleractinian corals are able to release bactericidal materials in response to mild mechanical stresses as the bites by predators (Geffen et al., 2004), which can serve as vectors to several coral disease (Sussman et al., 2003). Also mucus release on coral epidermis can both work as a mechanical and anticmicrobial barrier, acting as a first barrier (Mydlarz, et al., 2009; Rosenberg et al., 2007). Once these external barriers had failed, or together with them, corals can rely on their cellular adaptation abilities. One of the most important cellular defense mechanism for

corals is the rapid increase of the induction of a set of stress proteins called Heat shock proteins (Hsps) (Lindquist, 1986). This defense mechanism is ubiquitous and conserved throughout evolution occurring in all organisms from bacteria to humans (Fink, 1999; Kregel, 2002). Heat shock proteins are categorized into several families, named on the basis of their approximate molecular mass and specific functions: small Hsps, 40-kDa Hsp, 60-kDa Hsp, 70-kDa Hsp, 90-kDa Hsp and 110-kDa Hsp (Tab. 1).

Hsps play important physiological roles both in normal conditions and in situations involving systemic and cellular stresses (Kregel, 2002). Under normal cellular physiological conditions, the Hsps usually works as molecular chaperones. The chaperones are multicomponent molecular machines that promote folding through ATP- and cofactor-regulated binding and release cycles (Hartl et al., 2011). Hsps typically function as oligomers, as well as complexes of several different chaperones, co-chaperones, and/or nucleotide exchange factors (Feder & Hofmann, 1999).

Hsps are involved in a multitude of proteome-maintenance functions regulating the protein homeostasis (Parsell & Lindquist, 1993; Sanders, 1993; Fink, 1999). In fact, they participate in the folding and assembly of other proteins, such as *de novo* folding of proteins, refolding of stress-denatured proteins, prevention of oligomeric assembly, protein trafficking and assistance in proteolytic degradation, as well as in the intracellular protein transport and in the degradation of damaged proteins (Hightower, 1991; Gething & Sambrook, 1992; Vabulas et al., 2010; Hartl et al., 2011). Hsps are also involved in the breakdown and reorganization of tissues (Sanders, 1993; Rutherford & Lindquist, 1998), in gametogenesis (Dix, 1997), in apoptosis (Garrido et al., 2001), in signal transduction (Nollen & Morimoto, 2002), in translocation across membranes (Agarraberes & Dice, 2001), in pathological processes (Macario & de Macario, 2000) and in vertebrate immune response (Moseley, 2000; Pockley, 2003).

An induction and an up-regulation of the expression of Hsps constitutes an emergency response that allows the organism to improve the tolerance to harsh conditions (Parsell & Lindquist, 1993). Under stressful conditions cells increase either the amount or the activity of a transcription factor specific for the heat shock genes. which leads to an increase in the concentration of Hsps in the cell (Craig & Gross, 1991). High levels of specific Hsps are maintained throughout the exposure to stress. However, the magnitude of the response and its duration depend on the severity of the stress and the sensitivity of the organism (Lindquist, 1986; Feder, 1999).

Major family, and members	Intracellular localisation	Intracellular function
Small Hsps		
αB-crystallin	Cytoplasm	Cytoskeletal stabilisation
Hsp27	Cytoplasm/nucleus	Actin dynamics
Haem oxygenase, Hsp32	Cytoplasm	Haem catabolism, antioxidant of properties
Hsp40		
Hsp40	Cytoplasm/nucleus	Regulates the activity of Hsp70;binds non-native proteins
Hsp47	ER	Processing of pro-collagen; processing and/or secretion of collagen
Hsp60 (or chaperonins)		
Hsp60	Mitochondria	Bind to partly folded polypeptides and assist correct folding. Assembly of multimeric complexes
TCP-1	Cytoplasm	
Hsp70		
Inducible: Hsp70, Hsp70hom	Cytoplasm/nucleus	Bind to extended polypeptides. Prevent aggregation of unfolded peptides. Dissociate some oligomers. ATP binding. ATPase activity. Hsp70 downregulates HSF1 activity
Cognate/constitutive: Hsc70	Cytoplasm/peroxisome	
Grp78/BiP	ER	
mtHsp70/Grp75	Mitochondria	
Hsp90		
Hsp90 (α and β)	Cytoplasm	Bind to other proteins. Regulate protein activity. Prevent aggregation of refolded peptide. Correct assembly and folding of newly synthesised protein. Hsp90 assists the maintenance of the HSF1 monomeric state in non- stressful conditions.
Grp94/gp96/Hsp100	ER	
Hsp110		
Hsp110 (human)	Nucleolus/cytoplasm	Thermal tolerance
Apg-1 (mouse)	Cytoplasm	Protein refolding
Hsp105	Cytoplasm	

Tab. 1 - Heat shock protein families and their intracellular location and function. ER: endoplasmic reticulum. TCP-1: tailless complex polypeptide. Grp: glucose regulated protein. Hsp70 hom: testis-specific Hsp70. BiP: immunoglobulin heavy chain binding protein. Mt: mitochondrial. Apg-1: protein kinase essential for autophagy. From Pockley (2003)

1.4.1 Mitochondrial 60 kDa Heat shock protein (Hsp60)

The 60-kDa heat shock proteins (Hsp60) belong to the group of the chaperonins which are ring-shaped chaperones encapsulating non-native proteins in an ATP-dependent manner (Ritcher et al., 2010). Chaperonins are large double-ring complexes of approximately 800 kDa enclosing a central cavity. They occur in two subgroups that are similar in architecture but distantly related in sequence (Vabulas et al., 2010). Group I chaperonins (also called Hsp60s) occur in bacteria (Hsp60's bacterial homolog is called GroEL), mitochondria, and chloroplasts. The function of type I chaperonins is performed by the cooperation of two proteins, Hsp60 and Hsp10, as folding chamber and co-chaperone respectively, with the help of hydrolyzed ATP (Cheng et al., 1989, Bukau & Horwich, 1998). Group II chaperonins exist in archaea (thermosome) and in the eukaryotic cytosol (TRiC/CCT).

Hsp60 has been deemed essential in mitochondrial biogenesis, in the synthesis and transportation of essential mitochondrial proteins from the cell's cytoplasm into the mitochondrial matrix, playing a central role in the folding of newly imported and stress-

denatured proteins in the mitochondria (Martinus et al., 1995; Hood et al., 2003). Hsp60 possesses two main responsibilities with respect to mitochondrial protein transport: they catalyze the folding of proteins destined for the matrix and maintains protein in an unfolded state for transport across the inner membrane of the mitochondria (Koll et al., 1992). The hydrophobic portion of the Hsp60 is responsible for maintaining the unfolded conformation of the protein for trans-membrane transport (Koll et al., 1992). Cnidarian Hsp60 also functions to mature nuclear-encoded, mitochondrial imported proteins into their active state and is the major mitochondrial chaperonin (Down, 2006). Elevation of this protein signifies that there has been a general shift in the protein metabolic condition of the mitochondria, as chaperoning and degradation, implicating a possible change in the equilibria of many mitochondrial associated metabolic pathways (Papp et al., 2003; Down et al., 2006).
Finally, Hsp60 is also known to have anti-apoptotic ability and be able to protect against cell death by maintaining mitochondrial oxidative phosphorylation. In addition to its critical role in protein folding, Hsp60 is involved in the replication and transmission of mitochondrial DNA (Arya et al., 2007).

1.4.2 Cytosolic 70 kDa Heat shock protein (Hsp70)

The 70-kDa heat shock protein (Hsp70) is a cytosolic chaperonine which function is a crucial element in the maturation of newly made proteins to gain their active state. Hsp70 can also shuttle nascent protein within cells and renature degraded proteins. Hsp70s contain a peptide binding domain which is attached to the chaperoned protein and is released through ATPase activity carried out by their ATPase domain in order to prevent the uncorrect folding. (Moshe et al., 1999). In eukaryotic cells, the sequences are divided into four clusters, corresponding to their localization into the cell: cytosolic/nuclear cluster, endoplasmic reticulum (ER), mitochondrial cluster and, for plants, in the chloroplast. Accumulation of this protein generally represent a signal of a shift in the metabolic condition but the direction is often indeterminate by the examination of the Hsp70 alone and must considered in conjunction with other protein metabolic markers. The Hsp70 level seems to increase in reaction to high temperature variations (Fang et al., 1996; Brown et al., 2002) but response to chemical contaminant or xenobiotic is still uncertain as it increased for example within the exposure time to herbicide Irgarol 1050, (Down et al., 2005), while exposed to violent fuel oil spill it showed a down-regulation (Down et al., 2006). Elevation

of its expression could indicate that the process of protein maturation is up-regulated to compensate for a shift in protein degradation rates (Down et al., 2012; Papp et al., 2003)

1.5 Reaction to Oxidative stresses

1.5.1 Physiological conditions

All biological organisms maintain a redox environment into their cells throughout enzymes activities. Reactive oxygen species (ROS) are produced via the univalent pathway. H_2O_2 is formed by the continued reduction of O_2^-, then HO^+ is formed and reduced to hydroxyl ion and water (Lesser, 2006). As the production of ROS is directly and positively related to the concentration of O_2, oxidative stress are the result of imbalance between the physiological production of ROS and the ability of a biological system to detoxify them or to repair their damages (Lesser, 2006). The consequences of this imbalance can have toxic effects through the production of peroxides and free radicals, which cause important damages to all the components of cells, proteins, DNA and lipids included. Several cellular antioxidants, as enzymes Superoxide Dismutase (Sod), Catalase and Glutathione Peroxidase maintain this balance. There are several species of ROS, as the Singlet oxygen ($^1O2$) which is produced through several photochemical and chemical pathways, the most common are the photosensitization reactions or Superoxide radicals which are containing the superoxide anion (O_2^-), produced as a product of mitochondrial respiration.

Marine organisms use many metabolic pathways in order to maintain homeostasis, to growth and reproduce, which lead to the production of ROS and the subsequent antioxidant defenses required in order to prevent oxidative stress and cellular damage. Eukaryotic algae often exhibit a daily cycling of maximum Sod activities and other antioxidant enzymes. Many marine invertebrates produce ROS in response to xenobiotics, as bivalve mollusks (Lesser et al., 2006). In sea anemones and symbiotic cnidarians apoptosis and necrosis, following thermal stress, can be ROS mediated (Lesser, 2004). In corals, stressors such as heat stress, osmolarity, high light, and ultraviolet radiations can destabilize the photosynthetic electron-transport chain resulting in increased production rates of reactive oxygen species (ROS) (Down et al., 2002). Down and collegues (2002) proposed that algal-generated hydrogen peroxide can diffuse from the algal symbiont into the coral cytoplasm and inside the coral cytosol. It can be ‘neutralized’ by enzymatic and non-enzymatic antioxidant pathways or it

can converted into the hydroxyl radical by Fenton or Haber-Weiss chemistry (Halliwell & Gutteridge, 1999). In this prospective, if the ROS concentration is below a specific threshold, the antioxidant defenses of the coral may work and compensate the ROS damages. On the contrary, if the concentration is above the threshold, ROS will cause oxidative damage. It was also proposed that the corals might expel their endosymbiotic algae when the intensity of the oxidative damage is too much severe for the coral to overcome. This situation was named the "Oxidative Theory of Coral Bleaching" and it was proposed as coral's final defense against oxidative stress, eradicating the dominant source of ROS production. ROS are chemically reactive molecules containing oxygen. In aerobic organisms ROS are produced as a normal product of cellular metabolism in the mitochondria, for example during oxidative phosphorylation. In mitochondria there are two main sites of O_2^- generation, located in the inner mitochondrial membrane: the NADH-dehydrogenase at Complex I and the interface between ubiquinone and Complex III (Brand et al., 2004). After its generation O_2^- is then converted to H_2O_2 by spontaneous dismutation or by Sod in order to preserve the integrity of the inner membrane and the associated complexes, essential to oxidative phosphorylation. As an additional protection the inner membrane is also permeable to H^+ which, even causing some energy loss, reduces ROS production, a process regulated by specific uncoupling proteins. In mammals, ROS accumulation can decrease the organism's fitness because oxidative damage is a contributor to senescence and the accumulation of oxidative damage may also lead to cognitive dysfunction (Stadtman, 1992). Reactive oxygen species can be the responsible of diseases and cellular damages, while also participant in many normal cellular functions, playing an important regulatory role. ROS may react with lipids, proteins and even DNA causing serious damages to them. For example the reaction of ROS, especially HO•, with lipids is one of the most prevalent mechanisms of cellular injury. It is dependent on the degree of membrane fluidity, which depend by the saturation state of the lipid bilayer. The degradation products of lipid peroxidation are aldehydes and hydrocarbons. Lipid peroxidation in mitochondria resulted in multiple effects on enzyme activity and ATP production and on the initiation of apoptosis (Lesser, 2006). ROS reaction on protein results in site-specific amino acid modifications, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electrical charge, and increased susceptibility to removal and degradation. ROS can also damage or degrade a wide range of proteins and their amino acid building, and their accumulation in cells has been hypothesized to be part of the aging process (Lesser, 2006). Moreover the generation of ROS can induce many lesions in DNA finally resulting in

deletions, mutations, and other lethal genetic effects. In normal conditions ROS have important physiological role in cell signal transduction, called redox signaling (Schreck & Baeuerle, 1991), and in the defense from bacterial infections or other pathogens (Fang, 2004). ROS also have been proposed as second messengers, which levels are regulated by the antioxidant systems of cells (Lesser, 2006). Their role as second messengers included the expression of several transcription factors and other signal transduction molecules as heat shock–inducing factor, nuclear factor, mitogen-activated protein kinase (MAPK), (Apel & Hirt, 2004). Oxidative stress also plays a role in apoptosis through several mechanisms, as ROS are implicated as mediators (Hamptona & Orrenius, 1997). Oxidative stress can lead to cell death, through cellular necrosis and apoptosis. High levels of oxidative stress cause cell necrosis, while lower levels cause DNA damage and cell-cycle arrest or even initiate apoptosis (Apel & Hirt, 2004; Lesser, 2006). ROS are also involved in the mediation of morphogenic events associated with the onset of mutualistic symbiotic associations.

ROS are also an important component of plant defense systems against pathogens. The toxicity of ROS, or their derived compounds, may contribute to host cell death during the rapid tissue necrosis at the site of infection (Mehdy, 1994). Moreover O_2^- is directly involved in the apoptotic hypersensitive reaction of higher plants against pathogens, as well as strengthening the cell wall and preventing the spread of the pathogen to other parts of the plant, restricting its movement and reproduction (Jabs et al., 1996). Although the exact manner in which ROS defends the host from invading microbe is not fully understood, likely they may induce damages to microbial DNA. In the mammalian ROS is induced as an antimicrobial defense, for example in human respiratory epithelial cells were able to induce mitrochondrial ROS in response to influenza infection limiting viral replication by the induction of type III interferon and of an antiviral state, (Rada et al., 2008; Kim et al., 2013). Probably in the mechanism of defense ROS play a role by ROS- dependent signaling controls.

1.5.2 Reaction to Superoxide radicals: 25-kDa Sod

Superoxide is a compound containing the superoxide anion (O_2^-) and can be produced by the mitochondrial respiration. O_2^- can act as either an oxidant or a reductant in biological systems. There are two main sites of production, the NADH-dehydrogenase at Complex I and the interface between ubiquinone and Complex III (Brand et al., 2004). It can be also

product by microsomal NADPH-dependent electron transport involving cytochromes P450 (Zangar et al., 2004) or as a product of many enzymes such as Xanthine Oxidase which catalyze a reaction with purines in glyoxisomes of plants (Sandalio et al., 1988). Once produced, it is converted to H_2O_2 by spontaneous dismutation or by the enzyme Superoxide Dismutase (Sod). Superoxide radicals are among the major contributors to oxidative stress. Catalase and Superoxide Dismutase respectively convert hydrogen peroxide and superoxide into oxygen and hydrogen peroxide (which is later converted to water). However, this conversion is not 100% efficient, and residual peroxides persist in the cell. Manganese Superoxide Dismutase (MnSod) is known to be induced, in corals, following abiotic stress such as changes in-salinity conditions (Down et al., 2009). Shift in anti-oxidant defenses are often associated with cellular and tissue lesions following a decrease in the salinity. However also high level of salinity induced MnSod production even if without tissue or cellular major change in tissue morphologies visible. Also MnSod was not found to be produced in cnidarian cells during exposition to chemical contaminant, Irgarol 1051 (Down et al., 2007). Authors suggested that Irgraol may induce the generation of ROS and/or decreasing anti-oxidant defenses which lead to the inability to produce Sod. Accumulation and variations, in a single season, of MnSod and generally Sod, are observed also in relation to unusually high sea-surface temperatures. A strong correlation exhists between antioxidant enzymes and bleaching process, indicating that they may play an important role in preventing bleaching process (Down et al., 2002).

1.5.3 Porphyrin metabolism: the 32kDa Heme-oxygenase

Shifts in the porhyrin metabolic equilibrium reflect major shifts in the cellular metabolism (Down et al., 2009). Porphyrins are essential ligands used by proteins in many physiological processes, such as oxidative phosphoralylation and xenobiotic response (Down et al., 2009). Among the enzymes involved in this metabolism pathway the Heme- oxygenase-1 (HO-1) is essential and used also as a protective mechanism against oxidative stress. HO-1 is an enzyme that catalyzes the decomposition of heme to biliverdin, carbon monoxide and ferrous iron (Schwartzburd, 2001). The heme catabolism leads to the formation of low-molecular-mass redox-active iron, which is a more versatile catalyst of oxidative damage than heme (Lamb et al., 1999). An increase in protein levels of HO-1 suggests an increased demand in heme degradation. Heme contain a complex of iron with protoporphyrin IX

(PPIX) and is essential for the activity of all aerobic cells. It serves as the prosthetic group of numerous hemoproteins, as cytochromes, guanylate cyclase, and nitric oxide (NO) synthase, but can be also dangerous, particularly when released from proteins (Schwartzburd, 2001). HO-1 is known to be up-regulated in response to oxidative stress and toxicant exposure (Schwartzburd, 2001; Down & Down, 2006). Also in animal models the increase of Heme oxygenase expression is induced as a protective mechanism (Morse & Choi, 2002).
HO-1 in corals was found to be expressed more in presence of hypo-salinity stressful conditions (≥20ppt), although porphyrin synthesis pathway resulted unaltered, probably due to the need to increase bilirubin's anti-oxidant potential (Down et al., 2009). Moreover HO-1 increased its levels in response to chemical contaminants, as Irgaol 1051 or fuel-oil spill, suggesting an increase in the broken hemin and an increase in the production of porphyria species (Down et al., 2007; Down et al., 2006).

1.6 Site of study: the Republic of Maldives

The Repubblic of Maldives extends in the Indian Ocean from 7°06' N to 00°45' S of latitude and from 72°13' W to 73°45' E longitudinally and is located at about 700km from Sri Lanka. It is made up of a belt of 23 coral atolls, comprising 1190 coral islands, sand cays and faroes covering an area of 8,920 km^2 (Rajasuriya et al., 2004). Malidivian lagoons ranged from 40 to 60 m of deep, while islands ranged from 0,5 to about 2 km^2 (http://www.themaldives.com/). Island's soil is composed of coral sand (Bianchi et al, 1997) and the flora is scarce and composed of cocconut palm tree, mangroves and various species of salt-resistand plants (Naseer, 1997). Many atolls presented inside the lagoon a coral reef with the peculiar shape of a ring, called "*Faros"*. In the *Faros* is possible to found isolated small reef or patch reefs which are spotted colonies of corals. Also in less deep lagoon is possible to find meadows of phanerogams where often juveniles of many marine species found recovery (Shakeel & Hudha, 1997). The steep side of the coral reef which faced the open ocean or made the boundary of atolls, hosts in the upper part zooxanthellate corals while in the deeper parts there are branching eterotrophic corals without zooxanthellae. In the maldivian waters about 248 species of corals are present, belonging to 57 genera (Pichon & Benzoni, 2007). Maldivian weather is tropical hot and wet, characterized by two distinct seasons: the dry season occuring from November to April and the wet season, from

December to May. During the dry season weather is warmer, generally with higer light intensity with temperature reaching the maximum of 32°C, and moonson blowing from sud-est. In wet season temperature and light intensity are still high but lower if compared to dry season, also monsoon blows from nord-west and rainfall is higher (Edwards et al., 2001). Maldivian coral reef, rappresent the 5% of worlwide coral reefs, and are generally less threatened by human activities than in other parts of South Asia (Rajasuriya et al., 2004). Maldivian human population is low and there are two major economic sectors, tourism and fisheries. The tourism industry has become the largest income and it could presented a threat to the enviroment as inevitabily the constant request of natural resources had lead to a depletion of the biological diversity and the general degradation of the original ecosystem, leading to a damaged and often slow recovering ecosystem (Zhair, 2010). Also the coastal area is starting to be heavy impacted by human activities such as the construnction of seawalls, harbours, docks in the most hinabitat islands, as well as luxuryus and developed resorts (Shareef, 2010). Often the building of these structures involved the use of drastical impactant methods such as the dynamite with consequently disturction of the topography complexity, the biodiversity of reefs and associated fishes. Without the protection of coral reef, a natural barrier against waves actions, islands become less protected and more incline to be shallowed by the rising of the sea levels, another great problems of this area.

1.7 Thesis objectives

This study want to investigate directly *in situ* how scleractinian corals modulate the expression of some physiological and biochemical indicators of cellular stress, in both healthy and diseased colonies living in their natural environmental. In particular, different species of corals showing different morphological and ecological characteristics were analyzed, in order to assess the different susceptibilities of the different species and growth form to both biotic stressors (such as coral diseases) and abiotic stressors (such as temperature and light). The expression of a set of cellular stress biomarkers for detecting early signs of change in a coral's physiological state were used as diagnostic tool, including indicators of the protein homeostasis and the cellular oxidative status.

Despite the growing concerns about coral health in recent years, which however have prompted an increase in study in this field, to date few studies were conducted *in situ* on

marine organisms, monitoring the effect of heat stress in natural environments or as correlate of warming trend (e.g. Hofmann & Somero, 1995; Minier et al., 2000; Lejeusne et al., 2006). Most of the published works focused on the influence of acute or repetitive stresses on the physiology of corals raised or stressed in laboratory conditions. Although these studies provide valid information on specific biological responses to a given and controlled stress, they are less representative and sensitive than fieldwork ones (Chow et al. 2011; Lejeusne et al., 2006). Also despite the application of molecular techniques to address questions related to coral ecology have challenged researchers to try to identify and understand how corals counteract and tolerate different stresses, fewer biochemical indicators were used to asses biotic stress than to asses abiotic ones. Moreover, to date, to the best of our knowledge, any published studies on coral diseases have taken into consideration the oxidative stress as consequence of coral diseases as well as follow the successional changes of all biochemical indicators during the development of the disease. Finally, despite being well used as potential biomarkers of stress in corals, Heat shock proteins still represent a much debated topic, worth of being further investigated. Also their modulation and expression have been barely studied out of laboratory controlled conditions (Lejeusne et al., 2006) and moreover associated to biotic stress (as Seveso et al., 2012).

Specifically the objectives of this PhD thesis are:

- highlights the modulation of Hsp60 in the coral *Acropora muricata* subjected to Brown Band Disease (Chapter 2).
- Investigates the successional changes of different biochemical indicators during the development modulations of commonly used Hsps biomarkers, Hsp60 and Hsp70, together with oxidative stress biomarkers not yet used for biotic stressors: HO-1 and MnSod, to assess the cellular damages of the the Black Band Disease (Chapter 3).
- Investigate how seasonal and nictimeral temperature and light intensity variations influence the biomarkers expressions, Hsp60 Hsp70 and HO-1, in three species of corals: *Acropora tenuis, Poristes lobata,* and *Echinopora lamellosa* (Chapter 4).

