UNIVERSITA' DEGLI STUDI DI MILANO-BICOCCA

Facoltà di Scienze Matematiche, Fisiche e Naturali

Dipartimento di Biotecnologie e Bioscienze

Corso di Dottorato di Ricerca in Biologia XXV ciclo

Assessing the expression of Hsp60 in scleractinian corals subjected to biotic and abiotic stresses

Davide Seveso

Relatore: Dott. Paolo Galli

Coordinatore: Prof.ssa Giovanna Lucchini

Anno Accademico 2011-2012

"La lingua non è sufficiente a dire e la mano a scrivere

tutte le meraviglie del mare"

(Cristoforo Colombo)

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ABSTRACT

The reef health worldwide is seriously threatened by a multitude of factors such as abnormally elevated and low ocean temperatures, high UV radiations, changes in salinity, pollution and increasing incidence of diseases. Under adverse circumstances the equilibrium between the partners of the coral holobiont may be compromised and can lead to coral bleaching events. Bleaching refers to the loss in the coloration of the coral colony induced by the dissociation of the symbiosis between corals and their symbiotic algae. The extreme or unexpected environmental fluctuations could be very stressful for sessile marine organisms such as corals, causing important cell damage since corals lack any developed physiological regulatory system. One mechanism of reaction to deleterious conditions is the rapid increase of the induction of a set of stress proteins called Heat shock proteins (Hsps). Under normal cellular physiological conditions the Hsps mainly function as molecular chaperones and they are involved in a multitude of proteome-maintenance functions that regulate protein homeostasis in directing the folding and assembly of other proteins. They also are involved in the intracellular protein transport and in the degradation of damaged proteins. An up-regulation of the expression of Hsps constitutes an emergency response and confers tolerance to harsh conditions.

This study highlights the modulation of the expression of a vital but scarcely investigated group of Hsps, the mitochondrial Hsp60 which are essential for the vitality of the cell and whose induction represent one of the first reactions to stress. The overall objective of my dissertation is to elucidate the major aspects of Hsp60 modulation in various taxa of corals as a result of their exposure to different abiotic and biotic stress factors.

In the first study we investigated for the first time the effectiveness of the Hsp60 as indicator of biotic stress and competitive interaction in the coral *Acropora muricata*, focusing on two biological interactions such as a coral disease, the Skeleton eroding band (SEB) caused by the protozoan *Halofolliculina corallasia* and the algal overgrowth. The two different biological stresses trigger diverse responses on Hsp60 level. No detectable effect on Hsp60 modulation appeared in colonies subjected to algal overgrowth. On the contrary, corals displayed a robust up-regulation of Hsp60 in the fragments sampled just above the SEB dark band indicating that the aggressive behavior of the protozoan causes cellular damage also in coral portions neighboring and along the advancing front of the infection. Portions of coral sampled distant to the SEB band showed a Hsp60 level comparable to that observed in healthy colonies. We propose Hsp60 expression as a promising tool to evaluate physiological stress caused by coral disease in reef corals.

In the second study we examined the different modulation of Hsp60 in the coral *Seriatopora caliendrum* subjected to salinity stress, since that corals are generally considered stenohaline and osmoconformers. We analyzed the Hsp60 expression profiles of the coral polyps under three salinity scenarios (hypersalinity of 45 ppt, hyposalinity of 27 ppt and extreme hyposalinity of 15 ppt) during the time course of a 2 days period. Experiments were conducted at the Civic Aquarium of Milan using a flow-through aquaria system. *S. caliendrum* responds differently to hyper- and hyposaline conditions at morphological and cellular levels and the response of corals to osmotic stress reflects the severity and duration of the disturbance. The Western blot analysis showed for each salinity a similar strong up-regulation of Hsp60 after the first 6 h of stress, but subsequently Hsp60 exhibited for each salinity treatment specific patterns of expression. In hypersalinity

condition a negative trend of Hsp60 expression was observed, but the colonies showed a morphological appearance similar to healthy control colonies, suggesting a possible metabolic acclimation of corals to the stress. In *S. caliendrum* exposed to moderate hyposalinity, Hsp60 exhibited marked oscillation and the level of Hsp60 generally remained high over time indicating that cellular damages in the animal host were in progress. In extreme hyposalinity condition, a considerable gradual down-regulation of Hsp60 was detected until the end of the experiment. This was accompanied by extreme degradation and necrosis of coral tissues.

Finally, we focused on the responses of Hsp60 to thermal stresses, initially analyzing the susceptibility of three coral genera (*Montipora*, *Acropora* and *Seriatopora*) to a severe heat stress of 36°C for 12 h. Despite the Hsp60 trend appeared similar, each genus displayed a different persistence of the Hsp60 signal, and so a different threshold of tolerance and resistance. Secondly, the sensitive *S. caliendrum* was subjected to a cold shock of 21°C, a moderate heat shock of 29°C and a severe heat shock of 34°C. The modulation of the Hsp60 at lowered temperatures are similar to those involved in very elevated temperature stress with an up-regulation after 6 h followed by a down-regulation when the cellular damage become irreparable. This is accompanied by the appearance of bleaching events. The mild heat shock of 29°C did not significantly affect the normal Hsp60 oscillatory pattern.

With this study we proposed the application of the mitochondrial Hsp60 and the analysis of its modulation as an useful and accurate biomarker, to assess the effect of several types of stress in scleractinian corals, and to diagnose coral health prior that the coral bleaching occurred.

CONTENTS

– *Chapter 1* –

Background and General Introduction

1.1 Biology of the scleractinian corals

Scleractinian corals belong to one of the lowest metazoan phyla, the Cnidaria (class Anthozoa) and they represent the major building blocks of coral reefs which constitute some of the largest and most diverse ecological communities on earth. Coral reefs cover only 0.2% of the ocean but contain almost the 25% of the total marine species (Hoegh-Guldberg 1999) supporting an extraordinary biomass and diversity of life. Furthermore, coral reefs protects 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 exist in relatively stable environments (30° N and S of the equator) that are often oligotrophic and they play an essential role in shallow tropical oceans, in particular because of their capacity to efficiently deposit an external calcium carbonate skeleton modifying the substratum into the vast three-dimensional structures. These calcifying organisms, better known as stony corals, are typically colonial animals comprised of many genetically identical units called polyps. The extensive calcification by scleractinian corals result from the mutualistic symbiotic interactions between photosynthetic and microscopic unicellular dinoflagellate algae called zooxanthellae (*Symbiodinium* spp.) and the cnidarian coral host. *Symbiodinium* cells inhabit coral tissues in concentration of millions of cells per square centimeter and use sunlight to get energy via photosynthesis. The

zooxanthellae provide a metabolic benefit to their coral hosts by translocating carbohydrates, amino acids, and lipids to the host (Trench 1971) and representing the majority of daily carbon requirements for coral growth and skeletal deposition (Gates et al. 1995). In exchange, the host provides the algae with 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 commonly hosts only one or at most several individual varieties (or clades) of *Symbiodinium* at a time, and the host-symbiont specificity can be stable to perturbations (Baker 2003, Thornhill et al. 2006). In addition to the zooxanthellae, corals associate with a wide range of bacteria, fungi and viruses (van Oppen et al. 2009). Collectively, this complex is commonly referred to as the coral holobiont.

1.2 Coral reef under threats and the coral bleaching

The reef health worldwide is seriously threatened by a multitude of factors, both biotic and abiotic, including abnormally elevated and low ocean temperatures, high UV radiations, severe changes in salinity, destructive storms, pollution, predation outbreaks (e.g. crown of thorn starfish) and imminent sea level rise due to thermal expansion of seawater and glacial melting (Dustan 1999, Marshall & Schuttenberg 2006). Furthermore, increased concentrations of atmospheric and dissolved CO_2 resulted in a more acidic ocean chemistry, which leads to slowed deposition of calcium carbonate skeletons (Kleypas et al. 1999). Many coral species are also impacted by increasing incidence of disease (Cervino et al. 2004, Sutherland et al. 2004, Montano et al. 2012). According to a recent summary, 20% of the world's reefs have been irreparably damaged, and a further 24% are

threatened by anthropogenic pressures such as destructive fishing practices, coastal development, agricultural land-use, increased nutrients and sediment load from run-off and eutrophication (Wilkinson 2004, Wilkinson 2008).