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– Chapter 2 –

The modulation of the Hsp60 in response to the coral Brown Band disease

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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 until now many efforts have been made to identify the microbial communities associated with BrB as well as in the disease ecology, less attention has been given to the effect of the ciliate presence in coral physiology. For this reason, in *Acropora muricata* colonies affected by BrB in a Maldivian reef, the level of the mitochondrial Heat shock protein 60-kDa (Hsp60) was analyzed in the apparently healthy coral polyps located at different distances along the advancing front of the infection. Different Hsp60 levels were found in the different parts of the same colony. Starting from a basal protein level in the healthy control colonies, a down-regulation of the Hsp60 expression was detected near the ciliates band that indicates 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 level gradually returned to a state comparable to that found in the control showing that the cellular damage was confined near the infection. In conclusion, we propose the analysis of the Hsp60 modulation as a useful tool for examining the physiological variations not detected at morphological level in corals subjected to epizootic diseases, whilst providing new insights into the immune response of the corals.

Key words: Coral disease, Ciliates, Brown band disease, *Acropora muricata*, Hsp60, Maldives, coral immune system

2.1 Introduction

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

The representative macroscopic field sign of the syndrome is the presence of a distinctive brown zone of variable width located between the healthy coral tissue and the exposed white skeleton. This band is formed by a dense concentration of motile protozoan ciliates that feed on coral tissue and accumulate zooxanthellae (*Symbiodinium*) intracellularly resulting in the characteristic brown coloring and in rapid tissue loss (Willis et al. 2004, Ulstrup et al. 2007, Lobban et al. 2011). 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 performed in the identification of the microbial communities associated with it, less attention has been given to the effects of the ciliate presence on coral health and physiology, especially considering the high progression rate that has characterized the disease (Nicolet et al. 2013, Katz et al. 2014). In this context, the use of molecular biomarkers such as the Heat shock proteins (Hsps), whose levels can be indicative of a cellular stress due to the pathogen

activity, could clarify, on the one hand, some aspects regarding the typology and severity of the damage to coral tissues, and, on the other, provide helpful information about the coral stress response and defense mechanisms before visible signs of disease are apparent. In fact, as ubiquitous molecular chaperones and fundamental cellular protective systems, the Hsps are involved in preserving the physiological protein homeostasis and maintaining regular cellular functions, with a crucial role in protein folding, refolding/unfolding, aggregation, degradation, and transport (Sorensen et al. 2003, Mayer 2010, Hartl et al. 2011). In addition, in all organisms, as well as in corals, it is well known that the expression of Hsps is up-regulated in order to increase cellular repair and cellular 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 the cellular stress to immunophysiology (Pockley et al. 2008). In particular, Hsp60 and Hsp70, in addition to their primary function as molecular chaperones seem to play a dual role of stress biomarker and immune modulator which provides the opportunity to use them as potential therapeutic agents (Pockley et al. 2008, Tsan & Gao 2009, Quintana & Cohen 2011). With regard to corals, a recent paper suggests the involvement of the heat stress response gene, *hsp70,* in the immunological/defence response of *Acropora millepora* to microbial challenges (Brown et al. 2013). Furthermore, in the coral *A. muricata* infected by the ciliates responsible of the coral disease Skeleton Eroding Band (SEB), an up-regulation of Hsp60 as a defensive mechanism against the advancing infection was detected in the coral portions just above the SEB band, suggesting the implication of the Hsp60 in the coral immune response and proposing the Hsp60 as a tool to evaluate physiological stress caused by coral diseases (Seveso et al. 2012).

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

2.2 Materials and Methods

2.2.1 Study area and sampling design

In December 2013, after previous extensive surveys, a defined sampling area of approximately 200 m^2, located about 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 south east part of Faafu Atoll, Republic of Maldives, (Fig. 1). This site was selected because in the past it had been affected by episodes of BrB disease (Montano et al. 2012). Moreover, it represented an easy and short time access area suitable for rapid sampling activities. In addition, it extended along the same reef flat zone characterized by a similar depth (around 2-3 m) and thus subjected to the same environmental parameters. To confirm this, few days before sampling three HOBO pendant data loggers (Onset, UA-002-64) were placed and maintained until the end of the sampling in different locations within the whole area in order to measure sea temperature and light intensity. In parallel, seawater samples were collected for salinity measurements with a refractometer (Milwaukee Instruments, USA). Afterwards, colonies of the branching coral *A. muricata* of similar size and exhibiting symptoms of BrB disease were randomly selected, tagged and photographed (Canon G11 with Canon housing) by snorkeling. In order to ensure that only colonies displaying active disease were sampled, colonies were monitored every 2 days prior to the sampling activity and only those showing lesion progression were subsequently sampled and analyzed to study the modulation of the Hsp60. Furthermore, we selected colonies in which the BrB band was located at approximately the same distance from the tips of the coral branches.

2.2.2 Coral collection

Among the selected colonies, 6 were randomly chosen and for each of them 3 intact and apparently healthy small coral fragments were collected 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 approximately 1 cm from the site of infection, thus 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 in a range between 5-10 cm away from the site of infection were marked as distance -2 (D2). Finally the portions sampled far from the disease lesion (about 15-20 cm away) were marked as distance -3 (D3),

(Fig. 2A). In some situations a thin white zone of exposed skeleton was observed between the ciliate band and healthy tissue that may comprise bleached tissue and/or denuded skeleton (Willis et al. 2004, Lobban et al. 2011), (Fig. 2B). In these cases the collection of the coral fragments was started from the first portions of living tissue situated just above these white thin bands. Coral samples were excised from colonies of *A. muricata* with a hollow-point stainless steel spike (8 mm diameter) by applying constant rotational pressure in order to reduce the size of coral sampled and, at the same time, reduce the amount of sampling stress and limit 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 any contaminations. Afterwards, samples were immediately frozen at -80°C using an immersion cooler (FT902, JULABO, Labortechnick GmbH) placed in the MaRHE Centre laboratory of Maghodhoo Island which is located a few meters away from the sampling area.

All the coral samples were taken simultaneously at the same shallow depth, time (around 09:00 am) and during high tide (coral permanently submerged) to minimize seasonal and/or daily variations due to changes in water temperature and/or different UV intensity and salinity (Chow et al. 2009, 2012, Seveso et al. 2013). Furthermore, in order to confirm that in all the analyzed colonies the infection was effectively due to ciliate infestation, coral fragments were also collected in correspondence of the brown bands together with the samples D1, D2 and D3. In these fragments the infection caused by BrB ciliates was confirmed by microscopic analysis of coral fragments collected in correspondence of the band (Fig. 2B-D). Microphotographs of the infected tissues containing ciliates were obtained using a stereomicroscope Olympus SZ61 (Tokyo, Japan), paired with the Schott KL 300 LED cold light source and the Olympus LC 20 camera, and a microscope Zeiss Axioskop (Carl Zeiss, Jena, Germany). At the same time, considering that the samples D1, D2 and D3 should not contain protozoa in order to avoid interference during the analysis of Hsps60, the total absence of protozoa 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 meters away from the diseased ones. For each of them coral fragments were collected at 3 different distances, marked as C1, C2, C3, from the tip of the coral branch corresponding approximately to the same fixed distances of samples in the diseased branches.

2.2.3 Western analysis

The frozen coral fragments were powdered using mortar and pestle and the extraction of only the polyp proteins was performed as previously described (Seveso et al. 2013, 2014), in order to remove any *Symbiodinium* contamination from the extracts. All the 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 analysis. 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 used for quantification. Immunodecoration 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 with the Pierce ECL Western Blotting Substrate followed by X-ray films.

Densitometric analysis was performed on a Bio-Rad GS-800 calibrated imaging densitometer and quantified with the Image J 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 Hsp60 bands was normalized against the intensity of the β-Actin ones which at the different distances from the brown band did not display a significant modulation and consequently used as internal control of loading. In addition, in order to quantify the amount of Hsp60 expressed, for each blot the scanned intensity of the Hsp60 bands was normalized against the intensity of the Hsp60 protein standard. Data were expressed as the mean ± standard error of the mean (SEM). Data normality was verified using Shapiro–Wilk test. One-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc tests for pairwise comparison of means was performed for all the normalized Hsp60 intensity values obtained from the different groups of samples (C1,C2 C3 and D1, D2, D3).

2.3 Results and Discussion

In all the *A. muricata* colonies affected by the BrB disease, the microscopic 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 and C). As shown in Fig. 2D, the ciliates displayed an elongated, tube-like shape rounded at both the posterior and apical ends, similar to other described ciliate taxa responsible for the 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), to date little is known about the mechanisms employed by corals in order to fight and resist the ciliates' action. For this reason the expression of the 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 the Hsp60 level was detected in the coral fragments collected at different distances from the tip of the coral branch (Fig. 3A). In fact, as determined by densitometric analysis (Fig. 3B), in the coral fragments C1 (2,24 ± 0,18 ng Hsp60/µg proteins), C2 (2,09 ± 0,23 ng Hsp60/µg proteins) and C3 (2,12 ± 0,23 ng Hsp60/µg proteins) a similar basal level of Hsp60 was found (ANOVA, Tukey's HSD post hoc tests for pairwise comparison of means; $p \geq 0.05$ comparing C1, C2 and C3, Tab. 1), also confirming the important role of this chaperonin even under normal physiological conditions (Choresh et al. 2001, Chow et al. 2009, Seveso et al. 2014). Consequently, in Fig. 3C only a representative control sample (C1) is shown. On the contrary, our results showed a modulation of Hsp60 in *A. muricata* infected by BrB disease, with different Hsp60 levels 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 the Hsp60 expression was detected nearby the infected coral portions with the protein level that progressively increased moving away from the dark band and returning comparable to that found in the control only at the farthest distance from the band (D3). As shown in Fig 3D, the densitometric analysis confirmed that in the coral fragments sampled just above the dark band (D1), on the interface of ciliates progression,

the Hsp60 level approached values that were close to zero (0,48 ± 0,18 ng Hsp60/µg proteins), resulting significantly lower than those detected in the control, (ANOVA, Tukey's HSD post hoc tests for pairwise comparison of means; $p < 0.05$ compared to C1), (Tab. 1). At an intermediate distance (D2) the Hsp60 signal was slightly but significantly increased compared to D1 (1,12 ± 0,16 ng Hsp60/µg proteins). However, the protein level was still markedly lower than that of the healthy colonies ($p < 0.05$ compared to C1 and D1), (Tab. 1). Finally, in coral fragments sampled far from the dark band (D3) the Hsp60 level was almost twice compared to D2 (2,11 ± 0,14 ng Hsp60/µg proteins) and it was reverted close to that displayed in the control samples ($p \geq 0.05$ compared to C1, $p < 0.05$ compared to D1), (Fig. 3C, D and Tab. 1). In order to highlight that in the *A. muricata* colonies the Hsp60 trend is due only to the presence of the ciliates infection, it is essential to clarify that all the coral tissue samples displayed normal tissues pigmentation and did not show morphological differences, being free of necrosis and undamaged structurally. Furthermore, during the sampling time no significant changes in the temperature and light intensity values were observed within different locations of the sampling area and among different days (Tab. 2); seawater temperatures that were also in line with the regular mean seasonal trend (29,28 ± 0,26) recorded during the Northeast Monsoon season from December to April. In addition, no anomalies regarding 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 changes in the equilibria of mitochondrial-associated metabolic pathways (Papp et al. 2003). In addition, this Hsp is also involved in the immune response, in particular interacting with the Toll-like receptor (TLR) signaling pathway (Pocklet et al. 2008, Quintana & Cohen 2011). In corals many immune mechanisms have been described for resisting infections and maintaining tissue integrity, including TLRs, the melanin-synthesis pathway, component of the prophenoloxidase 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), but until now few reports have examined the involvement of a Hsp in the coral immune response in association with 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 coralliilyticus* has been proposed to be an element of the defense response of the coral, possibly by activating other constitutive components of the coral effector immune systems, such as the prophenoloxidase cascade (Brown et al. 2013). Similarly, Baruah et al. (2011) presented evidence that in the shrimp *Artemia* sp., the Hsp70 up-regulation increased the resistance to pathogens by priming and enhancing the expression of the prophenoloxidase system.

Interestingly, our results showed that the Hsp60 expression was higher in the coral portions that potentially can be infected than in those closer to the infection. This modulation of the Hsp60 could appear unexpected if compared to that of the same coral species infected by the ciliate *Halofolliculina corallasia*, responsible for the SEB disease, in the same geographic area (Seveso et al. 2012). In fact nearby the advancing front of the ciliate mass, a significant increase of Hsp60 has been observed in colonies affected by SEB if compared to the healthy control, while a decreased Hsp60 expression was observed in colonies affected by BrB. This extremely low level of Hsp60 could indicate that in such coral portions located at the front line the defense mechanisms were probably already exceeded 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 coral cells neighboring the infection and not yet directly infected the ciliate presence can cause 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 the SEB ciliates. In fact, while the SEB progression rate in *A. muricata* has been estimated to be at the maximum between 1 and 2 mm/day (Antonius & Lipscomb 2001, Page & Willis 2008), the mean rate of BrB progression on diseased branches of *A. muricata* in the field oscillates between 5 to 20 mm/day (Ulstrup 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 to the coral defense mechanism the time necessary to react, in the attempt to block and confine the infection.

Alternatively, since the BrB ciliates are only feeding on coral tissue without producing harmful secretions which could inhibit the 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 in corals subjected to BrB, bacteria as *Arcobacter* sp. and *Aeromonas* sp., could represent the primary disease causal agents, by invading the healthy tissue and leading to an impaired physiological condition that allows 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 factors secreted by these pathogenic bacteria.

In conclusion, the present study can provide new insights into the physiology of scleractinian corals subjected to epizootic disease. The results further support the notion that the Hsp60 expression may constitute a useful tool for checking specific variations in coral physiological and cellular parameters, which can not be detected simultaneously at morphological level. Furthermore, these data, if compared with those shown by Seveso et al. (2012), also suggest that different pathogens trigger a different and specific Hsp modulation. Since until now the ciliates feeding behavior and their role in the coral tissue mortality remain unclear (Yarden et al. 2007) further investigation is required by analyzing other coral diseases and other molecular biomarkers in order to elucidate major ecological and molecular aspects of the pathogen – host relationship.

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2.6 Figures and Tables

	C1	C2	C3	D1	D2	D3
C1	–	0.75	0.887	**0.001**	**0.006**	0.832
C2	0.75	–	0.999	**0.004**	**0.008**	0.999
C3	0.887	0.999	–	**0.003**	**0.007**	0.999
D1	**0.001**	**0.004**	**0.003**	–	**0.016**	**0.004**
D2	**0.006**	**0.008**	**0.007**	**0.016**	–	**0.009**
D3	0.832	0.999	0.999	**0.004**	**0.009**	–

Tab. 1 – Results of the 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). For each group N=6. The statistically significant differences ($p < 0.05$) are indicated in bold.

	DL 1		DL 2		DL 3		DL Mean (±SD)	
	T	LI	T	LI	T	LI	T	LI
Day 1	29,3	29467	29,14	27101	29,23	25135	29,22 (±0,09)	27234 (±2169)
Day 2	29,5	29654	29,32	27346	29,51	30457	29,44 (±0,11)	29152 (±1615)
Day 3	29,34	31357	29,18	28679	29,26	24146	29,26 (±0,09)	28060 (±3645)
Day 4	29,25	28368	29,49	30114	29,32	25513	29,35 (±0,13)	27997 (±2322)
Day 5	29,31	25345	29,37	27674	29,15	29653	29,27 (±0,11)	27557 (±2156)
5 Days Mean (±SD)	29,34 (±0,10)	28838 (±2226)	29,3 (±0,14)	28182 (±1235)	29,29 (±0,14)	26980 (±2864)		

Tab. 2 – Values of sea temperature T (°C) and light intensity LI (Lux) measured by the three data loggers (DL1, DL2, DL3) placed in different locations within the sampling area. Values measured during five representative days randomly selected during the sampling period (December 2013) are reported. The mean (±SD) of both the parameters recorded by the different data loggers and in the different days is also shown.

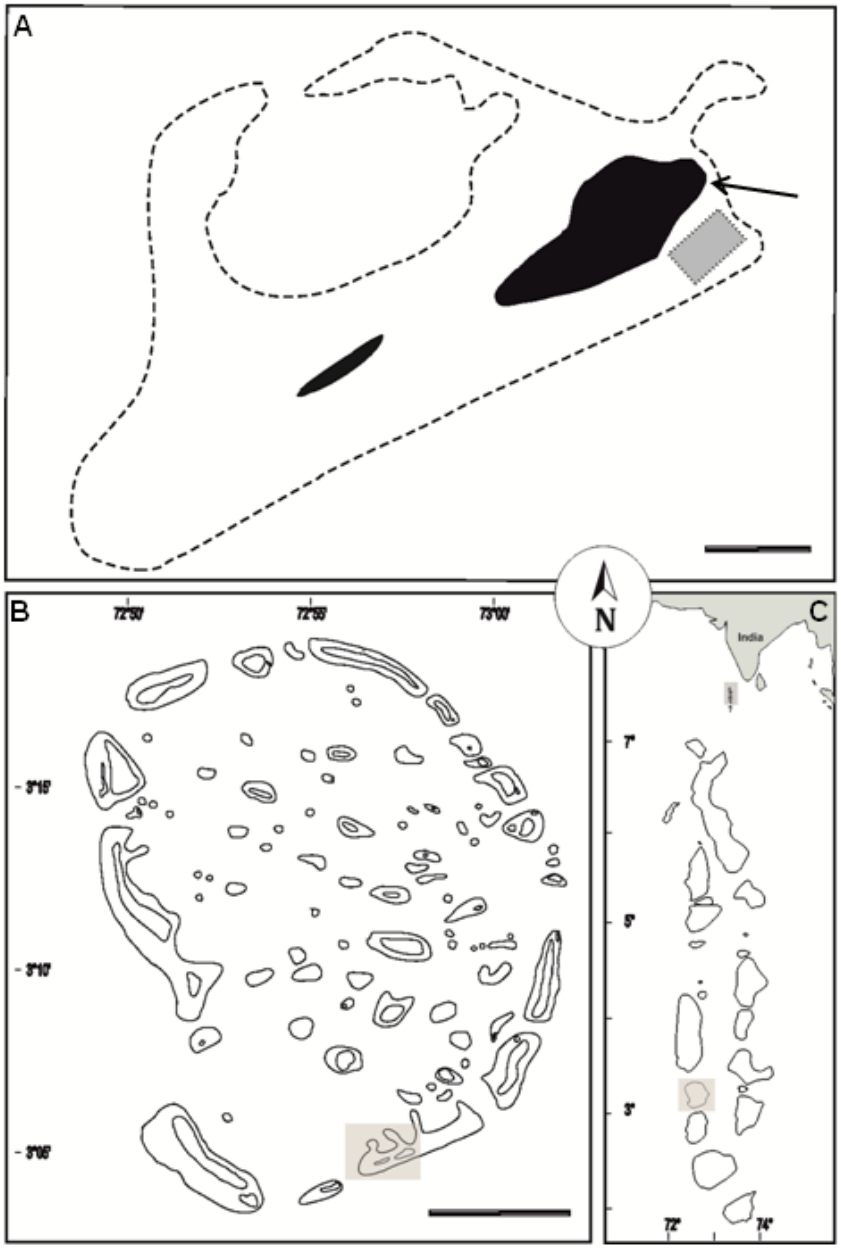

Fig. 1 – Map of Magoodhoo Island (A), located in the south east part of Faafu Atoll (B), Republic of Maldives (C). In A, the islands are indicated in black and the dotted line indicates the reef edges. The grey square indicates the area of approximately 250 m^2 and about 80 m distant from the shore in the Magoodhoo lagoon chosen as sampling site to create an easy and short time access area (see Materials and Methods). The coral patches inside this sampling area are located at a distance between them variable from 5 to 15 meters. Within the area three data loggers were placed in order to record the sea temperature and the light intensity. The black arrow indicates the position of the MaRHE Centre on the island. (Scale bars: A: ~ 150 m, B: ~ 4 km)

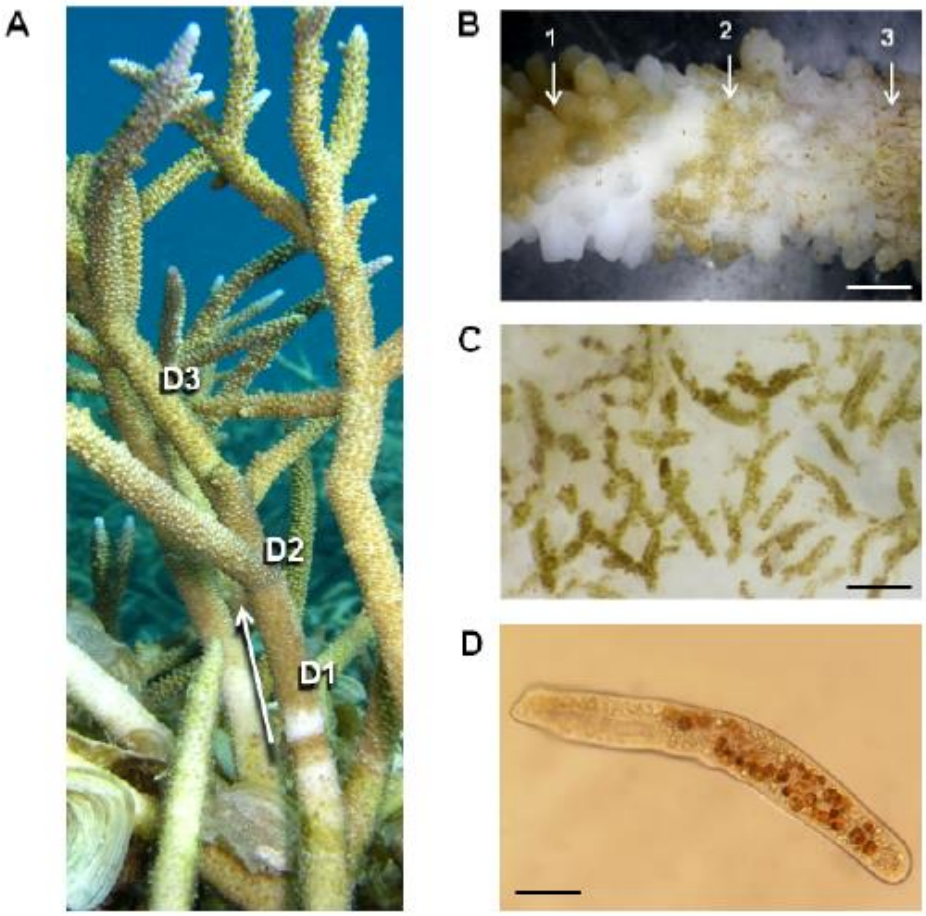

Fig. 2 – A. Colony of *A. muricata* affected by the Brown band (BrB) disease. The white arrow indicates the disease progression direction. Coral fragments sampled just above the ciliate mass (D1), in a range between 5-10 cm (D2) and 15-20 cm (D3) away from the site of infection are also indicated. B. Detail of the infected area on an *A. muricata* branch showing the macroscopic signs of the BrB disease including (1) healthy coral tissue following the ciliate band, (2) the mass of swarming ciliates responsible of the infection apparently wedged into the coral skeleton and (3) the exposed white coral skeleton already covered by algae preceding the brown zone. Scale bar: 70 mm. C. Micrograph of the ciliate clustering that constitutes the band and is embedded in the coral skeleton after ingesting the coral tissue. Ciliates appear as brown flecks on the white coral. The ciliate population appeared uniform and dominated by one morphologically distinct protozoan. Scale bar: 200 µm. D. Micrograph (x 40 magnification) showing the morphology of the brown band ciliate. To note the intact intracellular zooxanthellae (*Symbiodinium* sp.) inside the ciliate. Scale bar: 80 µm.