Under adverse circumstances the equilibrium between the partners of the holobiont may be compromised and often lead to the phenomenon known as coral bleaching, which is considered one of the main causes of degradation of coral reefs worldwide (Hughes et al. 2003). Bleaching refers to the loss in the overall coloration of the coral colony induced by the dissociation of the symbiosis between corals and their symbiotic dinoflagellates (Brown 1997). This is due to decrease in *Symbiodinium* cell density and/or reduction in photosynthetic pigments (Hoegh-Guldberg 1989, Glynn 1993, Douglas 2003).

Bleaching can be induced by various factors, including heat stress, cold stress, elevated irradiance, increased UV radiation, prolonged absence of light, low salinity, heavy sedimentation, general exposure to pollutants (herbicides, pesticides, heavy metals) starvation, and bacterial infection (Goreau 1964, Hoegh-Guldberg & Smith 1989, Lesser et al. 1990, Glynn 1991, Coles & Fadlallah 1991, Muscatine et al. 1991, Fang et al. 1995, Brown et al. 2000, Owen et al. 2002, Jones 2005).

The majority of reported bleaching events have been correlated with elevated sea surface temperatures (SST), (Hoegh-Guldberg & Jones 1999), considering that coral species are suggested to exist within a relatively narrow temperature range, often within 2-3°C from their upper thermal limit (Podesta & Glynn 1997, Berkelmans & Willis 1999). If temperature remains above a threshold for several weeks, the coral is unable to meet nutritional requirements through feeding alone, cannot retrieve or maintain sufficient densities of zooxanthellae, and mortality can occur (Brown 1997, Hoegh-Guldberg 1999, 2004).

Coral bleaching has become common since 1983, however, the 1997-1998 mass coral bleaching event remains the most geographically extensive and severe resulted in 90% mortality to 16% of reefs worldwide (Hoegh-Guldberg 2004). Large-scale bleaching events, caused primarily by heat stress which results in photo-oxidative stress in the organism (Lesser et al. 1990, Downs et al. 2002), have been associated with the El-Niño Southern Oscillation (ENSO) phenomenom (Wilkinson et al. 1999, Mumby et al. 2001). However, the events of coral bleaching are likely to increase in frequency and scale over time and become a yearly phenomenon by 2040. In fact with global climate change the surface temperature has increased approximately 0.2°C per decade in the past 30 years and will increase 1-3°C by 2050 (IPCC 2007, Hoegh-Guldberg 1999). Bleaching events have also occurred in the Pacific in 2000 and 2002 (Berkelmans et al. 2004), mild events in the Indian Ocean in 2005 and 2007 (McClanahan et al. 2007, Montano et al. 2010) and a severe event in the Caribbean in 2005 (Donner et al. 2007).

Bleaching of corals also has been attributed to high levels of solar radiation as these events usually coincide with periods of calm winds, resulting in increased penetration of solar radiation (Glynn 1996, Wilkinson 1998). The combination elevated SST and long summer days of intense UV exposure is commonly agreed to be the predominant trigger for mass bleaching episodes (Lesser et al. 1990, Gleason & Wellington 1993, Glynn 1993).

In some cases bleaching is a transient altered physiological state; the coral quickly recovers and the health of the individual is relatively un-impacted. Otherwise, if stressful conditions subside soon enough, zooxanthellae can repopulate coral's tissues and they can survive the bleaching event and recover their normal colour and metabolic activity (Marshall & Schuttenberg 2004).

The maintenance of photosynthetic algae within coral cells involves constant cellular communication between the two partners (Weis 2008), but the cellular mechanisms and molecular pathways involved in the intake and retention of unicellular algae are largely unresolved. Different hypotheses have been suggested regarding the breakdown of the symbiosis. They include, in situ degradation of the symbiont and its photosynthetic pigments (Brown et al. 1995, Fitt & Warner 1995, Ainsworth & Hoegh-Guldberg 2008), exocytosis of the symbiont cells (Brown et al. 1995), detachment of the symbiont-containing animal cell (Gates et al. 1992, Brown et al. 1995, Fitt et al. 2001), necrosis and apoptosis of both host and symbiont cells (Dunn et al. 2004, 2007, Lesser & Farrell 2004), and digestion of the symbiont by the coral host (Brown et al. 1995).