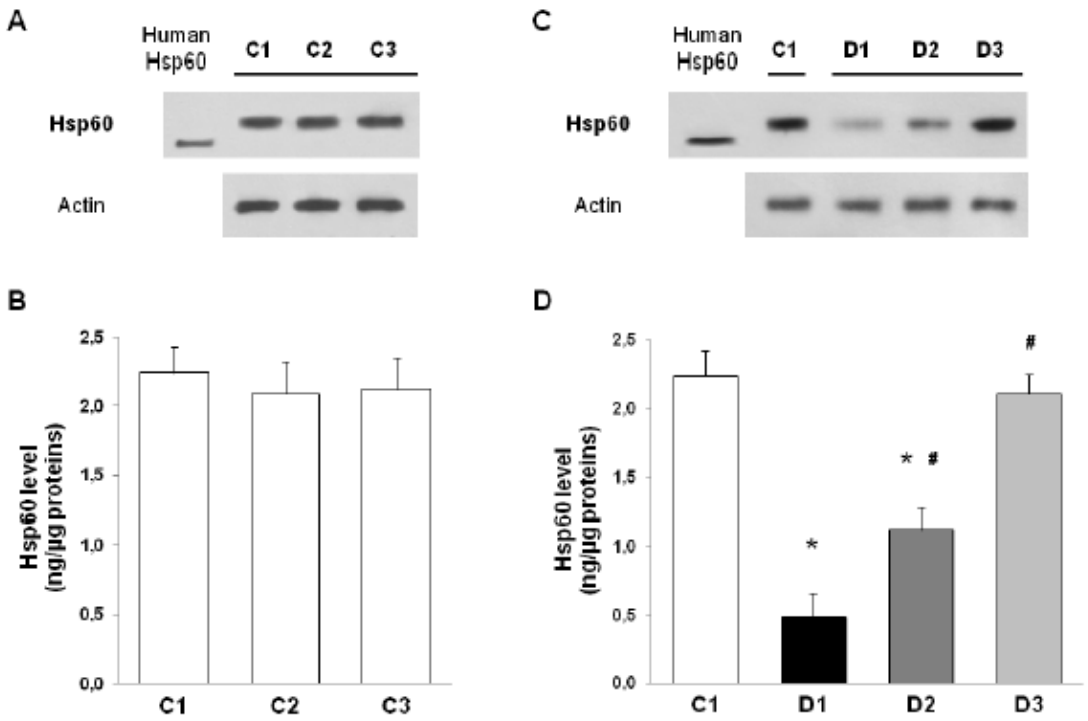

Fig. 3 – A. Hsp60 levels in healthy colonies of *A. muricata*. Samples of coral fragments located at 3 different distances from the tip of the coral branch (C1, C2 and C3) were subjected to Western analyses. Immunodecoration was performed with anti-Hsp60 and anti-β-Actin antibodies. Equal amounts of total protein were loaded in each lane. For each blot, the same amount of recombinant human Hsp60 was included. Filter representative of six experimental repeats (n = 6) is shown. B. Hsp60 levels were determined by densitometric analysis as described under Materials and Methods. Signals of six different blots were analyzed. Data are expressed as ng Hsp60/µg of total proteins and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pairwise comparisons).

C. Effect of BrB disease on Hsp60 modulation in the scleractinian coral *A. muricata*. Samples of coral fragments located at different distances from the brown band along the disease progression direction (D1, D2 and D3) were subjected to Western analyses and immunodecoration as in A. Samples prepared from healthy colonies (C1) are also shown. Filter representative of six experimental repeats (n = 6) is shown. D. Data of Hsp60 levels determined by densitometric analysis and expressed as ng Hsp60/µg of total proteins and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, * $p < 0.05$ compared to C, # $p < 0.05$ compared to D1).

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Modulation of Hsp60 in response to coral brown band disease

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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

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).

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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 m^2 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.

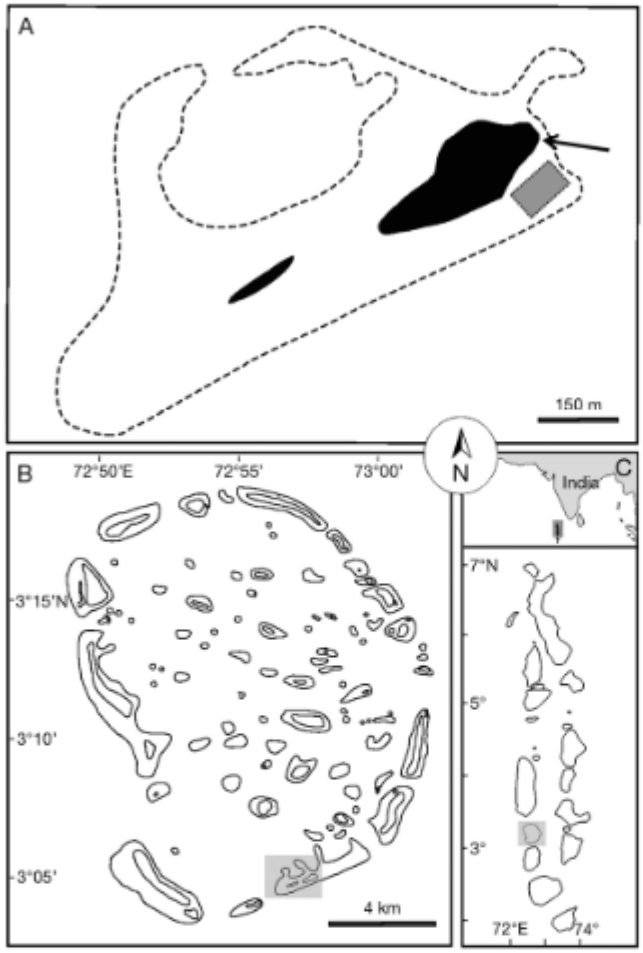

Fig. 1. Magoodhoo Island (A) located in the southeastern part of Faafu Atoll (B), Republic of Maldives (C). In A, black areas: the islands, black arrow: position of the MaRHE Centre on the island, dashed lines: the reef edges, grey rectangle: sampling site (area ~250 m^2, ~80 m from the Magoodhoo lagoon shore) chosen for fast and easy access (see 'Materials and methods'). Distance between coral patches inside this sampling area vary from 5 to 15 m. Within the area, 3 data loggers were placed to record seawater temperature and light intensity

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, Labortechnick) in the MaRHE Centre laboratory at Maghodhoo 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

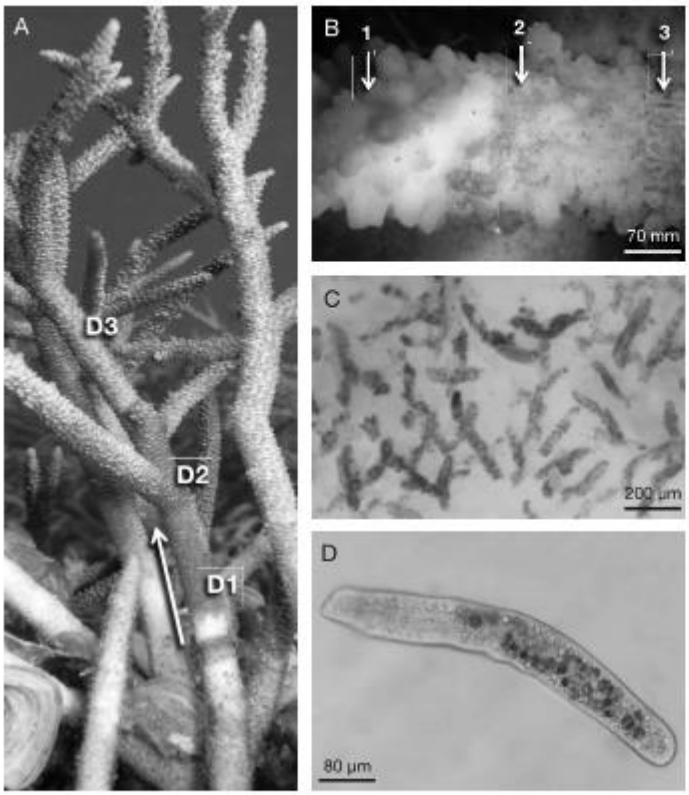

Fig. 2. (A) Colony of *Acropora muricata* affected by brown band (BrB) disease. White arrow: disease progression direction; D1, D2 and D3: sampling points along a coral fragment (just above the ciliate mass, at 5–10 cm and at 15–20 cm away from the site of infection, respectively). (B) Infected area on a branch showing the macroscopic signs of BrB disease including (1) healthy coral tissue in front of the advancing ciliate band, (2) the mass of swarming ciliates responsible for the infection, apparently wedged into the coral skeleton and (3) the exposed white coral skeleton (already covered by algae) following the ciliate infection. (C) Micrograph of the ciliate clustering that constitutes the band and is embedded in the coral skeleton after ingestion of coral tissue. Ciliates appear as brown flecks on the white coral. The ciliate population appeared uniform and was dominated by one morphologically distinct protozoan. (D) Micrograph (40× magnification) showing the morphology of the brown band ciliate. Note the intact intracellular zooxanthellae (*Symbiodinium* sp.) inside the ciliate

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 Hsps60, 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 comparison of means was performed for all the normalized Hsp60 intensity values obtained from the different groups of samples (C1,C2, C3 and D1, D2, D3).

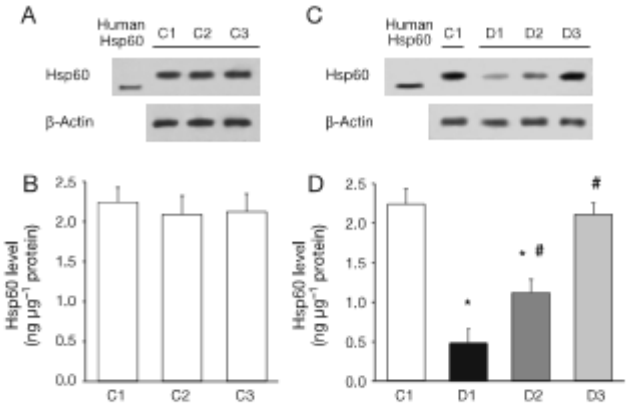

Fig. 3. (A) Hsp60 levels in healthy colonies of *Acropora muricata*. Samples of coral fragments located at 3 different distances from the tip of the coral branch (C1, C2 and C3) were subjected to Western blotting. Immunostaining was performed with anti-Hsp60 and anti-β-Actin antibodies. Equal amounts of total protein were loaded in each lane. For each blot, the same amount of recombinant human Hsp60 was included. 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). (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. (D) Hsp60 levels (mean ± SEM) were determined by densitometric analysis. *$p < 0.05$ compared to C1, #$p < 0.05$ compared to D1 (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 microscopic 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,

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

	C1	C2	C3	D1	D2	D3
C1	–	0.75	0.887	**0.001**	**0.006**	0.832
C2	0.75	–	0.999	**0.004**	**0.008**	0.999
C3	0.887	0.999	–	**0.003**	**0.007**	0.999
D1	**0.001**	**0.004**	**0.003**	–	**0.016**	**0.004**
D2	**0.006**	**0.008**	**0.007**	**0.016**	–	**0.009**
D3	0.832	0.999	0.999	**0.004**	**0.009**	–

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; $p \geq 0.05$ 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). At an intermediate distance (D2), the Hsp60 signal was slightly but significantly increased compared to D1 (1.12 ± 0.16 ng Hsp60 μg^{-1} proteins). However, the protein level was still markedly lower than that of the healthy colonies ($p < 0.05$ 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 ($p \geq 0.05$ 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 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

Day	DL1	DL2	DL3	Mean (±SD)
Temperature (°C)				
Day 1	29.3	29.14	29.23	29.22 (±0.09)
Day 2	29.5	29.32	29.51	29.44 (±0.11)
Day 3	29.34	29.18	29.26	29.26 (±0.09)
Day 4	29.25	29.49	29.32	29.35 (±0.13)
Day 5	29.31	29.37	29.15	29.27 (±0.11)
Mean (±SD)	29.34 (±0.10)	29.3 (±0.14)	29.29 (±0.14)	
Light intensity (lux)				
Day 1	29 467	27 101	25 135	27 234 (±2169)
Day 2	29 654	27 346	30 457	29 152 (±1615)
Day 3	31 357	28 679	24 146	28 060 (±3645)
Day 4	28 368	30 114	25 513	27 997 (±2322)
Day 5	25 345	27 674	29 653	27 557 (±2156)
Mean (±SD)	28 838 (±2226)	28 182 (±1235)	26 980 (±2864)	

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 prophenoloxidase 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 coralliilyticus* 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 prophenoloxidase 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 prophenoloxidase 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. muricata* 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. muricata* in the field varies from 5 to 20 mm d^{-1} (Ulstrup 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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– Chapter 3 –

Successional changes of different biochemical indicators during the progression of black band disease on the reef coral Goniopora columna

Abstract

Black Band Disease (BBD) is one of the most studied and widespread coral disease, ranging from the Atlantic Ocean to the Indo-Pacific, including the Red Sea and the Great Barrier Reef and infecting a wide range of scleractinian corals. It is characterized by a thick microbial mat, dominated by phototrophic cyanobacteria, which activities generate anoxic sulfide rich microenvironments and may enhance the BBD progression. In Maldives waters, one of the highest BBD prevalence was observed in colonies of *Goniopora columna*, which showed a very slow progression rate. For this reason, in these colonies the level of Heat shock proteins 60-kDa (Hsp60) and 70-kDa (Hsp70), Heme-oxygenase-1 (HO-1) and Manganese Superoxide Dismutase (MnSod) were analyzed in the apparently healthy coral polyps located at different distances along the advancing front of the infection, during the progression of the disease in two consecutive years (2013-2014). Our results showed that *G. columna* modulated several cellular processes in different way both within the colony and during the progression of the BBD. In fact, healthy colonies used as control, generally did not express significantly higher levels of cellular biomarkers. In particular, Hsp60 levels did not differ among the distances within the colony but down-regulated after one year, following the advance of the infection front. Hsp70 suffered an up-regulation in the diseased colonies in both periods of sampling, since in 2013 the distance closer to the necrotic band had lower expression than the other two farther distances, and in 2014 the father distance showed a decrease of Hsp70 expression. HO-1 was up-regulated with no difference within the diseased colonies. After one year the expression at the distance near the band in necrosis decreased significantly. MnSod, which was little expressed even in healthy colonies, was up-regulated in the diseased ones, with no differences within the colony, while after one year the first distance showed a down regulation. Generally, the results showed that the slow progression of BBD may give to the colonies the time to organize a shared strategy of defense as well as exhausting them. Moreover this study showed for the first time oxidative stresses induced by BBD. In conclusion, we propose a set of biomarkers as a useful tool for examining the physiological variations not detected at morphological level, in corals subjected to epizootic diseases, whilst providing new insights into the immune response of the corals.

3.1 Introduction

In the last few decades about the 27% of coral reefs have been destroyed worldwide (Frias-Lopez et al., 2004). One of the main causes of coral reef destruction has been a dramatic increase in coral disease (Rosenberg & Ben-Haim, 2002). Black Band Disease is one of the oldest and most studied coral disease. It is a widespread disease, ranging from the Atlantic Ocean to the Indo-Pacific (Barneah et al., 2007), including the Red Sea and the Great Barrier Reef (Page & Willis, 2006), infecting a wide range of corals. BBD is found predominately in scleractinian corals, till now it is described in 9 families (Acroporidae, Faviidae, Poritidae, Siderastreidae, Agariciidae, Fungiidae, Pocilloporidae, Mussidae and Dendrophylliidae) (Page & Willis, 2006; Montano et al. 2013). In Maldives waters BBD was found in 13 scleractinian genera belonging to 6 families (Acroporidae, Faviidae, Poritidae, Siderastreidae, Agariciidae and Fungiidae), but we did not detect it in soft or hydroid corals (Montano et al., 2013). BBD infection is known to be persistent in reef, contributing to the long term mortality of the infected coral species (Bruckner & Bruckner, 1997). In Maldives waters one of the highest prevalence was observed in *Goniopora columna*, which show a very slow progression rate (about 0.5 ± 0.11 mm day -1) (Montano et al., 2012, 2013). It is characterized by a thick microbial mat, which can migrate up to 2 cm per day across apparently healthy coral colonies, actively killing tissue and leaving the skeleton exposed behind. A microbial community, dominated by phototrophic cyanobacteria, is responsible of the disease virulence and create the characteristic dark band. This microbial mat include also sulfate-reducing *Desulfovibrio* species, sulfide-oxidizing *Beggiatoa* species, other heterotrophic microbes and marine fungi (Frias-Lopez et al., 2004) and exhibit a vertical and horizontal migration through the diurnal cycle (Richardson, 1996).

Despite about 40 years of research the identification of all this microbes is still uncertain as well as its complex developing and the primary causative agent. Even the presence of *Beggiatoa* species is in doubt as, in some cases, this species were not found in the mat, (Frias-Lopez et al., 2004; Cooney et al, 2002) leaving the oxidation of sulfide happening in the BBD mat in needs to be explained, probably by the activity of other organisms such as species of *ε-proteobacterium* which can oxidate sulfide into sulfate (Cooney et al., 2002). At least 64 different species of bacteria were identified living into this consortium (Frias-Lopez, 2004). The first pathogen identified was a filamentous nonheterocystous cyanobacterium, *Phormidium corallyticum* (Rützler & Santavy, 1983), but a recent study found that the most common cyanobacterium was not *P. corallyticum* but a member of the genus *Geitlerinema*, closely associated to *Oscillatoria* species (Myers et al., 2007).

However a lot of other bacteria have been proposed as possible primary causative agent of the disease in recent years, for example Cooney and colleagues (2002) found homology to *Cytophaga* species, to an *α-proteobacteria* already associated with Juvenile Oyster disease and to Oscillatoria coralline (which was, however, present also in non-BBD samples). Sato and colleagues (2010) found successional changes occurring during the development of the disease in *Montiopora hispida,* and suggested that bacterial pathogens which trigged the BBD infection may be very different from which caused the BBD virulence. Some cyanobacterium-infected lesions were found to evolved, in the 19% of the studied cases, into BBD with a change in cyanobacterium species involved. It is still unclear what factors govern whether a cyanobacterium-infected lesion develops in BBD or just loses the active microbial front. It is suggested that the bacterial pathogens that trigged the BBD may become less abundant during the subsequent successional changes and this may be why it is so difficult to identify the primary causative agents of BBD. Moreover Frias-Lopez and colleagues (2004) have found that the bacterial community associated with the BBD is distinct from the communities normally associated with healthy corals. It is known that cyanobacterial toxin can have a role in the pathogenicity of BBD (Richardson et al., 2007) and that the presence of high concentrate sulfide at the base of the BBD mat, in anoxic condition, cause the necrosis of coral tissue (Richardson, 1997). The high concentration of cyanobacteria, the presence of bacterial sulfur cycle with nitrogen, iron, phosphorus and carbon cycles tied to it, may promote further anoxic sulfide rich microenvironments and hence the BBD progression (Sato et al., 2010). Environmental factor as light, nutrients and temperature have also been correlated with the prevalence and progression of this disease (Sato et al., 2011; Boyett et al., 2007; Voss & Richardson, 2007; Muller & Van Woesik, 2011). Since to date, any of the proposed primary pathogens have fulfilled Koch's postulates (Richardson, 2004), and it can be probably that the entire BBD community is a whole pathogenic consortium, working as primary pathogen rather than an individual one.

Despite the increasing number of studies which focused onto the identification of the microbial mat and its succession, less attention is given to the phylogenic response of corals to the BBD attack. By now it is known that corals lack any developed physiological regulatory system but possess a well-developed cellular adaptation system which allow them to react to stressful conditions, caused by both abiotic and biotic stressors, enhancing the expression of several mechanisms involved in cellular repair and tolerance. Diagnostic cellular biomarkers, commonly used in human and veterinary medicine, can be used to asses health conditions (Down et al., 2000, 2005) as corals exhibit significant differences in cellular biomarker

profiles in relation to different stressors (Down et al., 2012). In this study were used three biomarkers two Heat shock proteins (Hsp60 and Hsp70) and Heme-oxygenase-1 (HO-1). Heat shock proteins (Hsps) work as molecular chaperones in order to preserve physiological protein homeostasis and maintain regular cellular functions. The Heme-oxygenase-1 catalyzes the decomposition of heme to biliverdin, carbon monoxide and ferrous iron (Schwartzburd, 2001). The heme catabolism leads to the formation of low-molecular-mass redox-active iron, which is a more versatile catalyst of oxidative damage than heme (Lamb et al., 1999). An increase in protein levels of HO-1 suggests an increased demand in heme degradation. HO-1 was chosen as a biomarker for BBD following Sato and colleagues (2010) study, as they suggested that the high concentration of cyanobacteria, together with the actions of all the bacterial consortium, may promote further anoxic sulfide rich microenvironments which promote the BBD progression.

3.2 Materials and Methods

3.2.1 Study area and sampling design

The sampling area, composed of several coral patches, of about 200 m^2 is located at 80 m from the in the lagoon of Magoodhoo Island (3°04'N; 72°57'E), in the southeastern part of Faafu Atoll, Republic of Maldives (Fig. 1) which permitted easy and quick access suitable for rapid sampling activities.

This site extends along the same reef flat zone characterized by similar depths (2−3 m) and is subjected to the same environmental conditions (Seveso et al. 2015).

Colonies of the massive coral *Goniopora columna* exhibiting symptoms of BBD disease were randomly selected, tagged and photographed (Canon G11 with Canon housing) by snorkelers.

Colonies were periodically checked in order to monitor the progression of the disease. Since the disease progression in *G. columna* was very slow, samples were taken in November 2013 and then a year later, in November 2014. In each year, at the same time (09.00 o'clock), the same colonies, which showed the same disease's progression state, were sampled and marked in order to allow the comparison among the same colony following the progression of the disease.

3.2.2 Coral collection

5 diseased large size (~30-40 cm high) colonies were selected randomly with other 5 intact and apparently healthy colonies. From each colonies small fragments were collected: for the diseased colonies samples were takend at 3 different fixed distances from the black band, along the disease progression direction (Fig. 2A). Distance 1 (D1) was sampled at ~1 cm from the black band, then distance 2 (D2) was sampled at proximately 5 cm away, along the advancing front of the microbial mat while distance 3 (D3) was sampled far from the black band, at about 10 cm. Size of colonies and sampling distances were chosen in order to allow the collection at the same distances from the necrotic tissue, even after one year of the disease progression. Coral samples were excised from colonies of *G. columna* using a hollow-point stainless steel spike (8 mm diameter) by applying constant rotational pressure to minimize the size of coral sampled, in order also to reduce the amount of sampling stress and limited excessive damage to the colonies, allowing a rapid coral recovery following the sampling (Bromage et al. 2009).

Each sample was collected with a new hollow-point spike to avoid contamination. Then

samples were immediately frozen at −80°C using an immersion cooler (FT902, JULABO, Labortechnick) in the MaRHE Centre laboratory at Maghodhoo Island, 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 when coral are permanently submerged, in order to minimize seasonal and/or daily variations in environmental factors such as water temperature, UV intensity and salinity (Chow et al., 2009, 2012, Seveso et al., 2013). After one year samplings are repeat with the same procedure in order to follow the slow disease progression (Fig. 2C, D) as highlighted in a previous study (Montano et al., 2014).

The 5 isolated and entirely healthy colonies of *G. columna* were randomly selected with in the same sampling area, considering only those located at least 10 m awayn from diseased colonies (Seveso et al., 2015).

3.2.3 Western blotting

As previously described by Seveso et al. (2013, 2014) once the coral fragments were collected and frozen they were pulverized using a mortar and a pestle. Always following Seveso et al methods, polyp proteins were extracted and *Symbiodinium* were removed.

Following the extraction all the protein samples were frozen at −20°C until used. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories) and aliquots of proteins were separated by SDS-PAGE on 8% polyacrylamide gels (Vai et al., 1986) with duplicate gels running in parallel.

After electrophoresis, one gel was stained with Coomassie Brilliant Blue to visualize the total proteins while the other was electroblotted onto nitrocellulose membrane for western blotting. Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters.

Immunostaing, as previously reported by Seveso et al. (2013), was performed with the following antibodies: anti-Hsp60 monoclonal antibody (IgG1 mouse clone LK-2, SPA-807, EnzoLife Sciences), anti-β-Actin monoclonal antibody (clone C4, MAB1501,Millipore), anti-Hsp 70 monoclonal antibody (IgG2a mouse clone BB70, SPA-822, EnzoLife Sciences), anti-HO-1 monoclonal antibody (IgG2b mouse clone HO-1-2, OSA-111, EnzoLife Sciences) and anti-MnSod polyclonal antibody (Rabbit, SOD-110, EnzoLife Sciences), and secondary anti bodies: anti-mouse IgG conjugated with horse radish peroxidase (Thermo Scientific) for anti-Hsp60, anti-Hsp70 and anti-HO-1, and Goat anti-rabbit IgG conjugate with horse radish peroxidase (EnzoLife Sciences) for anti-MnSod.