1.3 Environmental stresses and expression of Heat shock proteins

Marine environment vary in biotic and abiotic conditions and in the amplitude and frequency of fluctuations in these conditions (Brown 1997). Due to the physical characteristics of seawater, the aquatic environment can be extremely stressful to its inhabitants (Feder & Hofmann 1999). In particular, as sessile organisms, scleractinian corals inhabit several niches in the intertidal zone and undergo constant direct exposure to the surrounding environmental conditions. Extreme or unexpected short or long-term environmental fluctuations could be very stressful for marine organisms, causing cell damage. Organisms in nature have developed several mechanisms to withstand environmental stresses, such as behavioral adaptations, morphological changes, physiological regulations and biochemical and cellular specializations (Feder & Hofmann 1999). Nevertheless, corals lack any developed physiological regulatory system and

for this reason they are expected to possess well-developed cellular adaptation abilities (Brown 1997). One mechanism of reaction to deleterious environmental conditions is the rapid increase of the induction of a set of stress proteins called Heat shock proteins (Hsps), (Lindquist 1986). This is one of the most important defense mechanism, conserved throughout evolution as the Hsps are ubiquitous, occurring in all organisms from bacteria to humans (Fink 1999, Kregel 2002). Heat shock proteins are categorized into several families that are 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. 1.1).

There is substantial evidence that Hsps play important physiological roles both in normal conditions and also in situations involving systemic and cellular stress (Kregel 2002). Under normal cellular physiological conditions the Hsps mainly function as molecular chaperones. Chaperones are involved in a multitude of proteome-maintenance functions that regulate protein homeostasis in directing the folding and assembly of other proteins (Parsell & Lindquist 1993, Sanders 1993, Fink 1999). In particular, they participate broadly in *de novo* folding of proteins, refolding of stress-denatured proteins, prevention of oligomeric assembly, protein trafficking and assistance in proteolytic degradation. They also are involved 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). The chaperones are multicomponent molecular machines that promote folding through ATP- and cofactor-regulated binding and release cycles (Hartl et al. 2011). In fact, Hsps typically function as oligomers, as well as complexes of several different chaperones, co-chaperones, and/or nucleotide exchange factors (Feder & Hofmann 1999).

An induction and an up-regulation of the expression of Hsps constitutes an

emergency response and confers tolerance to harsh conditions (Parsell & Lindquist 1993). Cells respond to stress by increasing either the amount or the activity of a transcription factor that is specific for the heat shock genes. The result is increased transcription of 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).

Tab. 1.1 - 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)

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	

1.3.1 The mitochondrial 60-kDa Heat shock protein (Hsp60)

The 60-kDa heat shock proteins (Hsp60) belongs to the group of the chaperonins and in particular it is a mitochondrial chaperonin. Chaperonins are ring-shaped chaperones that encapsulate nonnative 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 (GroEL), mitochondria, and chloroplasts. The function of type I chaperonins is performed by the cooperation of two proteins, Hsp60 and Hsp10, that function with the help of hydrolyzed ATP, as folding chamber and co-chaperone, respectively (Cheng et al. 1989, Bukau & Horwich 1998). Group II chaperonins exist in archaea (thermosome) and in the eukaryotic cytosol (TRiC/CCT).

Through the extensive study of GroEL, Hsp60's bacterial homolog, 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. It functions to 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 HSP60 is responsible for maintaining the unfolded conformation of the protein for trans-membrane transport (Koll et al. 1992).

Cnidarian heat shock protein 60 is the major mitochondrial chaperonin and

functions to mature nuclear-encoded, mitochondrial-imported proteins into their active state (Ellis 1996). Elevation of this protein signifies that there has been a general shift in the protein chaperoning and degradation within the mitochondria and implies a change in the equilibria of mitochondrial-associated metabolic pathways (Papp et al. 2003).