Concentration for the antibodies were for all the primary antibodies 1:1000 while for secondary antibodies: anti-Hsp60 1:10000, anti-Hsp70 1:1000, anti-HO-1 1:7500 and anti-

MnSod 1:1000. Binding was visualized by Pierce ECL western blotting substrate followed by X-ray films.

Densitometric analysis was performed on a calibrated imaging densitometer (Bio-Rad GS-800) while band intensities were quantified using the Image J free software (http://rsb.info.nih.gov/ij/).

For each blot, the scanned intensity of each biomarker bands was normalized against the intensity of the β-Actin bands, which did not display a significant modulation at the different distances from the black band and throughout the infection time progression.

3.2.4 Statistical analysis

Data were expressed as the mean $\pm$ standard error (SEM) and their normality was verified using a Shapiro Wilk test. One-way analysis of variance (ANOVA) was performed for all the normalized biomarkers intensity values obtained from the different distance samples (C and D1, D2, D3) in each time year of sampling (2013 and 2014), followed by Tukey's HSD post hoc tests for pairwise comparison of means to asses significant differences ($p<0.05$). For the comparison among the two time periods Two-way analysis of variance (ANOVA) was performed.

3.3 Results

Hsp60 expressions

In the first period of sampling (2013) Hsp60 appears to not be produced in healthy samples, used as controls (C), while it is significantly up-regulated in the three distances from the Black Band (D1, D2,D3) ($F_{(3,16)}$= 11.79, p≤0,000), although any significant modulation was found within the three distances (Tab.1A). After one year of infection (2014) Hsp60 did not change its production patterns: healthy colonies still did not produce Hsp60 contrary to the three distances ($F_{(3,72)}$= 6.36, p=0,001), which Hsp60 levels, however, were not significantly different among the distances. A two-way Anova revealed a main effect, in Hsp60 expression, of the "time" ($F_{(1,84)}$=14.97, p≤0.000) (Tab.1B). Pairwise comparison showed there was a significant lowering of Hsp60 levels, after one year of infection, in samples from all the three distances but not in the healthy colonies sample (D1 p<0.05; D2 p≤0.01; D2 p=0.004) (Tab.3A).

Hsp70 expressions

Also Hsp70 was not expressed in the healthy sample although it was significantly expressed in the last two distances (D2 and D3) ($F_{(3,20)}$= 12.23, p≤0,000). ANOVA Tukey's HSD post hoc tests for pair-wise comparison of means showed any significant alteration in Hsp70 levels if the first distance is compared with the healthy colony, while Hsp70 levels showed significant differences within the three distances, rising its levels in the second distance (D2) if compared to the first (D1) (p≤0.05) (Tab.1C). Hsp70 levels pattern changed significantly after one year ($F_{(3,52)}$= 6.26, p≤0,001). Although healthy colonies showed no production of Hsp70, as one year previously, the three distances showed an up-regulation in protein levels. D2 from the Black Band had the higher levels compared with D1 (p≤0,05), however D2 Hsp70 levels were higher even if compared with D3 ones (p≤0.05) (Tab.1D). Two-way anova revealed Hsp70 expression to be effected by "distance" ($F_{(3,36)}$= 8.92, P≤0.001), but not by "time", although pairwise comparison showed a significant lowering at D3, after one year (p≤0.001) (Tab.3.B).

HO-1 expressions

HO-1 levels in all the three distances significantly differed when compared with the healthy samples ($F_{(3,16)}$= 11.79, p≤0,000) (Tab.2A), however no significant difference were found among the three distances. HO-1 was not expressed in the healthy colonies however, after three years of infection, but rise significantly in the three distances ($F_{(3,36)}$= 7.78, p≤0,000). Among the three distances HO-1 at D1 had significant lower level of expression than D2 (P≤0.05) and no significant difference with the healthy colonies (Tab.2B). HO-1 was revealed, by the two-way anova, to be effected by the "period of sampling" ($F_{(1,52)}$=20.15, p≤0.000)

which was dominated by the interaction between distances and period of sampling ($F_{(3,52)}$=6.19, p≤0.001). Pairwise comparison revealed a lowering in HO-1 expression at the first distance (D1) after 3 years (p≤0.000) (Tab.3C).

MnSod expressions

MnSod instead showed significant differences among the healthy samples and two distances (D1 and D3, p≤0.05), while the medial distance (D2) didn't show significant ($F_{(3,8)}$= 5.87, p≤0,05) (Tab.2C). However among the three distance in the first period there was any significant variation MnSod in control samples showed significant difference with the last two distances (D2 and D3, p≤0,01 and p≤0.05) while any difference with the first (D1) ($F_{(3,36)}$= 7.78, p≤0,000). A result near significance was showed for the comparison between D1 and D2, with less expression in the first distance (Tab.2D). Two-way Anova revealed a main effect of the distances ($F_{(3,16)}$=9.53, p=0.001) which was dominated by the interaction between distances and period of sampling ($F_{(3,16)}$=3.91, p≤0.05) (Tab.3D). Pairwise comparison revealed a lowering in MnSod expression levels at D1 after one year (p≤0.01).

3.4 Discussion and conclusion

Black Band Disease (BBD) is one of the oldest, most studied coral and widespread disease (Barneah et al. 2007; Page & Willis, 2006) which affect a wide range of corals (Page & Willis, 2006; Montano et al. 2013). Despite about 40 years of researches, the identification of all the microbial mat components, its complex developing, the primary causative agent are still uncertain (Frias-Lopez et al., 2004; Cooney et al, 2002) and even less attention is given to the physiological resistance of corals to the consortium attack. To date it is known that corals lack any developed physiological regulatory system but possess a well-developed cellular adaptation system which enhance the expression of several mechanisms involved in cellular repair and tolerance (Richardson et al., 2004). Our results show that *G. columna* modulated several cellular processes in different way both within the colony and during the progressive progression of the BBD. Differences also occurred between the healthy colonies and the diseased ones. Being clonal organisms, corals have local signals transmitted throughout the entire colony, relying on physiochemical barriers (as mucus) and its bacterial process as a first line of defense against invading pathogens (Mydlarz et al. 2009). Once the physical defense failed, they can relay onto cellular defense mechanisms as the rapid increase of the induction of stress proteins and enzymes. In our study although healthy colonies seem to no express any or not significant level of the proteins or enzymes used as cellular defense, the diseased

colonies significantly increased their expressions.

BBD is caused by a consortium of pathogens, with cyanobacteria that play an important role in the pathogenicity. Moreover, the presence of high concentrate sulfide, which follow the consortium activities, at the base of the BBD mat, in anoxic condition, is known to be the cause of the necrosis of coral tissue (Richardson et al., 1997). The high concentration of cyanobacteria and the presence of bacterial sulfur cycle could further promote the anoxic sulfide rich microenvironments and hence the BBD progression (Sato et al., 2010).

The Hsp60 showed an up-regulation in diseased colonies (compared to healthy ones) while its expression and modulation did not change among the three distances from the black band in both the two sampling periods. Elevation of this protein may indicate that there has been a change in the equilibria of many mitochondrial associated metabolic pathways (Papp et al. 2003, Down et al. 2006). After one year of infection the levels of expression at the three distances from the band were significantly lower (i.e. in 2014 Hsp60 levels did not differ among the distance but are lower expressed than in 2013, following the advance of the infection front). Moreover, Hsp60 is known to have anti-apoptotic ability and to be able to protect against cell death by maintaining mitochondrial oxidative phosphorylation (Arya et al. 2007). In this prospective the down-regulation that follow after one year of infection may indicate that the polyps could start be less able to react to the microbial mat attack with cellular repair mechanisms.

Also Hsp70 suffer an up-regulation in the diseased colonies in both periods of sampling. In fact in 2013 the distance close to the necrotic band had lower expression than the other two farther distances. Afterwards in 2014 the last distance lowered its expression, being the only significant difference among the two years. Elevation and/or accumulation of Hsp70 protein can be a signal of a shift in metabolic condition indicating that the process of protein maturation is up-regulated to compensate for a shift in protein degradation rates (Down et al., 2012; Papp et al., 2003), and this can explain the up-regulation in the diseased colonies. However, Hsp70 response to chemical contaminant or xenobiotic remain still unresolved as Hsp70 can be also be down-regulated while exposed to violent fuel oil spill (Down et al., 2006). Moreover, Hsp70 is also related to other metabolic and cellular pathways and it may be modulated in relation to cellular needs (Down et al., 2006). Consequently, a down-expression of this protein in the farther distance (D3) from the black band microbial mat's attack, when at the distance D1 the defence mechanisms were already exceeded and the second become the most active in the defence, may be related to the whole colony's defensive mechanisms organization.

Then the HO-1 and MnSod expressions could result to be the consequence of the anoxic sulfide rich microenvironments which are responsible of the tissue necrosis and, as proposed by Sato and colleagues (2010), may enhance BBD progression. HO-1 was not expressed in healthy colonies while showed up-regulation in the diseased ones. After one year of infection, the expression at the first distance (near the band in necrosis) lowered significantly, differing significantly also from the other two distances D2 and D3 of the same period. This may suggest that polyps near the BBD, being stressed by the disease for one year, are less or totally behind the ability of recovering, as HO-1 is known to be up-regulated in response to oxidative stress and toxicant exposure (Down et. al., 2006). Moreover healthy colonies showed a very low expression of MnSod which differed significantly from D1 and D3 in the first year, and from D2 and D3 after one year. During the first year there were no significant differences within the colony, while after one year the first distance showed a down regulation of MnSod. The MnSod down-regulation at the fist distance after one year of infection may suggest that polyps are less or totally behind the ability of recovering. In other studies cnidarian MnSod was not increased by erbicide exposure (Down et al., 2006) but increased significantly by hypo-salinity conditions (Down et al. 2009).

Also the Hsp60 modulation resulted different compared with its modulation in corals affected by other diseases, as Brown Band disease (BrB), (Seveso et al., 2015). This may be related to the different progression rates of the two diseases. In fact in *A.muricata,* the BrB ciliates spread with a mean between 5 to 20 mm/day (Nicolet et al. 2013, Seveso et al., 2015) while BBD in *G. columna* about 0.5 ± 0.11 mm/day (Montano et al., 2012, 2013). The slow progression in *G. columna* may give to the whole colony the time to organize the defense as well as exhausting the colony, as also suggested by the different expression of the anti-oxidant enzymes along the disease temporal progression. Being clonal organisms scleractinian corals have locals signals transmitted throughout the entire colony, inducing whole organism levels changes with relative freedom (Mydlarz et al. 2009). Also the Hsp70 modulation may suggest this, as the lowering of Hsp70 occurred at the farther distance after one year of progression, indicating that the second distance required more energy to face the bacterial attack consequently effecting the near polyps. These results showed for the first time that corals affected by BBD experienced oxidative stress as consequence of the microbial anoxic sulfide rich microenvironments that effected also the mitochondrial proteins, i.e. induction of MnSod, Hsp60 and HO-1 (Sato et al., 2010; Down et al.,2012), meaning that the regulation of oxidative phosphorylation and respiration has been altered (Kondoh et al. 2003) changing also during the progression of the disease.

In conclusion appear to be very important to asses an appropriate set of biomarkers in regards of the pathogenic mechanisms occurring in each disease, throughout field works, in order to better understand the disease progression and the coral resilience.

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3.7 Figures and Tables

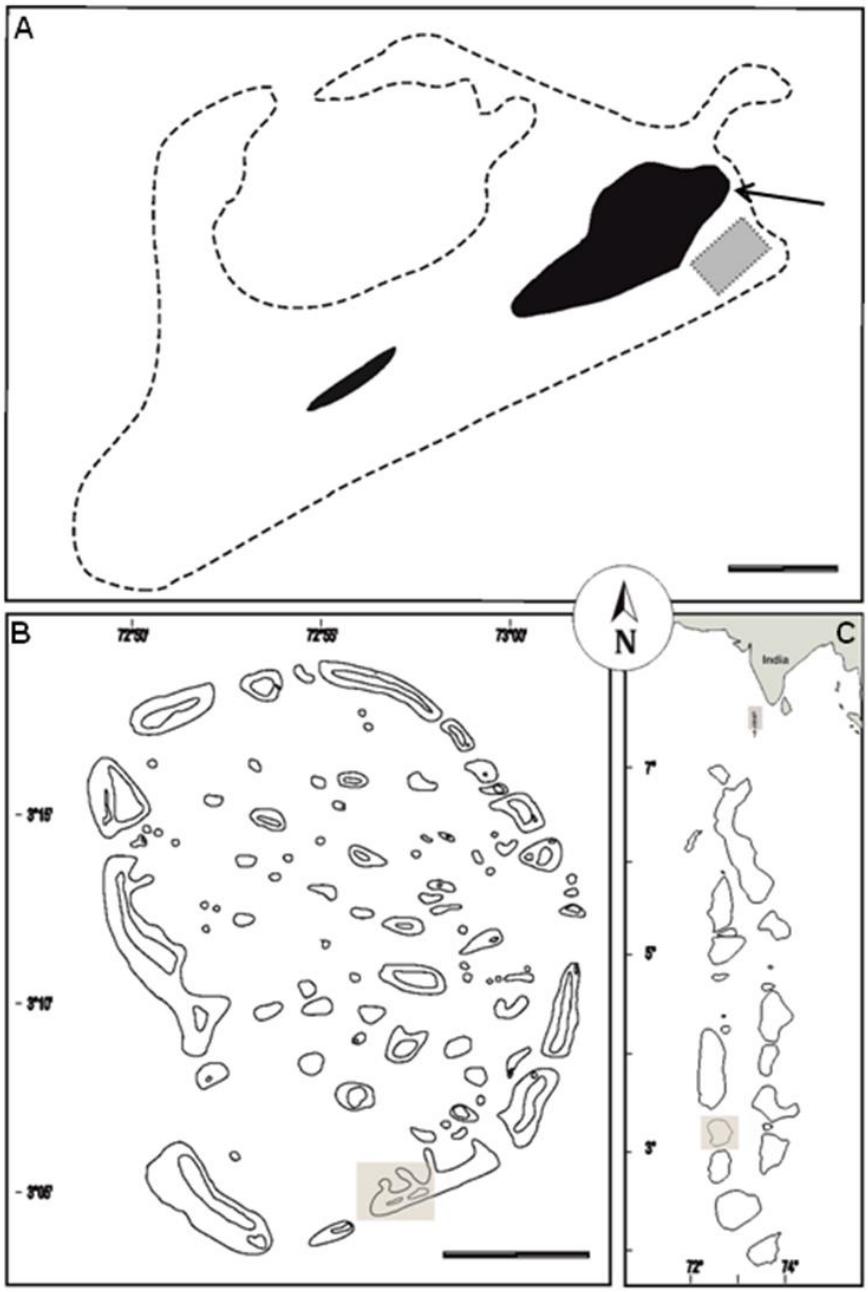


Figura 1. Map of Magoodhoo Island (A), located in the south east part of Faafu Atoll (B), Republic of Maldives (C). In A, the islands are indicated in black and the dotted line indicates the reef edges. The black arrow indicates the position of the MaRHE Centre on the island. (Scale bars: A: ~ 150 m, B: ~ 4 km)

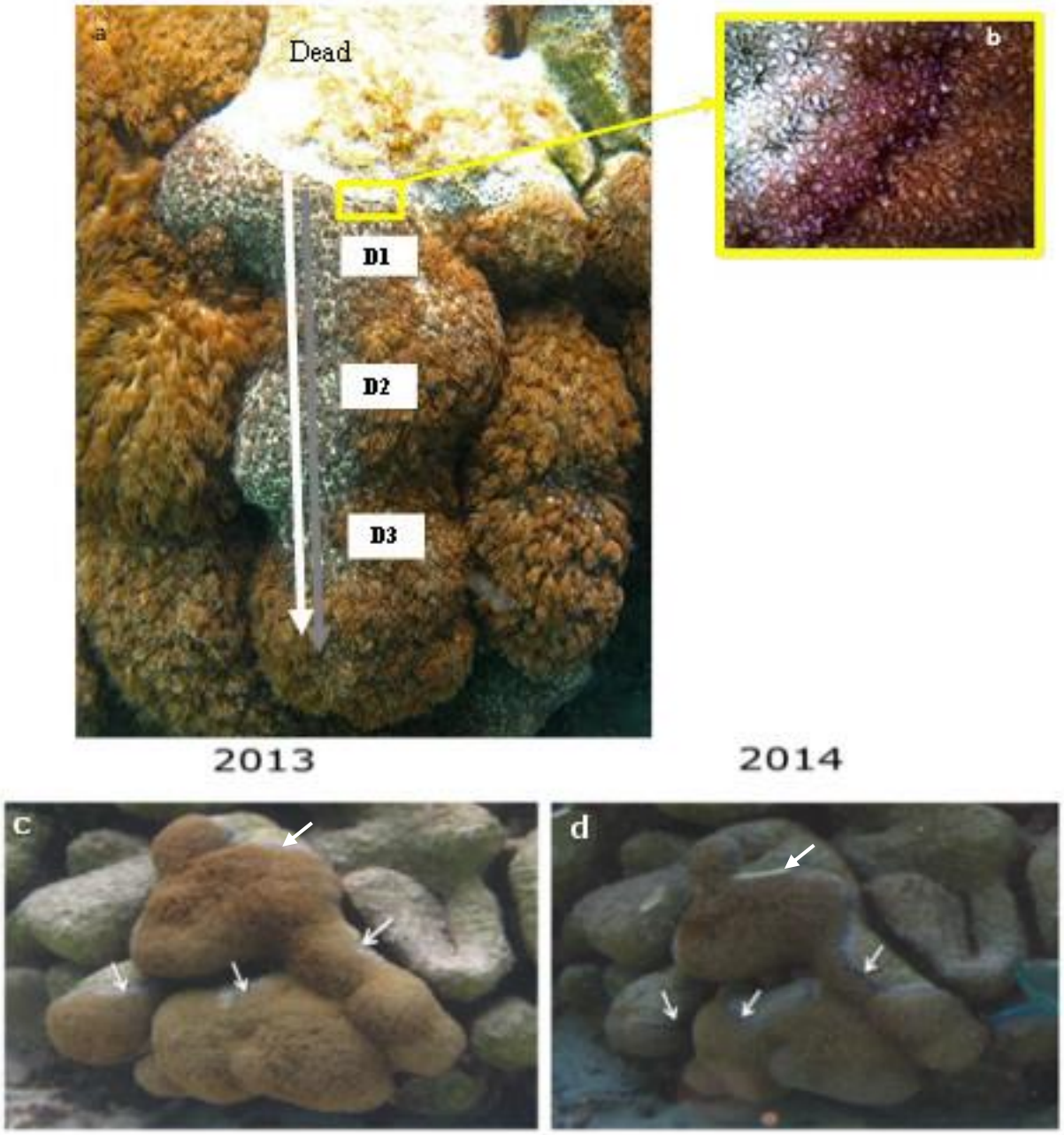


Figure.2. A- Large size colony (~30-40 cm high) of *G. columna* affected by the Black Band disease (BBD). The white arrow indicates the disease progression direction. Coral fragments sampled at ~1 cm below the black necrotic mass (D1), approximately at 5 cm (D2) and at ~10 cm (D3) away from the site of infection, are also indicated. B- Detail of the infected area on an *G. columna* showing the necrotic tissue of the BBD with apparently healthy coral tissue following the band. Scale bar: 70 mm. C- progression of the BBD in a colony before (2013) and after (D) one year of infection (2014), white arrows indicates the necrotic black band.

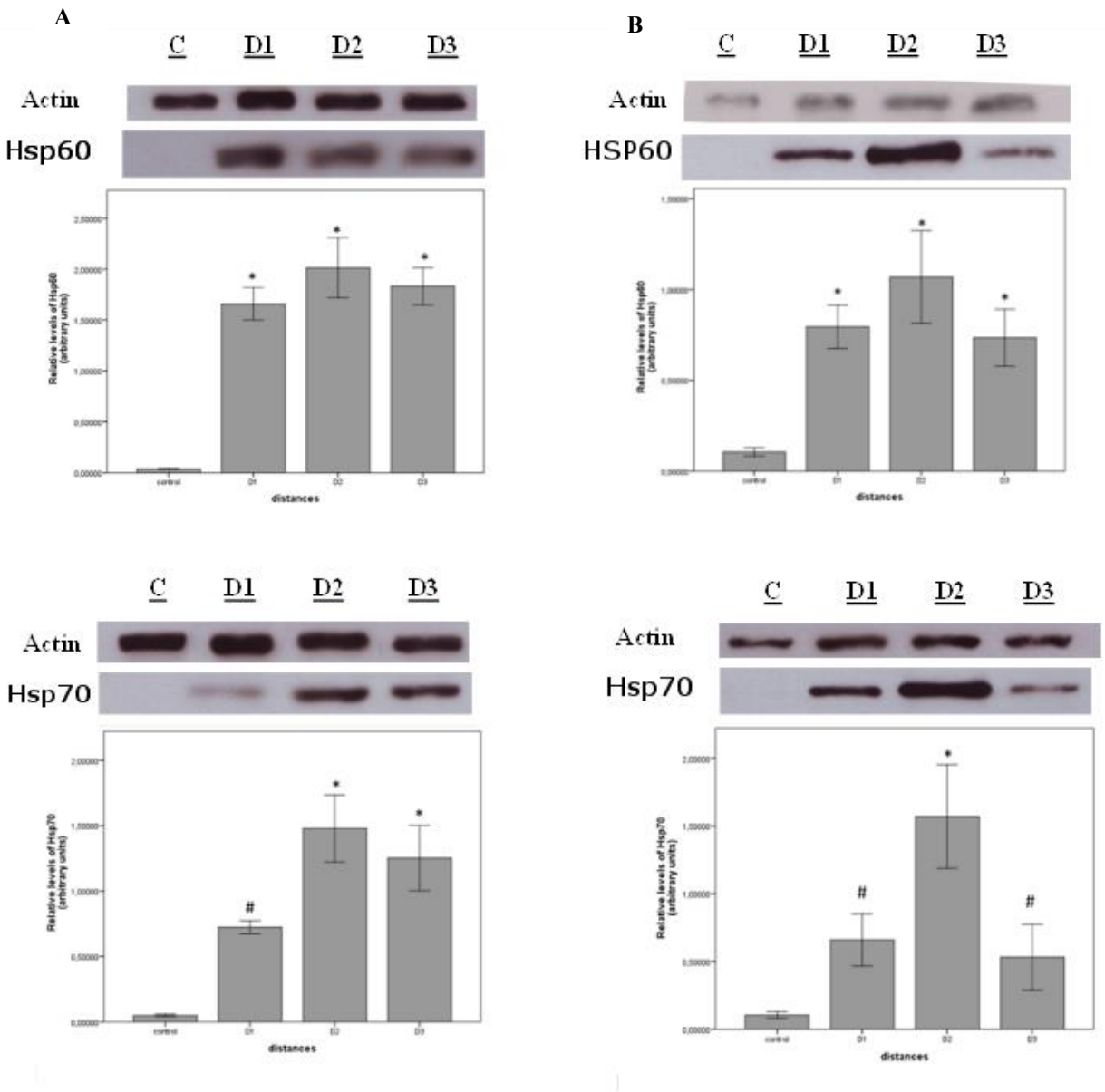


Table 1. Effect of BBD on Hsps modulations in the scleractinian coral *A. muricata* in 2013 (A) and in 2014 (B). Samples of coral fragments located at different distances from the brown band along the disease progression direction (D1, D2 and D3) were subjected to Western analyses and immunodecoration Samples prepared from healthy colonies (C1) are also shown. Filter representative of six experimental repeats (n = 6) is shown. D. Data of Hsps are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, * $p \leq 0.05$ compared to C, # $p \leq 0.05$ compared to D2).