Hsp60 has also anti-apoptotic ability and can 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 Thesis objectives

Escalating concerns about coral health have prompted an increase in effort put into understanding the complex organisms responsible for the formation and maintenance of these ecosystems. The study and the application of molecular techniques to address questions related to coral ecology is a continue challenging to identify and understanding how corals counteract and tolerate different environmental stresses. "Stress" is 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).

Biochemical indicators of stress are typically components of the cellular stress response, which are up-regulated as a consequence of exposure to environmental conditions that perturb cellular protein structure (Dahlhoff 2004). The Heat shock proteins are generally recognized to be ubiquitous, multi-functional, and act to protect the organism from the cell protein damage that can follow exposure to a wide variety of stressors, including

elevated temperature, cold shock, oxidative stress, increased UV, extreme pH, osmotic stress, pollutants (Lindquist 1986, Sanders 1993, Wiens et al. 1998, Feder & Hofmann 1999, Downs et al. 2002, Spees et al. 2002, Papp et al. 2003, Tetteh & Beuchat 2003). 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). Recently, the study of the heat-shock response of corals as a potential biomarker of stress represent a much debated topic. Sharp and colleagues (1994) were the first to find heat-induced Hsp70 in the coral *Goniopora djiboutiensis* by immunoblotting. As a result of this discovery, many other studies about the expression of Hsps in scleractinians subjected to different environmental stresses have been developed (Hayes & King 1995, Fang et al. 1997, Sharp et al. 1997, Downs et al. 2000, Brown et al. 2002, Downs et al. 2002, Kingsley et al. 2003).

This study highlights the modulation of the expression of a vital but scarcely investigated group of Hsps, the mitochondrial 60-kDa Hsp from cnidaria. The mitochondria are essential eukaryotic organelles that serve as a site for many vital metabolic pathways and are essential for the vitality of the cell. The mitochondria are also involved in a number of signal transduction events, including those that control the death of eukaryotic cells. This suggest that the mitochondrial Hsp60 play a central role in the functions of the organisms and that induction of mt-Hsp60 will be one of the first reactions to stress (Choresh et al. 2004). The overall objective of my dissertation is to elucidate the major aspects of Hsp60 induction and modulation in various taxa of corals as a result of their exposure to different

abiotic and biotic stress factors, with a particular focus on coral bleaching as the most prominent and highly observable stress response in corals. Specifically we set the following objectives:

- Investigate for the first time the effectiveness of the Hsp60 as indicator of biotic stress and competitive interaction in the widespread coral *Acropora muricata* (Chapter 2).
- Examine the different modulation of Hsp60 in the coral *Seriatopora caliendrum* subjected to different seawater salinity levels (Chapter 3).
- Test the susceptibility of various coral genera to heat stress using the Hsp60 analysis accompanied by morphological analyses and explore the thermotolerance of *S. caliendrum* (Chapter 4)

The potential outcome of this research is expected to be the establishment and testing of an appropriate biomarker, based on the accumulated knowledge of the expression of the mitochondrial Hsp60 in scleractinian corals, for diagnosing coral health prior that the damage becomes irreversible

1.5 References

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– Chapter 2 –

Up-regulation of Hsp60 in response to Skeleton eroding band disease but not by algal overgrowth in the scleractinian coral *Acropora muricata*

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2.1 Introduction

In nature, organisms have developed several mechanisms to withstand environmental stresses, such as physiological regulations and biochemical and cellular specializations (Brown, 1997). The increased importance of determining the effects of stress factors on the physiology of animals have led to an increase of studies investigating stress-inducible proteins in an ecological context (Feder and Hofmann, 1999; Dahlhoff, 2004).

Heat shock proteins (Hsps) are a highly conserved family of stress response proteins which represent one of the most important defense mechanisms of all organisms (Fink, 1999; Kumsta and Jakob, 2009). They function primarily as molecular chaperones, preventing protein aggregation, facilitating proper protein folding and complex assembly, targeting improperly folded proteins to specific degradative pathways and regulating stress-induced apoptosis (Mayer, 2010; Toivola et al., 2010; Vabulas et al., 2010). Hsps are expressed at low levels under normal physiological conditions, but their expression is up-regulated as a consequence of exposure to conditions that perturb cellular protein structure (Dahlhoff, 2004; Richter et al., 2010). High levels of specific Hsps are maintained throughout exposure and protect the organism from a wide variety of stressors.

In literature many works have focused on the expression of coral Hsps (Fang et al., 1997; Sharp et al., 1997; Branton et al., 1999; Robbart et al., 2004; Snyder and Rossi, 2004) particularly on the induction of the chaperonine 60-kDa heat shock protein (Hsp60) under environmental stress factors causing bleaching, such as high temperatures (Choresh et al., 2001; Brown et al., 2002; Kingsley et al., 2003; Chow et al., 2009, 2012), low temperatures (Kingsley et al., 2003), elevated light intensity (Downs et al., 2000; Chow et al., 2009, 2012) and xenobiotics (Downs et al., 2005).

Stress factors that trigger the heat shock response in reef building corals are usually considered to be abiotic. However, in the marine habitat the distribution patterns, spatial relations, growth and health of the populations are affected in a predictable manner not only by natural physical disturbances but also by interactions with other species in the community (Dayton, 1971). Abiotic and biotic stresses often work in concert with one another in driving the physiological ecology of intertidal communities and determining the structure and composition of benthic communities on coral reefs (Lang and Chornesky, 1990; Karlson, 1999). Nevertheless, not much research has been carried out with the aim of assessing the role of Hsps in relation to biotic factors. The study published by Rossi and Snyder (2001) has shown that stress proteins can also be induced solely through biological stressors, such as competition for space, in two Pacific cnidarians of the Actinaria group. However, to the best of our knowledge no information about Hsps and biotic stress on reef building corals (order Scleractinia) is presented in literature.

Among biotic stresses, coral diseases have been recognized as one of the cause of the coral reefs decline (Harvell et al., 1999; Weil, 2004). In particular, the protozoan disease known as the Skeleton eroding band (SEB) disease, has been the first coral disease described from an Indo-Pacific reef (Antonius, 1999) where it is now one of the most prevalent coral infection having the widest host range documented for any coral disease (Page and Willis, 2008). The organism associated with this syndrome has been identified as *Halofolliculina corallasia* (Antonius and Lipscomb, 2001), a species of folliculinid, heterotrich ciliate, which produces a black band (1–10 cm wide) at the interface between recently exposed skeleton and apparently healthy coral tissue (Antonius, 1999). In addition to coral diseases, another important cause of reef degradation has been attributed to the large increase

in the abundance of benthic algae which compete for space and light with scleractinian corals, often overgrowing on them (Jompa and McCook, 2003a). The coral-algal competition is widespread, but the interaction is highly variable in both process and outcome as reported in several studies (reviewed in McCook et al., 2001).

In this study, for the first time, the effectiveness of Hsps as an indicator of biological stress in scleractinian corals has been analyzed. To determine whether Hsp expression patterns could be related to competitive interactions in coral reef habitat, the staghorn coral *Acropora muricata* (Linnaeus, 1758) was chosen for this study, representing one of the most abundant coral species in the Indo-Pacific reef (Veron, 2000) expecially in the studied area, the lagoon of Magoodhoo Island, Republic of Maldives (Seveso, personal communication). In particular, we hypothesize that components of the stress response such as the Hsp60 could provide evidence of the intensity and the damage of competitive interactions between the whole holobiont of *A. muricata* and biological agents, such as the protozoan causing SEB disease and the turf/ macroalgae involved in overgrowth of corals.

2.2 Materials and Methods

2.2.1 Coral collection

The study was undertaken on coral patches inside 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.S1 Supplementary Data).