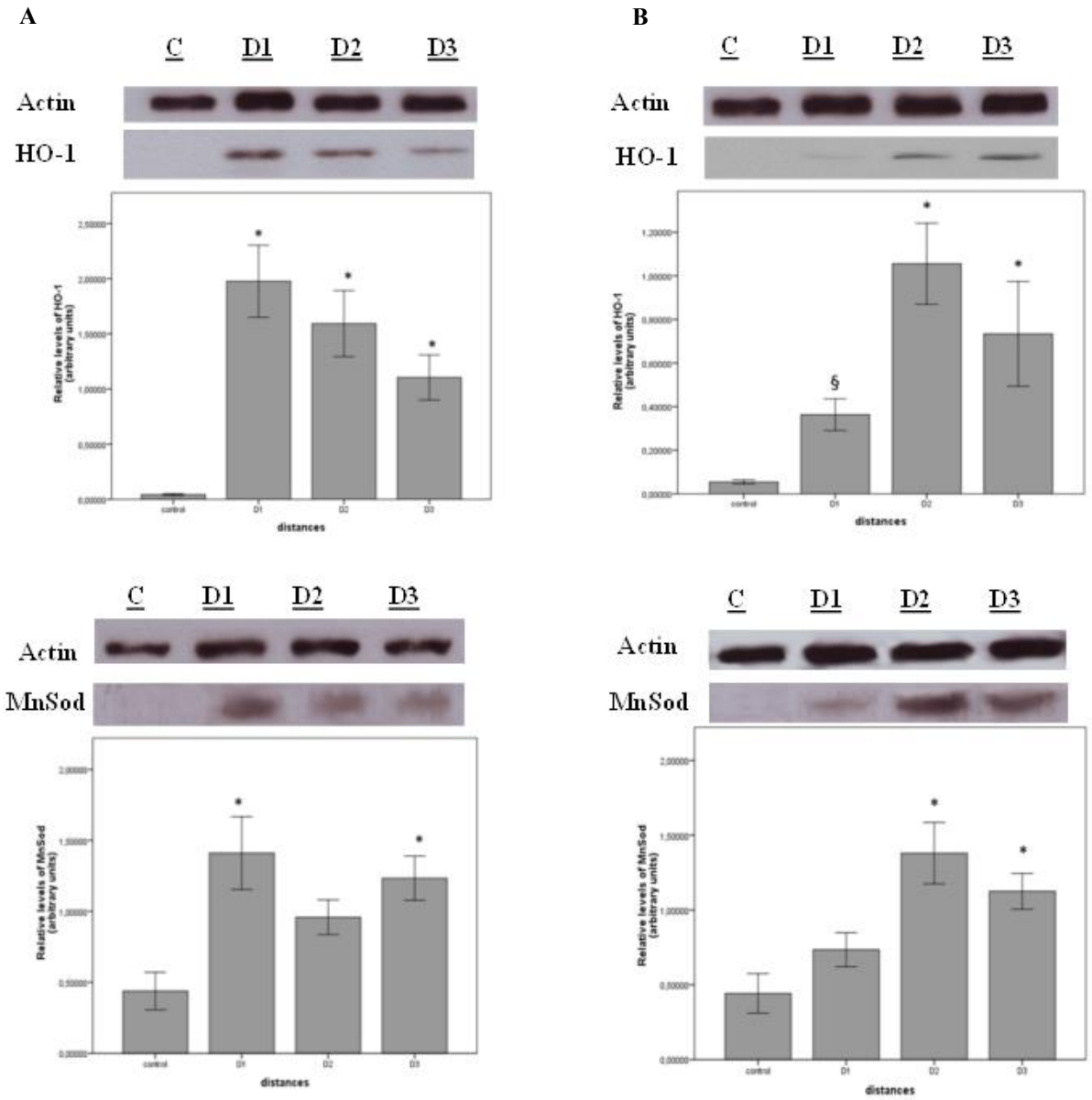
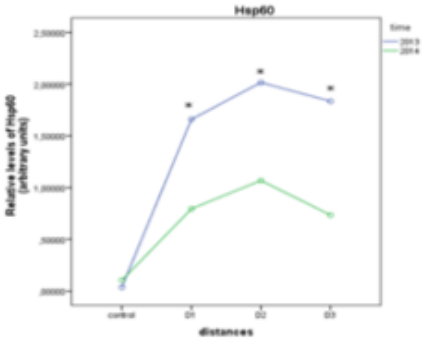


Table-2. Effect of BBD on HO-1 and MnSod modulations in the scleractinian coral *A. muricata* in 2013 (A) and in 2014 (B). Samples of coral fragments located at different distances from the brown band along the disease progression direction (D1, D2 and D3) were subjected to Western analyses and immunodecoration Samples prepared from healthy colonies (C1) are also shown. Filter representative of six experimental repeats (n = 6) is shown. Data of HO-1 and MnSod are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, * $p \leq 0.05$ compared to C, § $p \leq 0.05$ compared to D2).

A

Hsp 60

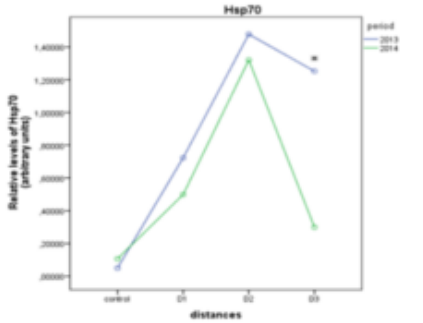
Factors	Sum sq	Df	Mean sq	F Value	Sign.
Time	9,331	1,000	9,331	15,773	0,000
Distance	0,743	2,000	0,371	0,628	0,537
Time * distance	0,093	2,000	0,047	0,079	0,924
Residuals	37,268	63,000	0,592		



B

Hsp 70

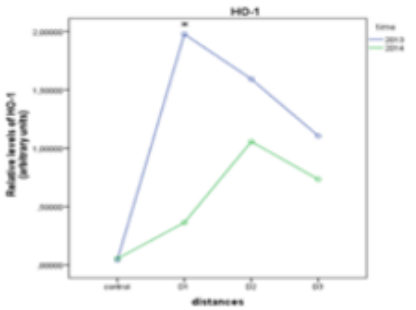
Factors	Sum sq	Df	Mean sq	F Value	Sign.
Time	0,426	1	0,426	0,636	0,428
Distance	17,938	3	5,979	8,922	0,000
Time * distance	1,820	3	0,607	0,905	0,443
Residuals	48,252	72	0,670		



C

HO-1

Factors	Sum sq	Df	Mean sq	F Value	Sign.
Time	7,061	1	7,061	20,328	0,000
Distance	1,114	2	0,557	1,603	0,214
Time * distance	3,033	2	1,516	4,366	0,019
Residuals	13,546	39	0,347		



D

MnSod

Factors	Sum sq	Df	Mean sq	F Value	Sign.
Time	0,049	3	0,049	0,626	0,441
Distance	2,247	1	0,749	9,533	0,001
Time * distance	0,922	3	0,307	3,912	0,029
Residuals	1,257	16	0,079		

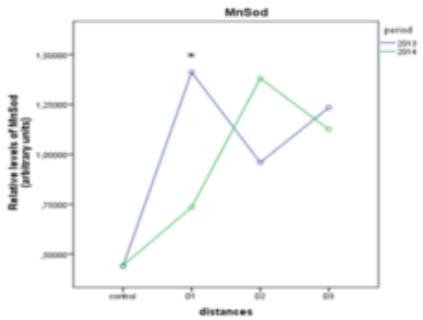


Figure 2. Results of two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons of means (showed on graphs), between years of sampling 2013 and 2014, of all biomarkers intensity values obtained at the three distances from the black band (D1, D2, D3) and the healthy colony used as control. Significative p-values are encircled. (A) Hsp60 intensity values, * $p \leq 0.05$; (B) Hsp70 intensity values, * $p \leq 0.000$; (C) H0-1 intensity values, * $p \leq 0.000$ (D) MnSod intensity values, * $p \leq 0.01$

– Chapter 4 –

Baseline expression of Heat-shock proteins and Heme-oxygenase in three coral species influenced by nictemeral and seasonal temperature and light fluctuations

Abstract

Marine sessile organisms such as corals are affected by daily fluctuations and oscillations, of abiotic factors like light intensity and temperature variations. In response to these moderate variations, organisms alter their behaviour and their physiology. Corals are known to experience large diel changes in their physiology between day and night showing different susceptibility depending mainly by their growth morphology and tissue tickness. Moreover, the Maldivian weather showed significant oscillation in temperature and light intensity being characterized by two season, dry and wet, which differed lightly in these factors. For these reasons, three scleractinian species (*A. tenuis, P. lobata* and *E .lamellosa*) showing different growing morphology were selected and sampled in both seasons in the lagoon of Maghodhoo Island (Faafu Atoll, Maldives) in six time points during the day. Three cellular stress biomarkers were analysed in order to assess the physiological pathways occurring in natural environment; the Heat Shock Proteins 60-kDa (Hsp60) and 70-kDa (Hsp70), and Heme-Oxygenase-1 (HO-1). Our results showed that alterations between light and darkness seems to play a key role in coral physiology, which was influenced by the different growing morphology and by the different seasons. The Hsp60 modulation appeared to be specie-specific in both seasons. The Hsp70 expression during the dry season showed quite any difference among species, since almost all the species displayed the same pattern of down-regulation in the darkness, while a great difference in the modulation was observed during the wet season. On the contrary, HO-1 was not expressed during both day and night in the two seasons and in each species. These results highlight the need of in field study to better understand both how corals normally react to environmental natural changes. In fact despite different growing morphology seems to be confirm to be crucial for coral susceptibility to environmental abiotic stresses, our results showed that other factors, as feeding behaviours, may be suggested to influence the coral physiological mechanisms.

4.1 Introduction

Since alterations in local environments associated with global change are becoming increasingly evident, the understanding of the physiological responses of organisms to these changes has become a priority. How organisms respond to new environments can enhance their survival (Mydlarz et al., 2009). The health and composition of corals reefs are dramatically influenced by the increasing of the average temperature around the world and by the changing in weather patterns, like the El Niño Southern Oscillation. Moreover climate change may also lead to local decreases in temperature or to greater variability in seasonal conditions (Hoegh-Guldberg & Fine, 2004). Corals are extremely sensitive to environmental factors and, not having the ability to relocate, have developed a physiological plasticity with relative limits (Harvell, 1991). However corals are normally exposed to changes in both light and temperature intensity, during the day, even if the magnitude of climate change seems to overwhelm their ability to acclimate as reefs are disappearing with an alarming rate. About 27% of the coral reefs are already destroyed (Frias-Lopez et al., 2004) while about 33% is facing possible extinction (Carpenter et al., 2008). The loss of Indo-Pacific reefs is estimated around an average rate of 2% per year (Mydlarz et al., 2009). In the case of reef corals diseased or experiencing maximum or minimum temperature variations, knowledge of their diel variation in responses related to temperature and irradiance, would be useful. Most reef-building scleractinian corals live near their upper thermal limits and may experience heat stress with an increase of only a few degrees centigrade (Fitt et al., 2001). Recent data report that since the mid-1970s the sea surface temperature across the tropical region increased by 0.4-1°C (Cantin et al., 2010). The global mean surface temperature change for the period 2016–2035 will likely be in the range 0.3°C to 0.7°C (IPCC 2014), while global temperatures are predicted to rise at least 2°C by 2050-2100 (IPCC 2007). So, all model projections show that the thermal tolerance of reef-building corals is likely to be exceeded within the next few decades. In a context of global warming is of fundamental importance to estimate and quantify the baseline expression of biomarkers normally used to assess acute stressors. To date few studies were conducted in situ, on marine organisms, monitoring the effect of heat stress in natural environments or as correlate of warming trend (e.g. Hofmann & Somero, 1995; Minier et al., 2000; Lejeusne et al., 2006). Most of the published works focused on the influence of acute or repetitive stress on the physiology of corals raised or stressed in laboratory conditions. Although these studies provide valid information on specific biological response to a given and controlled

stress, they are less representative and sensitive than fieldwork ones (Lejeusne et al., 2006). The daily rotation of the earth effects organisms with predictable oscillations, through the day, of abiotic factors like light intensity, temperature variation and tides as well as biotic factors like predation and prey availability. In response to these moderate variations organisms often have to alter their behavior and their physiology. Corals experience large diel changes in their physiology between day and night, for example they shift from light and high UV radiation in daytime, with consequent ROS production by meanly *Symbiodinium,* to hypoxic conditions and decrease in pH levels within the coral tissues during the night-time, as a result from respiration of the holobiont (Hemond et al., 2015). Being sessile organisms coral have developed both physiological and behavioral changes, including tentacle extension and retraction, which often display diel patterns (Levy et al., 2006). Their ability to integrate environmental signals into organismal-level responses, due to their clonal nature and colony wide fluid conducting system, allows to transmit local signals throughout the entire colony (Mydlarz et al., 2009). Diel patterns of gene expression and subsequent protein production have been observed in corals in response to both light and circadian regulation (Levy et al., 2007, 2011; Reitzel et al., 2010; Brady et al., 2011). These diel patterns is probably a way for the coral to cope with cyclic patterns of stress, as UV, temperature and ROS, and also to facilitate digestion of molecules given by symbionts (Hemond et al., 2015). Corals response to stressors is dependent on a wide repertoire of mechanisms, with significant variation in the expression of these mechanisms (Mydlarz et al., 2009). Many strategies are mediated through the production of various protective proteins, of antioxidant, of ROS, of membrane stabilizing compound and cellular repair mechanisms (Mydlarz et al., 2009). Heat shock proteins (Hsps) are one of the first protective proteins produced in response to thermal stress, responding also to a wide range of stressors, in order to stabilize proteins and assist cellular functioning (Mydlarz et al., 2009; Lejeusne et al., 2006). Some studies indicate that an increase in Hsps productions is a response to both high irradiance and elevated temperatures often correlated with increased resistance to bleaching (Mydlarz et al., 2009; Brown et al., 2002). Heat shock proteins are often over-expressed during or after environmental stress while showing a constitutive expression in normal condition, expressed with a circadian rhythmicity (Lejeusne et al., 2006; Levy et al., 2011). Antioxidant free-radicals, as the Superoxide Dismutase (Sod) and the Catalase are the first to by produced as defence against the overproduction of reactive oxygen species (ROS). These overproduction of ROS is a consequence of the disruption of photosystems and damage to photosystem II of zooxanthellae, following high temperature and radiance stress.

When ROS are accumulated in a high consistent amount that can damage a wide range of cellular components, antioxidant free-radicals are produced to avoid those damages and the subsequent breaks down of the coral symbiosis and eventual the coral death (Downs et al. 2002; Lesser et al., 2006). Also oxidative stress-protein Heme-Oxygeanase-1 (HO-1) appears to be induced and also increased in its expression by high UV radiation and cellular oxidative stress. It also suggested that heat shock proteins may be involved in Heme-Oxygenase-associated oxidative stress mechanism, following high temperature increasing (Fang et al., 1996). Corals response to stress appears to be highly species/genus specific (McClanahan et al., 2007; Montano et al. 2010; Seveso et.al, 2014.), particularly related to different growing morphology. The differences among species in their susceptibility represent a critical aspect of community dynamics and species diversity (Hughes & Connell, 1999). Branching corals, such as *Acropora* species, appear to have higher mortality rates after bleaching, suggesting that, having thinner tissue, they are less able than other morphologies to sequester protective proteins and to prove shelter from stressors (Loya et al., 2001; Mydlarz et al., 2009). Massive corals, as *Porites* species, on the contrary appear to be less susceptible showing more resistance and recovery than other morphologies (Marshall & Baird, 2000; Mydlarz et al., 2009; Loya et al., 2001).

For these reasons in this study we examined and compared the responses to natural daily thermal variation of three different coral species *Acropora tenuis*, *Porites lobata* and *Echinopora lamellosa* belonging to different growing morphologies.

In each species the expressions and modulations of Hsp60, Hsp70 and HO-1 were analyzed, representing the commonly used biomarkers in coral health assessment (Fang et al., 1996: Downs et al., 2012). Hsps were chosen to asses cellular tolerance to normal environmental condition and HO-1 to verify if oxidative stress could be induced even in normal condition.

4.2 Materials and Methods

4.2.1 Study area and sampling design

The sampling area, composed of several coral patches, of about 200 $m^{2,}$ is located at 80 m offshore from the in the lagoon of Magoodhoo Island (3°04'N; 72°57'E), in the southeastern part of Faafu Atoll, Republic of Maldives (Fig. 1) which permitted easy and quick access suitable for rapid sampling activities. This site extends along the same reef flat zone characterized by similar depths (2−3 m) and is subjected to the same environmental conditions (Seveso et al., 2015).

Colonies of different corals species belonging to three different species, chosen for their different growing morphology and susceptibility to stress: the branching coral *Acropora tenuis*, the massive *Porites lobata* and laminar *Echinopora lamellose.* Colonies were periodically checked in order exclude the presence of diseases or other biotic stressors. Samples were taken in November for the wet season and March for the dry season in order to made a comparison between the two seasons. During each selected month corals were sampled in six time intervals while temperature and light intensity were measured by data-loggers attached to the colonies.

4.2.2 Coral collection

4 apparently healthy colonies for each species were selected randomly. From each colonies small fragments were collected at six time intervals, for each seasons: 02.00, 06.00, 09.00, 13.00, 16.00 and 20.00. All coral samples were taken at the same shallow depth and during high tide when coral are permanently submerged, in order to minimize environmental factors apart from temperature and light intensity (Chow et al., 2009, 2012; Seveso et al., 2013).

Coral samples were excised from colonies using a hollow-point stainless steel spike (8 mm diameter) by applying constant rotational pressure to minimize the size of coral sampled, in order also to reduce the amount of sampling stress and limited excessive damage to the colonies, allowing a rapid coral recovery following the sampling (Bromage et al., 2009). Each sample was collected with a new hollow-point spike to avoid contamination. Then samples were immediately frozen at −80°C using an immersion cooler (FT902, JULABO, Labortechnick) in the MaRHE Centre laboratory at Maghodhoo Island, located a few meters away from the sampling area.

4.2.3 Western blotting

As previously described by Seveso et al. (2013, 2014) once the coral fragments were collected and frozen they were pulverized using a mortar and a pestle. Always following Seveso et al methods, polyp proteins were extracted and *Symbiodinium* were removed.

Following the extraction all the protein samples were frozen at −20°C until used. Protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories) and aliquots of proteins were separated by SDS-PAGE on 8% polyacrylamide gels (Vai et al., 1986) with duplicate gels running in parallel.

After electrophoresis, one gel was stained with Coomassie Brilliant Blue to visualize the total proteins while the other was electroblotted onto nitrocellulose membrane for western blotting. Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters.

Immunostaing, as previously reported by Seveso et al. (2013), was performed with the following antibodies: anti-Hsp60 monoclonal antibody (IgG1 mouse clone LK-2, SPA-807, EnzoLife Sciences), anti-β-Actin monoclonal antibody (clone C4, MAB1501,Millipore), anti-Hsp 70 monoclonal antibody (IgG2a mouse clone BB70, SPA-822, EnzoLife Sciences) and anti-HO-1 monoclonal antibody (IgG2b mouse clone HO-1-2, OSA-111, EnzoLife Sciences), and secondary anti bodies: anti-mouse IgG conjugated with horse radish peroxidase (Thermo Scientific) for anti-Hsp60, anti-Hsp70 and anti-HO-1.

Concentration for the antibodies were for all the primary antibodies 1:1000 while for secontati antibodies: anti-Hsp60 1:10000, anti-Hsp70 1:1000 and anti-HO-1 1:7500. Binding was visualized by Pierce ECL western blotting substrate followed by X-ray films.

Densitometric analysis was performed on a calibrated imaging densitometer (Bio-Rad GS-800) while band intensities were quantified using the Image J free software (http://rsb.info.nih.gov/ij/).

For each blot, the scanned intensity of each biomarker bands was normalized against the intensity of the β-Actin bands, which did not display a significant modulation at the different distances from the black band and throughout the infection time progression.

4.2.4 Statistical analysis

Data were expressed as the mean $\pm$ standard error (SEM) and their normality was verified using a Shapiro Wilk test. One-way analysis of variance (ANOVA) was performed for all the normalized biomarkers intensity values obtained from the different hours samples in the

same season, followed by Tukey's HSD post hoc tests for pairwise comparison of means to asses significant differences ($p \leq 0.05$). For the comparison among the two season periods a Two-way analysis of variance (ANOVA) was performed. To illustrate how biomarkers differed in modulation among species General Linear Models (GLMs) were performed. GLMs take in account the relationships of the predictor variables with responses on the dependent variables, but also the relationships among the multiple dependent variables, in our case the relationships between the three species, the biomarkers data, the hours and the seasons of sampling.

4.3 Results

General overview:

Western analysis revealed that, as regards the morphological appearance of the corals, each of the three coral genera displayed a different modulation for all biomarkers in both season, at different times/hours (Wilk's Lambda =0.55, $F_{(20,\ 154)}$= 2,71 p≤0.000). Although these variations in biomarkers occurred, they were always not accompanied by temperature and light intensity significant variations among hours in the same season and among the two seasons. Meanly is light intensity which have the highest variation during the day alternating darkness to daylight and to which each species seems to react greatly.

Temperature and light variations:

Temperature showed some significant daily variations (Tab.1) in both dry season ($F_{(5,24)}$= 32.67, p≤0,000) and wet season ($F_{(5,31)}$= 19.9, p≤0,000). Also light intensity showed significant differences (Tab.2) in both dry season ($F_{(5,24)}$= 8.23, p≤0,000) and wet season ($F_{(5,31)}$= 12.98, p≤0,000).

Seasonal circadian variations

Heme-oxygenase-1 expression:

HO-1during both seasons and in each species, showed any expression during both day and night. Western Blot analysis didn't detect any signals of HO-1. To test if the problem was not related to the antibody or other external factor, analysis were performed even with a sample from *Goniopora columna*, known from previous experiment to express HO-1. Results for *A.tenuis, P. lobata* and *E. lamellosa* still showed any expression for HO-1.

Hsps expressions:

Dry season vs Wet season:

Hsp60 in *E. lamellosa* showed any modulation throughout the day during the dry season while a one-way Anova revealed a variation during the day in wet season ($F_{(5,12)}$= 4.33, p≤0,05), (Tab.3, Fig2A). *A. tenuis* in the dry season showed significant modulations for Hsp60 throughout the day ($F_{(5,30)}$= 5.60, p≤0,001) whereas during the wet season the Hsp60 expression was not significantly modulated (Tab.4, Fig.2B). *P. lobata* during the dry season Hsp60 is significantly modulated throughout the day ($F_{(5,30)}$= 2.64, p≤0,05) and even during

the wet season Hsp60 levels were modulated through the day ($F_{(5,18)}$= 3.84, p≤0,05), (Tab.5,Fig.2C). Hsp70 in *E.lamellosa*, during the dry season, had a significant variation in its expression along the day ($F_{(5,18)}$= 3.41, p≤0,05) and also in the wet season Hsp70 expression is significantly modulated during the day ($F_{(5,12)}$= 4.33, p≤0,05), (Tab.3, Fig2D). In *A.tenuis*, during the dry season, Hsp70 had a significant variation in its expression along the day ($F_{(5,6)}$= 7.41, p≤0,05) and during the wet season Hsp70 expression is significantly modulated during the day ($F_{(5,12)}$= 10.64, p≤0,000), (Tab.4, Fig2E). Finally in *P.lobata*, during the dry season, Hsp70 didn't exhibit any significant variation in its expression along the day while on the contrary in the wet season Hsp70 expression is significantly modulated during the day ($F_{(5,12)}$= 5.42, p=0,003), (Tab.5, Fig2F).

Circadian variations:

In the morning hours, between 06.00 to 09.00, in dry season when temperature rose significantly (+1.5°C; p≤0.001) both Hsps expressions showed no variations as showed in Figures.3B,C,D and 4B,C,D. On the contrary in wet season there was a variation in Hsp60 and Hsp70 expressions in *P. lobata* with an down regulation at 09.00, compared to 06.00 (p≤0.01), when temperature and light rose significantly (+1.3°C and 29668,2 lx; p≤0.01;), Fig.5D-6D and Tab.5. *P. lobata* also exhibited an down regulation of only Hsp60 at 09.00 when compared to 16.00 (p≤0.05) although temperature and light intensity didn't show variations, Fig.5D and Tab.5.

In both season the highest peak of temperature and light intensity were at 13.00. In dry season (≥ 1.1°C and ≥ 32515.8 lx; Tab.1) quite any significant variations occurred in both Hsp60 and Hsp70 expressions for all the three genera at this hours (Fig.3-4), with just two exceptions: Hsp70 expression in *E. lamellosa* lowered if compared to 16.00 (p≤0.05) with just light intensity significantly different between the two time (+56542 lx; p≤0.01) (Fig.3B, Tab.3) and *A. tenuis* which had higher expression of Hsp60 compared to 20.00 (P≤0.05) with both temperature and light significantly different (+3.2°C and +71231,6 lx; p≤0.000) (Fig.3C, Tab.4). However in the wet season Hsp60 and Hsp70 expressions changed at this hour while both temperature and light intensity lowered significantly (-1.4°C and -38934 lx; p≤0.000, Tab.1-2). In wet season *E. lamellosa* showed higher level of Hsp60 compared to 09.00 (p≤0.05) when temperature differed significantly (-0.9°C; p≤0.05), (Fig.5B, Tab.3). *A. tenuis* showed Hsp70 levels rising at 13.00 compared to 02.00 (p≤0.01) and 06.00 (p≤0.05) when temperature and light intensity rose (≥ 2.2°C and ≥37475.7 lx; p≤0.000) as showed in Fig.6 and Tab.4. Finally *P. lobata* had lower Hsp70 levels at 13.00 compared to 06.00

($p \leq 0.004$) and to 16.00 ($p \leq 0.05$), when temperature and light intensity rose (+2.2°C; +314339 lx; $p \leq 0.000$ and 1.2°C; +37476 lx; $p \leq 0.001$ respectively), (Fig.6, Tab.5).

In the night (20.00 to 02.00) no light is recorded (0 lx) and temperature quite drastically lowered (≤ 3.7°C for dry and ≤ 2.5°C for wet season) as showed in Tab.1 and 2. In dry season *E. lamellosa* had no variation in Hsp60 levels while exhibited a down-regulation for Hsp70 levels at 02.00 compared to 16.00 ($p \leq 0.05$) when temperature significantly differed (-2.3°C; $p \leq 0.000$), (Fig.3-4B, Tab3). *A. tenuis* had a down regulation of Hsp60 at 20.00 compared with the other hours: with 06.00 ($p \leq 0.01$), 09.00 ($p \leq 0.001$) when temperature significantly differed (-1.3°C; $p \leq 0.05$), 13.00 ($p \leq 0.05$) when both temperature and light intensity differed (-3-3°C and -71231,6 lx; $p \leq 0.000$) and 16.00 ($p \leq 0.05$) when temperature differed (-2.3°C; $p \leq 0.000$) with the exception of 02.00 which has similar temperature (-0.1°C) and no light (0 lx), as showed in Fig.3C and Tab.4. Also Hsp70 exhibited a down regulation of at 02.00 ($p \leq 0.01$) and 20.00 ($p \leq 0.05$) again when temperature was lower (-1.3°C and -1.2°C respectively; $p \leq 0.05$) compared to 09.00, (Fig.4C, Tab.4); *P. lobata* instead had an up-regulation in Hsp60 expression at 02.00 compared to 06.00 ($p \leq 0.05$) although any temperature and light variations occurred, as showed in Fig.3D and Tab.5 In wet season *E. lamellosa* showed an up regulation in Hsp60 at 02.00 and 20.00 ($p \leq 0.05$; Fig.5, Tab.3) and an up-regulation of Hsp70 at 02.00 ($p \leq 0.05$; Fig.6, Tab.3) compared to 09.00 when both temperature and light intensity differed (-1.5°C, $p \leq 0.000$; -31516,9 lx, $p \leq 0.001$ and -1.3°C, $p \leq 0.01$; -31516,9 lx, $p \leq 0.001$ respectively) As showed in Fig.6 and Tab.4, *A. tenuis* exhibited a different modulation during the night compared to the other hours: a down-regulation in Hsp70 levels at 02.00 compared to the afternoon hours when temperature and light rose (13.00 $p \leq 0.001$; -2.5°C,-39324,3 lx $p \leq 0.001$ and 16.00 $p \leq 0.05$; -1.3°C $p \leq 0.01$) and also to 20.00 even if temperature and light intensity were not significantly different. Then Hsp70 were up-regulated at 20.00 ($p \leq 0.01$) if compared to 09.00 with a significant difference in temperature and light intensity (+1.3°C, $p \leq 0.01$ and +39324.3 lx, $p \leq 0.001$). Finally *P. lobata* did not show variation in both Hsps between night and day.

Seasonal variations:

Consequently between the two season there were differences among Hsps expression at 13.00 (difference of 1.4°C and 38934 lx, lowering in wet): *E. lamellosa* had Hsp60 and Hsp70 expression higher in the wet season, as showed in Fig.2 A,D, *A. tenuis* rose Hsp70 expression in wet season (Fig.2E) while *P. lobata* showed higher Hsp70 expression in dry season (Fig.2C). Other differences, between the two seasons, in Hsps modulations occurred

although temperature and light intensity didn't show any significant variation among the two seasons. *E. lamellosa* showed significant differences between seasons at 09.00 ($p \leq 0.05$) when Hsp60 expression was lower in wet season, at 02.00 ($p \leq 0.05$) when Hsp60 was higher in wet season and at 20.00 ($p \leq 0.01$) when has higher Hsp60 expression in wet season, as showed in Fig.2A. Also this species had higher Hsp70 expression at 02.00 ($p \leq 0.000$) during wet season (Fig.2D). *A.tenuis*, instead, despite exhibiting a quite similar Hsp60 modulation in both seasons, showed a significant difference in Hsp60 expression between dry and wet season at 20.00 ($p \leq 0.01$) when Hsp60 expression decreased during the dry season (Fig.2B). Also Hsp70 expressions were significantly different between seasons for this species at 09.00($p \leq 0.05$) when levels rose in dry season, at 16.00 ($p \leq 0.01$) when its expression lowered in dry season and at 20.00 ($p \leq 0.000$) when in Wet Hsp70 reached its maximum daily expression while in dry season continued to lower, as showed in Fig.2E. *P. lobata* then, showed a significant difference at 02.00 ($p \leq 0.05$) when Hsp60 expression was higher in dry season than in wet season, at 06.00 ($p \leq 0.05$) when Hsp60 expression is higher in wet season, as showed in Fig.2C and Hsp70 showed an higher expression during the dry season at 09.00 ($p \leq 0.05$) (Fig.2F).

Comparison among the three species:

During the dry season *P. lobata* showed the highest variations: it had the highest Hsp60 levels at 02.00 (Wilk's Lambda =0.83) when the lower temperature and no light intensity were recorded and at 20.00 (Wilks'Lambda=0.81) when temperature and light intensity were similar to 02.00 and 06.00 (Fig.3A, Tab.6). As showed in Fig.3A and Tab.6 Hsp60 was also higher in *P. lobata* compared to *A. tenuis* at 06.00 ($p = 0.01$) when temperature is lower compared to the morning and afternoon hours, and light intensity is lower only if compared to 13.00, and to *E. lamellosa* at 13.00 ($p \leq 0.05$) when temperature rose of about $\geq 2°C$ and light intensity ≥ 71231.6 lx (except 09.00). Finally *P. lobata* have lower expression at 16.00 compared to *A. tenuis* ($p \leq 0.05$) when temperature is higher of about $\geq 2.2°C$ and had no significant light differences, with exception of 09.00 and 13.00 (which differed of just about 1°C, lowering and rising respectively), (Fig.3A, Tab.6). At 09.00 is *A. tenuis* which had the highest Hsp60 levels (Wilks'Lambda=0.81) when temperature were higher compared to morning and night ($\geq 1.2°C$) and lower than the afternoon ($\leq 2.1°C$) while light intensity showed no significant difference with other hours of the day (Fig.3A, Tab.6). While Hsp60 appeared to be differently modulated during the day in the three genera, As showed in Fig.3A and Tab.6, Hsp70 expressed significant variation just in *A. tenuis* compared to *E.*

lamellosa, at 16.00 ($p \leq 0,05$), when its levels were lower. In wet season, as showed in Tab.6 and Fig.5A-6A, there was the quite opposite situation, being Hsp70 the most differently modulated with Hsp60 showing highest levels only in *A. tenuis* at 06.00 (Wilks'Lambda Value =0.77) when temperature and light intensity were lower than daily hours ($\leq$ 2.2°C and $\leq$ 37475.7 lx) and similar to night ones, and at 09.00 (Wilks'Lambda Value =0.89), when temperature rose and light intensity ($\geq$1.3°C; $\geq$23631.5 lx) than night and early morning hours and lowered than 13.00 of 0.9°C and 7807.5 lx. At 06.00 (Wilks'Lambda Value =0.77) *P. lobata* had the highest Hsp70 expression. *P. lobata* had then the lower Hsp70 expression at 13.00 (Wilks'Lambda Value =0.79) when both temperature and light intensity had their peak ($\geq$ 0.9 to 2.5°C and $\geq$ 31439 lx). Then at 02.00, when temperature were lower of morning and afternoon ($\leq$ 2.5°C) and no light, *E. lamellosa* had the highest Hsp70 production (Wilks'Lambda Value =0.77). *A. tenuis* had the highest Hsp70 expression at 20.00 (Wilks'Lambda Value =0.80) when both temperature and light intensity were lowering ($\leq$ 2.3°C and 39324.3 lx).

4.4 Discussion and conlusion

Alterations in light intensity and between light and darkness periods seems to play a key role in coral physiology, as previously demonstrated in laboratory studies (Chow et al., 2012). In addition, in field studies, these variations appeared to be affected by seasonal weather fluctuations. Even though the seasonal environmental differences occurred just at 13.00 when both temperature and light intensity increased in dry season, at 16.00 when only the temperature increased, and then after 06.00 when light started to rising later in dry season than in wet season (respectively 97 lx vs 1812 lx). Corals are known to shift from phototrophic activity during the day, when their endosymbionts produced energy, to heterotrophic processes at night, when they mostly prey plankton by their tentacles, thus experiencing diel changes in their physiology as consequence (Hemond et al., 2015). Behavioural activities could explain some of our results and for example, the Hsp60 variation of *A. tenuis* between 06.00 and 20.00 in the wet season, when a significant light and temperature variation occurred. Moreover, since a reaction to circadian variations seem to be specie-specific, our results appeared to be more representative of corals in general than those obtainable by laboratory experiments. In this study all the colonies of *P. lobata*, *E. lamellosa* and *A. tenuis* showed a down-regulation of Hsp70 during the darkness period in dry season and in additionthis down-regulation also occurred in *P. lobata* and *A. tenuis* in the wet season with the exception of the 20.00 for *A. tenuis*. Also Hsp60 appeared down-expressed during the night in *A. tenuis* in both season while is up-expressed Hsp6 in both seasons in the other two species *P. lobata* and *E. lamellosa*. Another factor which seems to influence the Hsps expressionappeared to be the seasonal variation characterizing the Maldivian weather which differently could influence the different coral species and the different typology of Hsps considered. In this context, the Hsp70 in dry season did not show significant difference among species, since almost all the species displayed the same Hsp70 patterns. On the contrary the Hsp70 showed the greater difference during the wet season. The Hsp60 modulation, instead, seemed to be more specie-specific in both seasons and this could be related to the location of Hsp60 into the mitochondria and their cellular work. Specifically during the dry season *E. lamellosa* didn't modulate Hsp60 both during night and day, always keeping its levels high, while during wet season *A. tenuis* showed this pattern. This may be related to the different susceptibility to environmental stress of the two coral genera characterized by two different growth form and morphology, since *Echinopora* generally is considered as a moderate tolerant genus while *Acropora* a highly susceptible

genus (Marhall & Baird, 2000). The diversity among species in their susceptibility to disturbances represents a critical aspect of community dynamics, since changes in the community structure and species composition determined the long-term persistence of coral reefs (Seveso et al., 2014). Coral susceptibility is known to be related to the morphology growth, to metabolic rates, tissue thickness and host CO_2 supply strategies.

In this context *A. tenuis* may suffer more the diel variations during the hottest and brightest season (dry) than *E. lamellosa* which instead may be less tolerant when temperature and light lowered (wet). However, during dry season in the morning all the species didn't show any significant modulation of Hsps keeping their level high, with the exception of *P. lobata* that had low level of Hsp70 in the morning. In wet season, instead, the Hsps expression profiles changed although temperature decreased from dry season just at 13.00 and 16.00, and light intensity at 13.00,. During the morning, both Hsps showed variation in their modulations. *P. lobata* had an up-regulation in early morning of both Hsps and followed by a decrease and a final increase in the night . Then when temperature and light intensity rose to reach the peak at 13.00, and seasons differed significantly, in dry season again quite any significant modulations occurred in Hsps, with the exception of Hsp70 in *E. lamellosa,* which is drastically down-regulated. However, some lowering during the seasonal peak can be detected also in the other species, although not statistically significant. Instead in wet season *P. lobata* down-regulated Hsp70, keeping high the overall level of Hsp60. On the contrary, *E. lamellosa* up-regulated Hsp60 keeping high the general level of Hsp70. *A. tenuis* up-regulated Hsp70, keeping high and not modulating the Hsp60 levels. When temperature and light started to decrease, in dry season *E. lamellosa* increased Hsp70 level to then lowered it during the night regaining the paths followed by the other two species which lowered their Hsp70 expression into the darkness. Instead in wet season *E. lamellosa* kept high levels of Hsp60 and up-regulated Hsp70 in the night. *A. tenuis* instead, keeping Hsp60 still high, up-regulated Hsp70 level till the start of darkness, then down-regulated it in late night, keeping it at low levels till the up-regulation of 13.00. Opposite to the other species *P. lobata* didn't modulated both Hsps during the night, keeping their levels high. Instead in dry season during the night the three species showed different modulations patterns of Hsp60 with *E. lamellosa* which did note modulate it keeping the high levels of the day, *A. tenuis,* down-regulating Hsp60 while *P. lobata* up-regulating it.

E. lamellosa and *P. lobata* behaviour during the night in dry season and those of *E. lamellosa* and *A. tenuis* in wet season (i.e. having high levels of Hsp60) may be explained by the metabolic explanation given by Hemond and colleagues (2015) since the food become

more available, at night, in both season, when coral become heterotrophic and the difference occurring between season may be due to specie-specific adaptive strategies. Also in other studies, the elevation of this protein has been associated with a probable change in the equilibria of many mitochondrial metabolic pathways, although often in stressed conditions (Papp et al., 2003; Down et al., 2006). During dry season, the high levels that all the species kept during the day may be explained with the major request of energy following the calcification increasing in the light, a process known as "light-enhanced calcification" (LEC) (Hemond et al., 2015). This process required energy and thus major work for chaperone proteins, which can differ their expression following the different growing velocity of each species. Despite the LEC can explain the rise of Hsps during the daylight, *P. lobata*, a massive coral which is expected to grow at a slower rate than the other morphologies (Marhall & Baird, 2000), showed the highest Hsp60 modulation during dry season, with the exception of 09.00 and 16.00 when is "beaten" by *A. tenuis.* These rise during daylight in *A. tenuis* may be related with LEC as branching corals are expected to grow at a faster rate during the day (Marhall & Baird, 2000). For Hsp70 the three species didn't show, in dry season, variations among them in its expression, keeping it high during the day (with the down-regulation at 13.00), probably expressing it for normal cellular pathways and not as reaction to stress, till light and temperature reached their extremes, higher peaks and darkness-low temperature. Indeed Hsp70 trend could be related to many cellular pathways and it may be modulated to compensate for shifts in protein degradation rates and/or in relation to other cellular needs (Down et al., 2012; Papp et al., 2003; Down et al., 2006). In wet season Hsp70 appeared to be the most differently modulated among the three species while Hsp60 has higher levels just in *A. tenuis* during the morning, probably mirroring the dry season LEC needs. Hsp70 had the highest levels in *P. lobata* during early morning for then showing the lowest level in correspondence of 13.00. In the night *A. tenuis* displayed the highest level of Hsp70 (20.00) for then "giving in to"*E. lamellosa* that had the highest level at late night (02.00). *P. lobata* showed a very different behaviour in Hsp70 expression and the "shift" occurring between the two season for the down-regulation, may indicate that during wet season *P. lobate,* despite being a massive coral and thus more resistant (Marshall & Baird, 2000; Mydlarz et al., 2009; Loya et al., 2001), after the increasing of light and temperature, may request a suspension or a reduction in Hsps production. However, Hsp60 resumed its high levels at 13.00. This may be related to the different location and utilization of Hsp60 and Hsp70, with the first more related to respiration and metabolic pathways then the latter. *A. tenuis* showed an opposite modulation for Hsp70 during the two seasons with

down-regulation and up-regulation quite inverted. This indicated that differences in temperature and light intensity between seasons may play a role in the behaviour of this species. Finally *E. lamellosa* appeared to be the species with a marked oscillatory pattern in both Hsps expression that changed to the quite opposite depending in which season it occurred. This is in particular evidenced by the reaction of both Hsps during the seasonal environmental peaks, which changed significantly between seasons (±1.4°C and 38934lx),. and could be related to the laminar morphology of this coral and thus with a moderate tolerance to environmental changes.

This study results highlighted the need of *in situ* study directly in the natural environment to better understand both how corals normally react to environmental natural changes and to stressors. Our results appear to confirm that different growing morphology were crucial for coral susceptibility (McClanahan et al., 2007; Montano et al., 2010; Hughes & Connell 1999), as showed by *E.lamellosa.* However the massive *P. lobata* showing the highest levels of both Hsps may indicated that this species exhibit these high basal levels for reasons that exceeded resistance. Moreover, comparing the *P. lobata* results with another study in the same genera but different species, *P. asteroids,* we found that they are quite contrast, at least for Hsp60 (Kenkel et al., 2011), since in this work Hsp60 were up-regulated during thermal stresses in the afternoon when corals were full exposed to the sun, while *P. lobata* up-regulated Hsp60 during the night, when temperature lowered. However, Kenekl and colleagues registered a rise of 7-8°C which is much more than Maldives maximal peak of 1.4°C which could not represent a thermal stress thus leading corals to modulate Hsps in reaction just to normal physiological cellular pathways. In conclusion this study highlights how each species seems to have a basal specie-specific pathway of Hsps expression, as confirmed by laboratory studies (Brown & Down, 2002; Kingsley et al., 2003). In the Maldivian environment, the basal pathway is indeed influenced by seasonal changes and may be due to a different adaptation to the same environmental habitat. Also the metabolic explanation of the reaction of Hsps to light and darkness could be integrated with those of Chow and colleagues (2012) confirming that Hsp60 are regulated by an oscillatory mechanism.

4.5 Acknowledgments

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4.6.Reference

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4.7. Figures and Tables

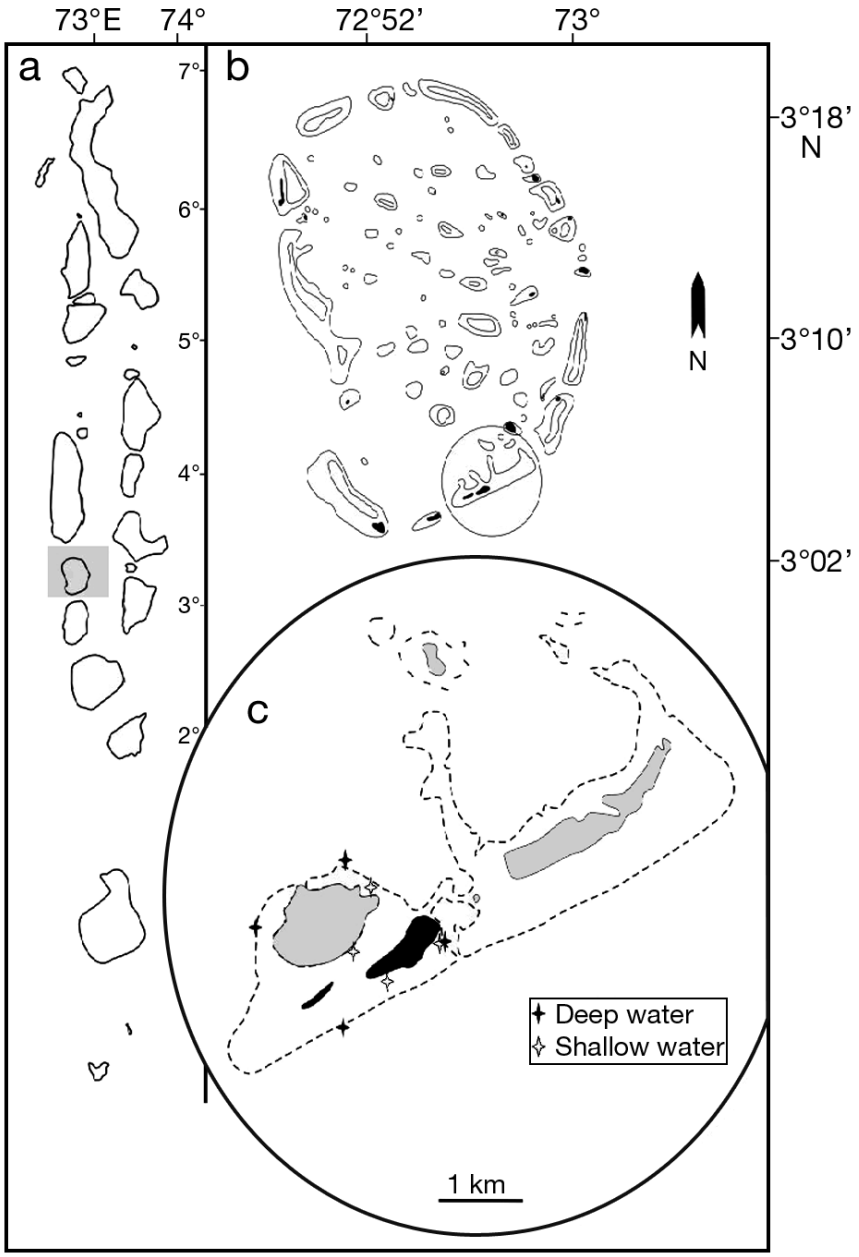


Figure 1 Map of Magoodhoo Island (C), located in the south east part of Faafu Atoll (B), Republic of Maldives (A). In B and C, the islands are indicated in black and gray. In C the dotted line indicates the reef edges the stars indicate the water levels. In black is island hosting the MaRHE Centre.

Dry season						Wet season					
hours	mean °C	hours	mean's difference	St.err.	Sign.	hours	mean °C	hours	mean's difference	St.err.	Sign.
02.00	28.97	06.00	0,42	0,36	0,855	02.00	28.4	06.00	-0,26	0,31	0,958
		09.00	-1,26	0,36	**0,021**			09.00	-1,52	0,31	**0,000**
		13.00	-3,31	0,36	**0,000**			13.00	-2,46	0,31	**0,000**
		16.00	-2,25	0,36	**0,000**			16.00	-1,26	0,30	**0,002**
		20.00	-0,09	0,36	1,000			20.00	-0,19	0,31	0,988
06.00	28.6	02.00	-0,42	0,36	0,855	06.00	28.7	02.00	0,26	0,31	0,958
		09.00	-1,68	0,36	**0,001**			09.00	-1,26	0,31	**0,003**
		13.00	-3,73	0,36	**0,000**			13.00	-2,20	0,31	**0,000**
		16.00	-2,67	0,36	**0,000**			16.00	-1,00	0,30	**0,021**
		20.00	-0,51	0,36	0,727			20.00	0,07	0,31	1,000
09.00	30.2	02.00	1,26	0,36	**0,021**	09.00	29.9	02.00	1,52	0,31	**0,000**
		06.00	1,68	0,36	**0,001**			06.00	1,26	0,31	**0,003**
		13.00	-2,05	0,36	**0,000**			13.00	-0,94	0,31	**0,045**
		16.00	-0,99	0,36	0,107			16.00	0,26	0,30	0,952
		20.00	1,17	0,36	**0,037**			20.00	1,32	0,31	**0,002**
13.00	32.3	02.00	3,31	0,36	**0,000**	13.00	30.9	02.00	2,46	0,31	**0,000**
		06.00	3,73	0,36	**0,000**			06.00	2,20	0,31	**0,000**
		09.00	2,05	0,36	**0,000**			09.00	0,94	0,31	**0,045**
		16.00	1,06	0,36	0,073			16.00	1,20	0,30	**0,004**
		20.00	3,22	0,36	**0,000**			20.00	2,27	0,31	**0,000**
16.00	32.2	02.00	2,25	0,36	**0,000**	16.00	29.6	02.00	1,26	0,30	**0,002**
		06.00	2,67	0,36	**0,000**			06.00	1,00	0,30	**0,021**
		09.00	0,99	0,36	0,107			09.00	-0,26	0,30	0,952
		13.00	-1,06	0,36	0,073			13.00	-1,20	0,30	**0,004**
		20.00	2,16	0,36	**0,000**			20.00	1,07	0,30	**0,012**
20.00	29.1	02.00	0,09	0,36	1,000	20.00	28.6	02.00	0,19	0,31	0,988
		06.00	0,51	0,36	0,727			06.00	-0,07	0,31	1,000
		09.00	-1,17	0,36	**0,037**			09.00	-1,32	0,31	**0,002**
		13.00	-3,22	0,36	**0,000**			13.00	-2,27	0,31	**0,000**
		16.00	-2,16	0,36	**0,000**			16.00	-1,07	0,30	**0,012**

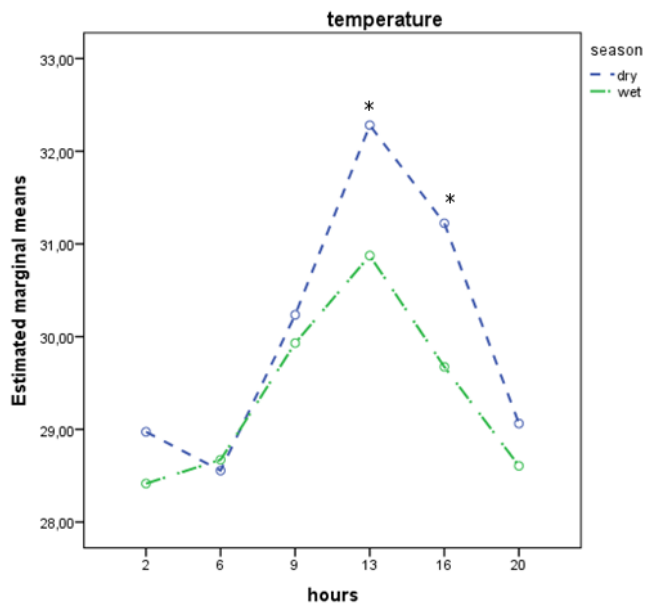


Table 1. (A) Tukey's HSD post hoc tests for pair-wise comparison of means for dry and wet season daily values of sea temperature T (°C) measured by data loggers at each sampling hour, significant p-value (≤0.05 and ≤0.001) are in bolt. (B) Comparison between seasons of sea temperature values T (°C) (two -way ANOVA followed by Tukey's HSD multiple pairwise comparisons, * p ≤ 0.000).

Dry season						Wet season					
hours	mean lx	hours	mean's difference	St.err.	Sign.	hours	mean lx	hours	mean's difference	St.err.	Sign.
02.00	0	06.00	-3594,10	14051,44	1,000	02.00	0	06.00	-1.848,68	6.861,56	1,000
		09.00	-38715,80	14051,44	0,101			09.00	-31.516,88	6.861,56	**0,001**
		13.00	-71231,60	14051,44	**0,000**			13.00	-39.324,33	6.861,56	**0,000**
		16.00	-14689,60	14051,44	0,898			16.00	-7.885,34	6.611,97	0,837
		20.00	0,00	14051,44	1,000			20.00	0,00	6.861,56	1,000
06.00	96.9	02.00	3594,10	14051,44	1,000	06.00	1812.4	02.00	1.848,68	6.861,56	1,000
		09.00	-35121,70	14051,44	0,164			09.00	-29.668,20	6.861,56	**0,002**
		13.00	-67637,50	14051,44	**0,001**			13.00	-37.475,65	6.861,56	**0,000**
		16.00	-11095,50	14051,44	0,967			16.00	-6.036,66	6.611,97	0,940
		20.00	3594,10	14051,44	1,000			20.00	1.848,68	6.861,56	1,000
09.00	31689.1	02.00	38715,80	14051,44	0,101	09.00	40214.2	02.00	31.516,88	6.861,56	**0,001**
		06.00	35121,70	14051,44	0,164			06.00	29.668,20	6.861,56	**0,002**
		13.00	-32515,80	14051,44	0,227			13.00	-7.807,45	6.861,56	0,862
		16.00	24026,20	14051,44	0,539			16.00	23.631,54	6.611,97	**0,014**
		20.00	38715,80	14051,44	0,101			20.00	31.516,88	6.861,56	**0,001**
13.00	78258.3	02.00	71231,60	14051,44	**0,000**	13.00	49428.1	02.00	39.324,33	6.861,56	**0,000**
		06.00	67637,50	14051,44	**0,001**			06.00	37.475,65	6.861,56	**0,000**
		09.00	32515,80	14051,44	0,227			09.00	7.807,45	6.861,56	0,862
		16.00	56542,00	14051,44	**0,006**			16.00	31438,99	6.611,97	**0,001**
		20.00	71231,60	14051,44	**0,000**			20.00	39.324,33	6.861,56	**0,000**
16.00	18186.8	02.00	14689,60	14051,44	0,898	16.00	8404.5	02.00	7.885,34	6.611,97	0,837
		06.00	11095,50	14051,44	0,967			06.00	6.036,66	6.611,97	0,940
		09.00	-24026,20	14051,44	0,539			09.00	-23.631,54	6.611,97	**0,014**
		13.00	-56542,00	14051,44	**0,006**			13.00	-31.438,99	6.611,97	**0,001**
		20.00	14689,60	14051,44	0,898			20.00	7.885,34	6.611,97	0,837
20.00	0	02.00	0,00	14051,44	1,000	20.00	0	02.00	0,00	6.861,56	1,000
		06.00	-3594,10	14051,44	1,000			06.00	-1.848,68	6.861,56	1,000
		09.00	-38715,80	14051,44	0,101			09.00	-31.516,88	6.861,56	**0,001**
		13.00	-71231,60	14051,44	**0,000**			13.00	-39.324,33	6.861,56	**0,000**
		16.00	-14689,60	14051,44	0,898			16.00	-7885,34	6611,97	0,837

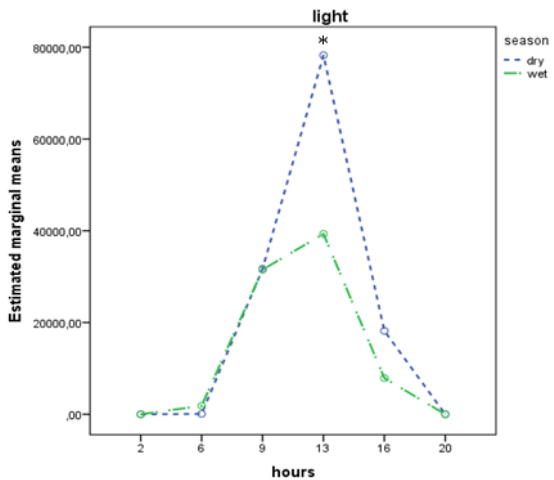


Table 2. (A) Tukey's HSD post hoc tests for pair-wise comparison of means for dry and wet season daily values of lighy intensity LI (Lux) measured by data loggers at each sampling hour, significant p-value (≤0.05 and ≤0.001) are in bolt.
(B) Comparison between seasons of sea temperature values LI (Lux) (two -way ANOVA followed by Tukey's HSD multiple pairwise comparisons, * p ≤ 0.000).

Hsps	season	Hsp	E. lamellosa	02	06	09	13	16	20	Temp	light
Hsp60			**Btw species**								
02.00	dry		↓ 0.038	-	-↑	-↓↓	-↓↓	-↓↓	-↓	29	*0*
06.00	dry		-	-↓	-	-↓↓	-↓↓	-↓↓	-↓	28,6	*97*
9	dry		↑ 0.015	-↑↑	-↑↑	-	-↓↓	-↓↑	-↑↑	30,2	*31689*
13	dry		↓ 0.013	-↑↑	-↑↑	-↑↑	-	-↑↑	-↑↑	**32,3**	***78258***
16	dry		-	-↑↑	-↑↑	-↑↓	-↓↓	-	-↑	31,2	*18187*
20	dry		↓ 0.006	-	-↑	-↓↓	-↓↓	-↓↓	-	29,1	*0*
Hsp60											
2	wet	Up	↑ 0.038	-	-↓↓	0.041↓↓	-↓↓	-↓↓	-↓↓	28,4	*0*
6	wet		-	-↑	-	-↓↓	-↓↓	-↓↓	-↓	28,7	*1812*
9	wet	Dw	↓ 0.015	0.041↑↑	-↑↑	-	0.014↓↓	-↑↑	0.039↑↑	29,9	*40214*
13	wet	Up	↑ 0.013	-↑↑	-↑↑	0.014↑↑	-↑↑	-↑↑	-↑↑	**30,9**	***49428***
16	wet		-	-↑↑	-↑↑	-↓↓	-↓↓	-↑	-↑↑	29,6	*8404*
20	wet	Up	↑ 0.006	-↑↑	-↓	0.039↓↓	-↓↓	-↓↓	-	28,6	*0*
Hsp70											
2	dry	Dw	↓ 0.000	-	-↑	-↓↓	-↓↓	0.039↓↓	-↓	29	*0*
6	dry			-↓	-	-↓↓	-↓↑	-↓↓	-↓	28,6	*97*
9	dry			-↑↑	-↑↑	-	-↓	-↓↑	-↑↑	30,2	*31689*
13	dry	Dw	↓ 0.003	-↑↑	-↑↑	-↑↑	-	0.049↑↑	-↑↑	**32,3**	***78258***
16	dry	Up		0.039↑↑	-↑↑	-↑↓	0.049↓↓	-	-↑	31,2	*18187*
20	dry			-	-↑	-↓↓	-↓↓	-↓↓	-	29,1	*0*
Hsp70											
2	Wet	Up	↑ 0.000	-	-↓↓	0.04↑↑	-↓↓	-↓↓	-↓↓	28,4	*0*
6	wet			-↑	-	-↓↓	-↓↓	-↓↓	-↓	28,7	*1812*
9	wet	Dw		0.04↑↑	-↑↑	-	-↓↓	-↑↑	-↑↑	29,9	*40214*
13	wet		↑ 0.003	-↑↑	-↑↑	-↑↑	-↑↑	-↑↑	-↑↑	**30,9**	***49428***
16	wet		-	-↑↑	-↑↑	-↓↓	-↓↓	-	-↑↑	29,6	*8404*
20	wet		-	-↑↑	-↓	-↓↓	-↓↓	-↓↓	-	28,6	*0*

Table 3. Effect of Temperature and light intensity on Hsps modulation in the scleractinian coral *E. lamellosa* in dry and wet seasons. (Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons) and multiple comparison among species. Just significant p-value (≤0.05 and ≤0.001) and sea temperature T(°C) and light intensity LI(Lux) mean values are reported, highest seasonal values are in bolt. Arrows in orange (LI) and black (T) indicate environmental parameters patterns, green arrows the modulation of the Hsps.

Hsps	season	Hsp	A. tenuis	02	06	09	13	16	20	Temp	light
Hsp60			**Btw seasons**								
02.00	Dry	-	↓ 0.001	-	-↑	-↓↓	-↓↓	-↓↓	-↓	29	*0*
06.00	Dry	Up		-↓	-	-↓↓	-↓↓	-↓↓	- 0.008↓↑	28,6	*97*
9	dry	Up		-↑↑	-↑↑	-	-↓↓	-↓↑	0.001↑↑	30,2	*31689*
13	dry	Up		-↑↑	-↑↑	-↑↑	-	-↑↑	0.018↑↑	**32,3**	***78258***
16	dry	Up		-↑↑	-↑↑	-↑↓	-↓↓	-	0.043↑↑	31,2	*18187*
20	dry	Dw		-	0.008↑↓	0.001↓↓	0.018↓↓	0.043↓↓	-	29,1	*0*
Hsp60											
2	wet	-	↑ 0.001	-	-↓↓	↓↓	-↓↓	-↓↓	-↓↓	28,4	*0*
6	wet	-		-↑	-	-↓↓	-↓↓	-↓↓	-↓	28,7	*1812*
9	wet	-		↑↑	-↑↑	-	↓↓	-↑↑	↑↑	29,9	*40214*
13	wet	-		-↑↑	-↑↑	↑↑	-↑↑	-↑↑	-↑↑	**30,9**	***49428***
16	wet	-		-↑↑	-↑↑	-↓↓	-↓↓	-↑	-↑↑	29,6	*8404*
20	wet	-		-↑↑	-↓	↓↓	-↓↓	-↓↓	-	28,6	*0*
Hsp70											
2	Dry	Dw		-	-↑↓	0.01↓↓	-↓↓	-↓↓	-↓	29	*0*
6	dry	-		0.01↓↑	-	-↓↓	-↓↓	-↓↓	-↓↑	28,6	*97*
9	dry	Up	↑ 0.015	-↑↑	-↑↑	-	-↓↓	-↓↑	0.031↑↑	30,2	*31689*
13	dry	-	↓ 0.012	-↑↑	-↑↑	-↑↑	-	-↑↑	-↑↑	**32,3**	***78258***
16	dry	-	↓ 0,011	-↑↑	-↑↑	-↑↓	-↓↓	-	-↑↑	31,2	*18187*
20	dry	Dw	↓ 0.000	-	-↑↓	0.031↓↓	-↓↓	-↓↓	-	29,1	*0*
Hsp70											
2	Wet	Dw		-	-↓↓	↓↓	0.012↓↓	0.019↓↓	0.001↓↓	28,4	*0*
6	wet	Dw		-↑	-	-↓↓	0.038↓↓	-↓↓	0.004↓	28,7	*1812*
9	Wet	Dw	↓ 0.015	-↑↑	-↑↑	-	-↓↓	-↑↑	0.006↑↑	29,9	*40214*
13	wet	Up	↑ 0.012	0.012↑↑	0.038↑↑	-↑↑	-↑↑	-↑↑	-↑↑	**30,9**	***49428***
16	wet	Up	↑ 0,011	0.019↑↑	-↑↑	-↓↓	-↓↓	-	-↑↑	29,6	*8404*
20	wet	Up	↑ 0.000	0.001↑↑	0.004↓	0.006↓↓	-↓↓	-↓↓	-	28,6	*0*

Table 4. Effect of Temperature and light intensity on Hsps modulation in the scleractinian coral *A. tenuis* in dry and wet seasons. (Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons) and multiple comparison among species. Just significant p-value (≤0.05 and ≤0.001), and sea temperature T(°C) and light intensity LI(Lux) mean values are reported, highest seasonal values are in bolt. Arrows in orange (LI) and black (T) indicate environmental parameters patterns, green arrows the modulation of the Hsps.

Hsps	season	Hsp	P. lobata	02	06	09	13	16	20	Temp	*light*
Hsp60			**Btw seasons**								
02.00	Dry	Up	↑ 0.054	-	0.049↑↓	-↓↓	-↓↓	-↓↓	-↓	29	*0*
06.00	Dry	Dw	↓ 0.044	0.049↓↑	-	-↓↓	-↓↓	-↓↓	-↓↑	28,6	*97*
9	dry			-↑↑	-↑↑	-	-↓↓	-↓↑	-↑↑	30,2	*31689*
13	dry			-↑↑	-↑↑	-↑↑	-	-↑↑	-↑↑	**32,3**	***78258***
16	dry			-↑↑	-↑↑	-↑↓	-↓↓	-	-↑↑	31,2	*18187*
20	dry			-	-↑↓	-↓↓	-↓↓	-↓↓	-	29,1	*0*
Hsp60											
2	wet		↓ 0.054	-	-↓↓	↓↓	-↓↓	-↓↓	-↓↓	28,4	*0*
6	wet	Up	↑ 0.044	-↑	-	0.017↓↓	-↓↓	-↓↓	-↓	28,7	*1812*
9	wet	Dw		↑↑	0.017↑↑	-	↓↓	0.053↑↑	-↑↑	29,9	*40214*
13	wet			-↑↑	-↑↑	↑↑	-↑↑	-↑↑	-↑↑	**30,9**	***49428***
16	wet	Up		-↑↑	0.053↑↑	-↓↓	-↓↓	-	-↑↑	29,6	*8404*
20	wet			-↑↑	-↓	↓↓	-↓↓	-↓↓	-	28,6	*0*
Hsp70											
2	Dry			-	-↑↓	-↓↓	-↓↓	-↓↓	-↓	29	*0*
6	dry			-↓↑	-	-↓↓	-↓↓	-↓↓	-↓↑	28,6	*97*
9	dry		↑ 0.032	-↑↑	-↑↑	-	-↓↓	-↓↑	-↑↑	30,2	*31689*
13	dry		↑ 0.054	-↑↑	-↑↑	-↑↑	-	-↑↑	-↑↑	**32,3**	***78258***
16	dry			-↑↑	-↑↑	-↑↓	-↓↓	-	-↑↑	31,2	*18187*
20	dry			-	-↑↓	-↓↓	-↓↓	-↓↓	-	29,1	*0*
Hsp70											
2	Wet			-	-↓↓	-↓↓	↓↓	↓↓	↓↓	28,4	*0*
6	Wet	Up		-↑	-	0.014↓↓	0.004↓↓	-↓↓	↓	28,7	*1812*
9	Wet		↓ 0.032	-↑↑	-↑↑	-	-↓↓	-↑↑		29,9	*40214*
13	wet	Dw	↓ 0.054	↑↑	0.004↑↑	-↑↑	-↑↑	0.047↑↑	-↑↑	**30,9**	***49428***
16	wet	Up		↑↑	-↑↑	-↓↓	0.047↓↓	-	-↑↑	29,6	*8404*
20	wet			↑↑	↓	↓↓	-↓↓	-↓↓	-	28,6	*0*

Table 5. Effect of Temperature and light intensity on Hsps modulation in the scleractinian coral *P .lobata.* in dry and wet seasons. (Two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons) and multiple comparison among species.Just significant p-value (≤0.05 and ≤0.001 and sea temperature T(°C) and light intensity LI(Lux) mean values are reported, highest seasonal values are in bolt. Arrows in orange (LI) and black (T) indicate environmental parameters patterns, green arrows the modulation of the Hsps.

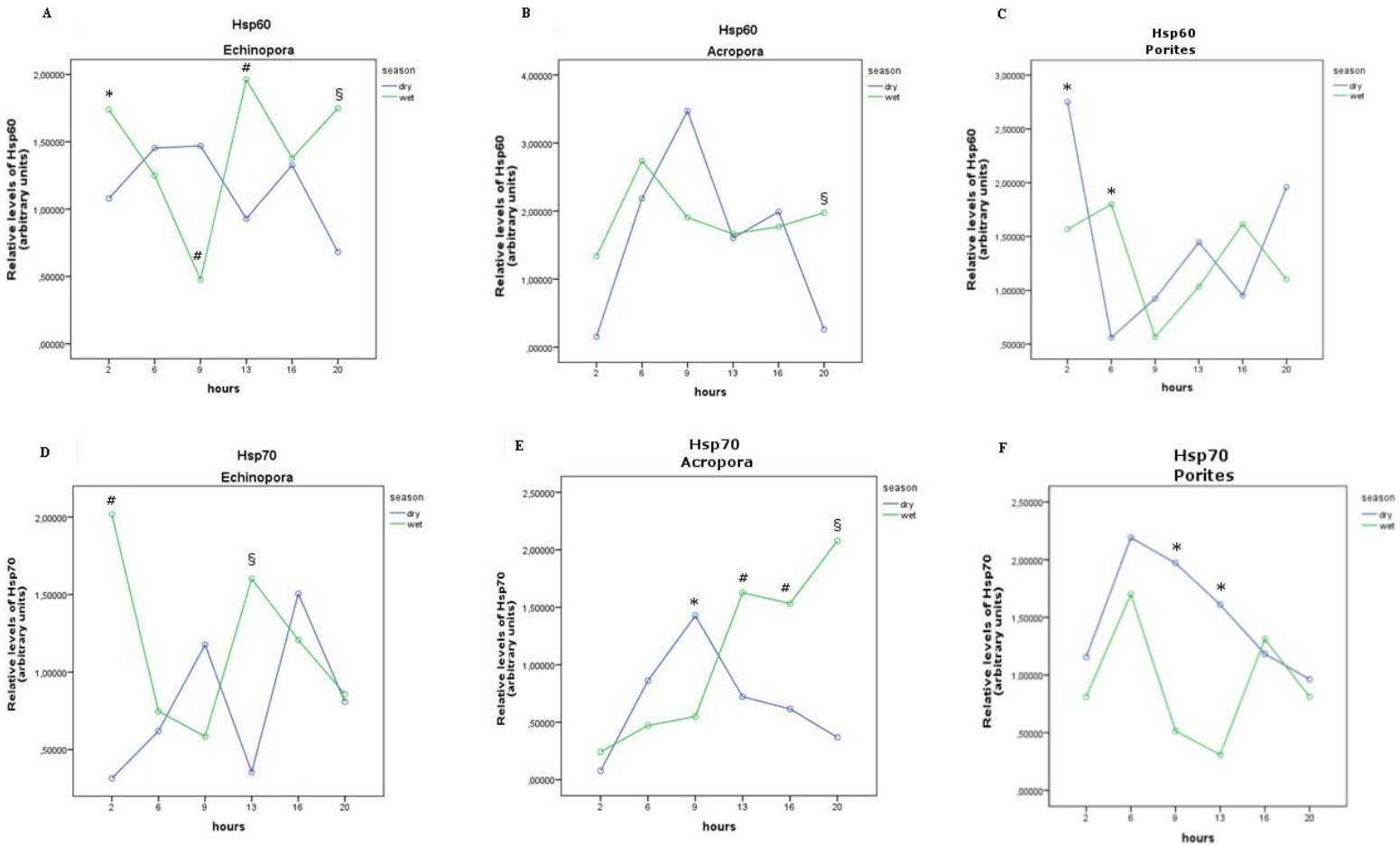
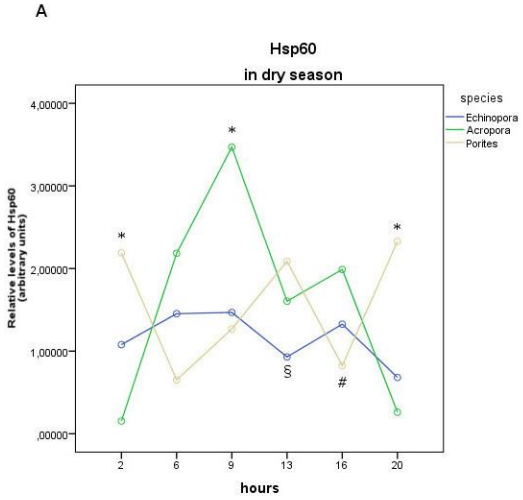


Figure 2. Comparisons between seasons of Hsp60 and Hsp70 for each specie. Data of Hsp are expressed as arbitrary units and as mean ± SEM (two -way ANOVA followed by Tukey's HSD multiple pairwise comparisons, * p≤0.05; #p≤0.01; § p≤0.000).



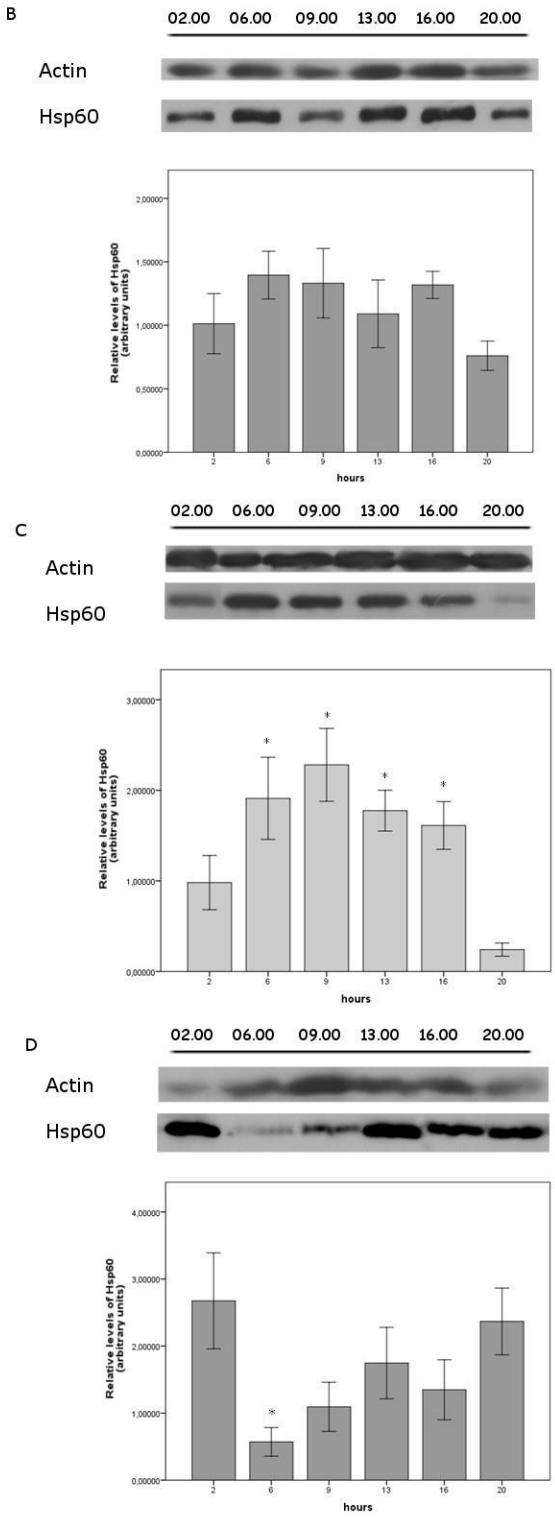
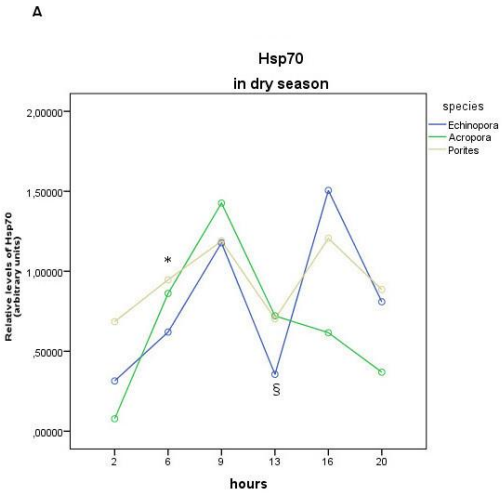
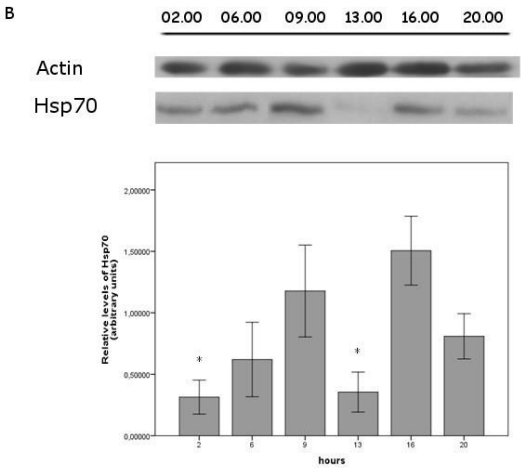
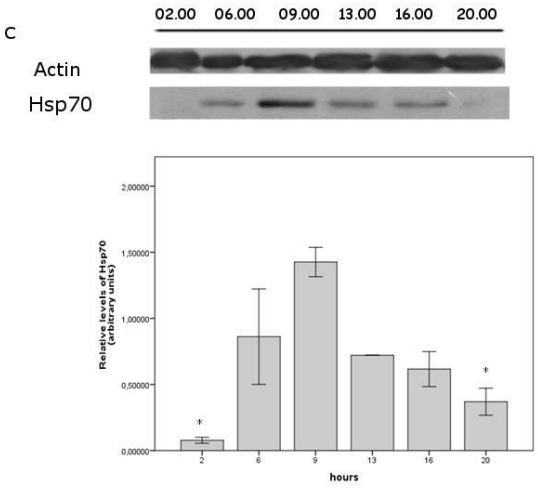


Figure 3. Effect of sea temperature and light intensity on Hsp60 modulation in the three species of scleractinian corals in dry season. Comparison among the species (A) and in *E. lamellosa* (B). *A. tenuis*(C) and *P.lobata*(D). Samples of coral fragments located at different hours during the day (02.00,06.00, 09.00, 13.00,16.00, 20.00) were subjected to Western analyses and immunodecoration Data of Hsp60 are expressed as arbitrary units and as mean ± SEM (two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, (A)* $p < 0.05$ among species, § $p < 0.05$ *P.lobata*.compared to *E.*lamellosa; # $p < 0.05$ *P.lobata*. compared to *A.tenuis;* (C) * $p < 0.05$ compared to 20.00; (D) * $p < 0.05$ compared to 02.00).







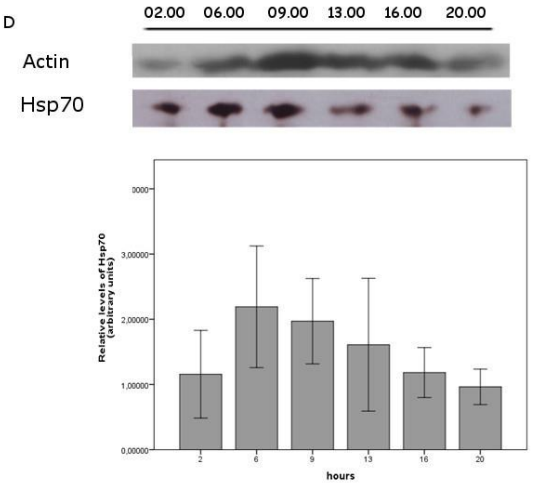


Table 4. Effect of sea temperature and light intensity on Hsp70 modulations in the three species of scleractinian corals in dry season. Comparison among the specis (A) and in *E. lamellosa* (B). *A. tenuis*(C) and *P.lobata*(D). Samples of coral fragments located at different hours during the day (02.00,06.00, 09.00, 13.00,16.00, 20.00) were subjected to Western analyses and immunodecoration Data of Hsp70 levels are expressed as arbitrary units and as mean ± SEM (two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, (A)* $p < 0.05$ among species, § $p < 0.05$ *P.lobata*.compared to *E.lamellosa*; (B) * $p < 0.05$ compared to 16.00; (C) * $p < 0.05$ compared to 09.00).

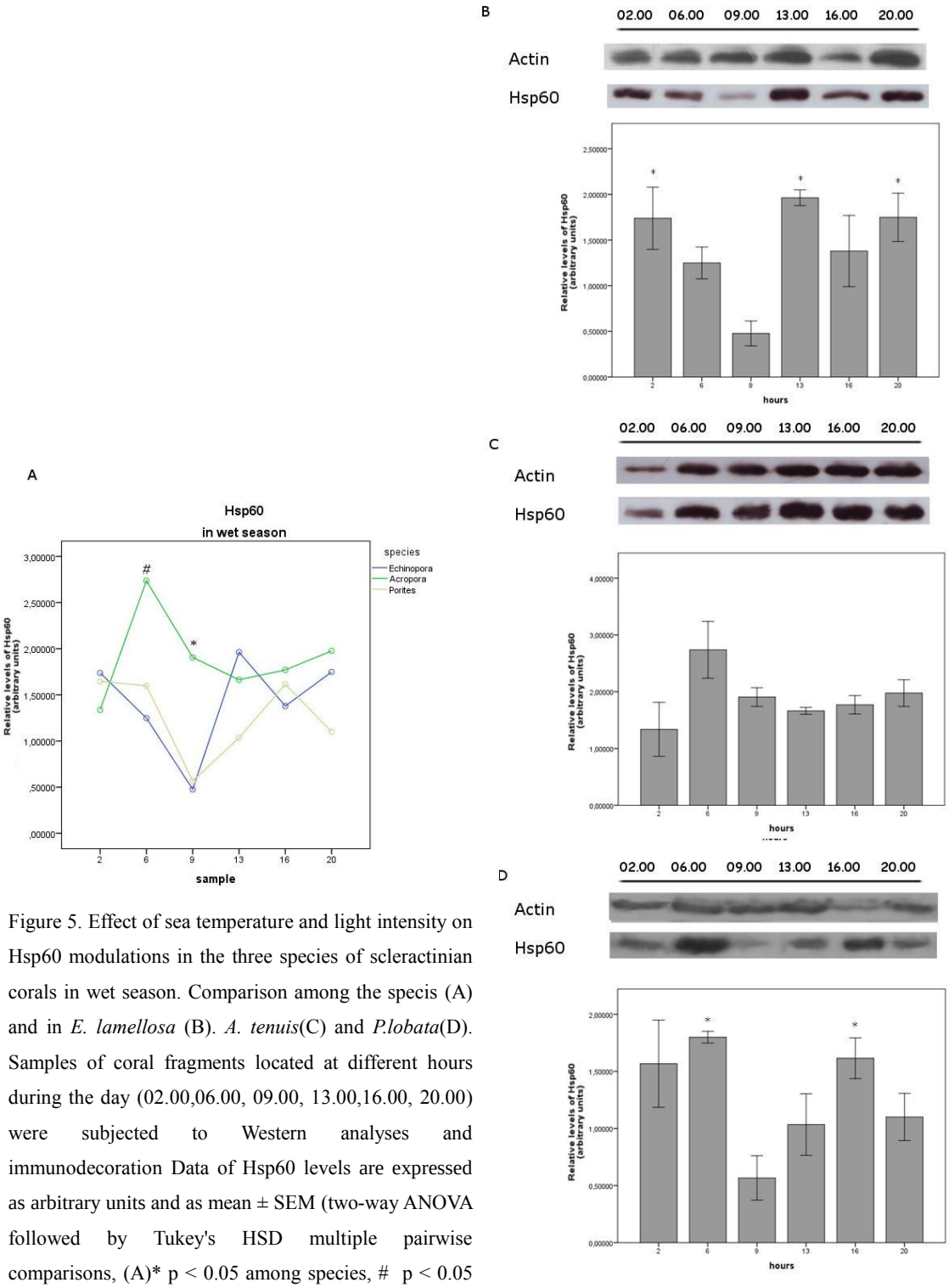


Figure 5. Effect of sea temperature and light intensity on Hsp60 modulations in the three species of scleractinian corals in wet season. Comparison among the specis (A) and in *E. lamellosa* (B). *A. tenuis*(C) and *P.lobata*(D). Samples of coral fragments located at different hours during the day (02.00,06.00, 09.00, 13.00,16.00, 20.00) were subjected to Western analyses and immunodecoration Data of Hsp60 levels are expressed as arbitrary units and as mean ± SEM (two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, (A)* $p < 0.05$ among species, # $p < 0.05$ *A.tenuis* compared to *E.lamellosa*;(B) * $p < 0.05$ compared to 09.00; (D) * $p < 0.05$ compared to 09.00).

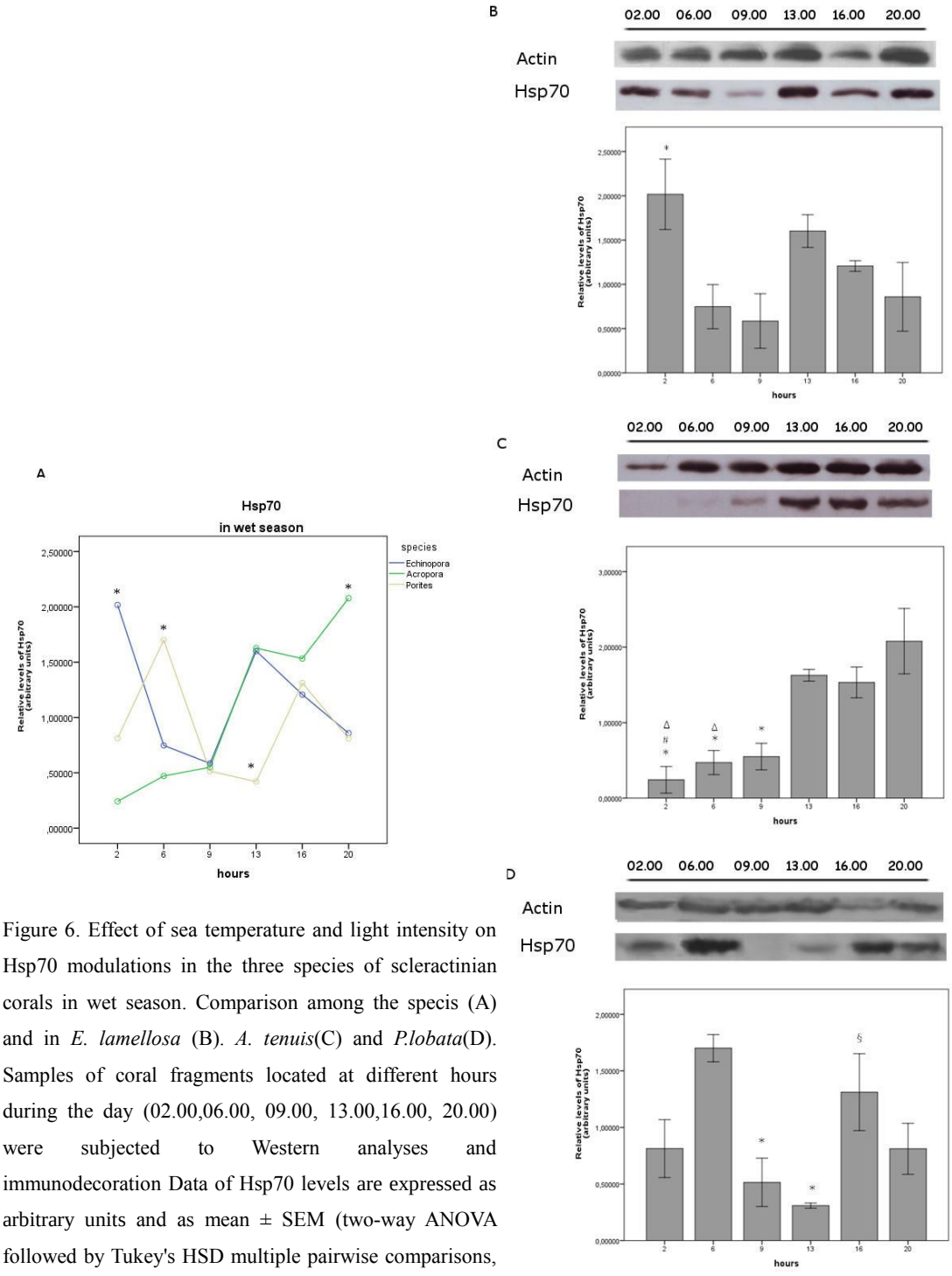


Figure 6. Effect of sea temperature and light intensity on Hsp70 modulations in the three species of scleractinian corals in wet season. Comparison among the specis (A) and in *E. lamellosa* (B). *A. tenuis*(C) and *P.lobata*(D). Samples of coral fragments located at different hours during the day (02.00,06.00, 09.00, 13.00,16.00, 20.00) were subjected to Western analyses and immunodecoration Data of Hsp70 levels are expressed as arbitrary units and as mean ± SEM (two-way ANOVA followed by Tukey's HSD multiple pairwise comparisons, (A)* $p < 0.05$ among species, # $p < 0.05$ *A.tenuis* compared to *E.lamellosa*;(B) * $p < 0.05$ compared to 09.00; (C) * $p < 0.01$ compared to 20.00. Δ $p<0.05$ compared to 13.00, # compared to 16.00; (D) * $p<0.01$ compared to 06.00, § $p<0.05$ compared to 13.00).

Hsps	season	higer	λ	Echinopora	Acropora	Porites	Temp	light
Hsp60								
02.00	dry	porites	0.82	0.025	0.001	-	29	0
06.00	dry	Porites	0.81	-	0.01	-	28,6	97
9	dry	acropora	0.80	0.001	-	≤0.000	30,2	31689
13	dry	porites	0.86	0.02	-	-	**32,3**	**78258**
16	dry	acropora	-	-	-	0.049	31,2	18187
20	dry	porites	0.82	0.001	0.001	-	29,1	0
Hsp60								
2	wet	-	0.86	-	-	-	28,4	0
6	wet	acropora	0.83	-	0.005		28,7	1812
9	wet	acropora	0.89	0.007	-	0.007	29,9	40214
13	wet	-	0.85	-	-	-	**30,9**	**49428**
16	wet	-	-	-	-	-	29,6	8404
20	wet	-	0.87	-	-	-	28,6	0
Hsp70								
2	dry	-		-	-	-	29	0
6	dry	Porites	0.81	0.002	0.025	-	28,6	97
9	dry	-		-	-	-	30,2	31689
13	dry	Porites	0.86	0.012	-	-	**32,3**	**78258**
16	dry	-	-	-	-	-	31,2	18187
20	dry	-	0.82	-	-	-	29,1	0
Hsp70								
2	wet	Echinopora	0.86	-	0.001	0.015	28,4	0
6	wet	porites	0.83	0.053	0.013	-	28,7	1812
9	wet	-	0.89	-	-	-	29,9	40214
13	wet	Porites (lower)	0.85	0.009	0.008	-	**30,9**	**49428**
16	wet	-	-	-	-	-	29,6	8404
20	wet	acropora	0.87	0.021	0.011	-	28,6	0

Table.6. General Linear Model multiple comparison of means, with Wilk's Lambda values for dry and Wet season daily values of Hsps levels of expression among species, and sea temperature T(°C) and light intensity LI(Lux) mean values, highest seasonal values are in bolt.

– Chapter 5 –

5.1 General discussion and conclusion

The reef health worldwide is seriously threatened by both biotic and abiotic factors which in the last few decades have led to the disappearance of about the 27% of coral reefs worldwide (Frias-Lopez et al., 2004). Although reef corals have a remarkable ability to acclimatize to changes in solar irradiance (Anthony & Hoegh-Guldberg, 2003) abnormally perturbations, which can lead to environmental changes, as elevated and low ocean temperatures, high UV radiations, microbial attacks, pollutants, salinity changes and imminent sea level rise (Dustan 1999; Marshall & Schuttenberg, 2006) contribute to weaken corals health. The increasing of anthropogenic activities also contribute to threat the reefs health (Wilkinson, 2004, 2008). Under adverse circumstances, the equilibrium between the partners of the holobiont may be compromised, leading to the breakage of the symbiosis and generally to the death of the coral colony.

In addition, in recent years coral diseases have been reported to be increasing worldwide in both the occurrence of known diseases and in the incidence of newly reported infections (Sutherland et al., 2004; Croquer et al., 2006; Weil et al., 2006, 2012; Montano et al., 2013, 2014). In this context, several reports of Brow Band disease highlighted its ongoing spread across the Indo-Pacific Ocean, especially affecting corals of the genus *Acropora* (Raymundo et al., 2009; Lamb & Willis 2011; Montano et al., 2012). Another important coral disease is the Black Band Disease, which is known to be one of the most widespread disease, ranging from the Atlantic Ocean to the Indo-Pacific, including the Red Sea and the Great Barrier Reef (Barneah et al.. 2007; Page & Willis, 2006), affecting a wide range of corals. To date, although these diseases are well studied, less attention has been given to the effects of the pathogenic microbial presence on coral health and physiology. Understanding how pathogens alter and affect the coral physiology as well as understanding the mechanisms by which these animals respond to their highly fluctuating environment is becoming increasingly important. Furthermore, in the prospective that the effects of climate change cascade through the biosphere may magnify the impacts of stressors (both biotic and abiotic), especially for organisms that occur in variable habitats (Dahlhoff, 2004) such as corals, the establishment of a set of biomarkers as a diagnostic tool to understand the effects of diseases and stresses, could help the management of coral reefs as well as found a way to contrast the ongoing grow of coral diseases. Moreover, it appears important to analyze the correct biomarkers for each diseases, based on the pathogenic mechanisms which take place during the disease development. To date few researches had investigated the effects of coral diseases with a wide range of biochemical indicators and

considering the pathogenic mechanisms which occur during the infection, as well as the coral physiology *in situ* in both natural and stressed conditions. In this prospective my PhD research aimed at improving the current knowledge about the response of a wide range of biomarkers as well as their specie-specific physiological patterns of expression in natural conditions. In this contest the most important feature of this work is the analysis directly in field, which allow to observe these responses in coral natural environment thus giving a more realistic impression of what effectively is happening in coral reefs physiology. In this study we examined the natural pathways of expression, in different coral species, of several biomarkers we choose also for investigate coral diseases.

Our results highlight the need of *in situ* study to better understand how corals normally react to environmental natural changes and then, consequently, how they change their modulation in response to stress. In fact, despite the different growth morphology of corals seems to be crucial for coral susceptibility to environmental stresses (McClanahan et al., 2007; Montano et al., 2010; Hughes & Connell, 1999), our results show that susceptibility not always correspond to high level of expression of the cellular biomarker or to high variations in its modulation. Hsp60 and Hsp70 were modulated in the three analysed coral species in response to both circadian natural light/darkness alternation as well as seasonal changes in light and temperature values. On the contrary, HO-1 was not modulated or even expressed in any of the three species observed as well as in healthy colony of a fourth species (*G. columna*), confirming the use of this biomarkers as a response to an oxidative stress (Down et al., 2006). In general our findings also suggested that behavioural aspects, like feeding strategies, can influenced the Hsps modulation.

Our study regarding the coral physiological response to disease highlight as appear important to analyse the cellular response occurring at different distances from the front of infection, in order to understand how the whole colony react to the pathogens attacks. In fact. being clonal organisms. scleractinian corals have locals signals transmitted throughout the entire colony, inducing whole organism levels changes with relative freedom (Mydlarz et al., 2009). In this prospective we first analyse the Hsp60 expression during the infection of BrB disease in *A. muricata.*The mitochondrial chaperonin Hsp60 is essential in mitochondrial biogenesis and in several mechanisms essential for proteins, as assisting in the folding of stress-denatured proteins in the mitochondria (Hood et al., 2003). Generally, its up-regulation implies a general shift in the protein chaperoning and degradation within the mitochondria accompanied by changes in the equilibria of mitochondrial-associated metabolic pathways (Papp et al., 2003). Interestingly, our results showed that the Hsp60 had lower expression in the coral portion closer to the infection of BrB ciliates (D1) than in the coral portions that potentially can be infected, indicating that the defence mechanisms were

probably already exceeded 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. The BrB ciliates have been observed to infected and progress along the coral branches much faster than another ciliates, responsible to another disease (SEB) and pathogens, as the BBD microbial consortium. We speculate that the rapid migration of the BrB ciliates would not give to the coral defence mechanisms the time necessary to react, in the attempt to block and confine the infection. This appeared to be confirmed by the BBD Hsp60 results which did not show a modulation in coral portions along the disease progression. However, the Hsps level appeared decreased on the whole colonies after one year of disease progression, which probably exhausted the whole colony. Also Hsp70 expression patterns may be consequence of this exhausted condition of the whole colony. Our results also showed, for the first time, that corals affected by BBD experienced oxidative stress as consequence of the microbial anoxic sulfide rich microenvironments that affected also the mitochondrial proteins, as demonstrated by the induction of MnSod and HO-1 in the diseased colonies. The regulation of oxidative phosphorylation and respiration has been altered also changing during the progression of the disease. One of the characteristics of BBD is the high concentrate sulfide, which follow the photogenic consortium activities and, in anoxic condition, is known to be the cause of the necrosis of coral tissue, promoting further the anoxic sulfide rich microenvironments and enhance the BBD progression (Richardson et al., 1997; Sato et al., 2010). HO-1 and MnSod expression patterns may be consequences of this anoxic sulfide rich microenvironments. Furthermore, HO-1 appeared always not expressed in all the healthy colonies while showed up-regulation in the diseased ones for then decreasing after one year of infection near the band in necrosis. Also this may suggest that polyps near the band, being stressed for long time, are less or totally behind the ability of recovering, as HO-1 is known to be up-regulated in response to oxidative stress and toxicant exposure (Down et. al., 2006). At the same time also MnSod, although healthy colonies showed a very low expression and diseased colony first didn't expressed it with significant difference along the colony, after one year of infection exhibited a down regulation at the first distance. In conclusion, the present study can provide new insights into the physiology of scleractinian corals subjected to epizootic disease as well as their physiological mechanisms into normal environmental conditions. Furthermore, these data highlight the need of further investigations which required the analyses of other coral diseases and possibly increasing the set of molecular biomarkers available, in order to elucidate major ecological and molecular aspects of the pathogen – host relationship.

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