To study Hsp60 expression in corals subjected to SEB disease, infected colonies of *A. muricata* were located in the lagoon and photographed (Canon A710IS with Ikelite housing). The presence of *A. muricata* colonies infected

by SEB ciliates was confirmed by microscopic analysis (Leica EZ4D, Leica Microsystems, Germany) of coral fragments collected at the dark band (Fig. 2.1A and B). Seven of these colonies were selected and for each colony two different coral fragments were sampled and marked as: "healthy" (H), fragment of a healthy coral branch far from the dark band in an infected colony and "diseased" (D), fragment sampled just above the ciliate dark band, along the disease progression direction, in an infected colony (Fig. 2.1C). All the coral portions were collected using hammer and chisel. To avoid artifacts which might occur when coral fragments were transported under stressful conditions, specimens were immediately frozen at -80°C in the field using an immersion cooler (FT902, JULABO, Labortechnick GmbH). Both the coral fragments of samples H and D should not contain protozoa to avoid interference during the analysis of Hsps, so the total absence of protozoan has been carefully verified by microscopic examination of each frozen sample prior to their homogenization.

To study Hsp60 expression in corals subjected to algal overgrowth, colonies of *A. muricata* which presented some branches overgrown by filamentous and mixed-species algal turfs were located in the lagoon and photographed. These colonies had some branches with dead coral tissue covered by a thick algal turf which became less dense at the coral tips revealing the living tissue below. To get a confirmation of this, these branches have been carefully examinated by microscopic analysis. Other branches of the same colonies were free of algae. Seven colonies were selected and for each colony two different coral fragments were sampled and marked as: "without algae" (WA), fragment of a living coral branch free of algae in a coral colony overgrown by algae, and "algal overgrowth" (AO), fragment of living coral tissue sampled just next to the algal interface that was the area where the turf started to be thinned out (Fig. 2.2A). All the coral portions were collected

and stored as described for samples H and D. For both experiments, as control (C) seven isolated and entirely healthy colonies of *A. muricata* were likewise sampled in the same zone of the lagoon.

All the coral samples for the controls and the two biotic stresses were collected simultaneously in October 2010 at the same depth, at the same early morning time (08:00 am) and during high tide (coral permanently submerged) to minimize seasonal and/or daily differences in cnidarian behavior and in Hsp60 expression due to changes in water temperature and/or different UV intensity (Chow et al., 2009, 2012), fluctuations in salinity and pH, and other effects that are typical of the intertidal environment. An HOBO pendant data loggers (Onset, UA-002-64) were used to measure temperature of specific locations and seawater samples which were collected in tubes were used for salinity measurements with a refractometer (Milwaukee Instruments, USA).

2.2.2 Coral species identification

To confirm that the coral species under investigation was *A. muricata*, coral DNA was extracted using DNeasy® Tissue kit (QIAGEN, Qiagen Inc., Valencia, CA, USA) and a rDNA region of about 500 bp (spanning the entire ITS1, 5.8S, ITS2 and a portion of 28S and 18S) was amplified and sequenced. Amplification was performed using the coral-specific primer A18S (5' GATCGAACGGTTTAGTGAGG 3'), (Takabayashi et al., 1998) and the universal primer ITS4 (5' TCCTCCGCTTATTGATATGC 3'), (White et al., 1990). Sequences were compared with known scleractinian corals sequences in GenBank using the BLAST nucleotide search (http://www.ncbi.nlm.nih.gov/BLAST/). BLAST searches showed 94% identity with rDNA sequences of *A. muricata*.

2.2.3 Protein extracts and Western analysis

In the laboratory, 1 g of each frozen coral fragment was powdered using mortar and pestle. Proteins of the holobiont were extracted by homogenizing the tissue powder in 400 µl of SDS-buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol) containing 1mM phenylmethylsulfonyl fluoride (Sigma) and Complete EDTA free cocktail of protease inhibitors (Roche Diagnostic). Samples were boiled for 10 min and skeleton fragments were removed by a single step of centrifugation (15 min at 12000 rpm, 4°C). Supernatants were clarified (5 min at 12000 rpm) and then frozen at -20°C until used. Aliquots of the supernatants were used for protein concentration determinations using the Bio-Rad protein assay kit (Bio-Rad Laboratories, California, USA). Protein samples were separated by SDS-PAGE on 8% polyacrylamide gels (18 cm x 16 cm) (Vai et al., 1986). The same amount of proteins (80 µg) was loaded on each lane of the gel. Pre-stained protein markers (range 7-175 kDa, New England Biolabs) were also loaded. Duplicate gels were run in parallel. After electrophoresis, one gel was stained with Coomassie Brilliant Blue to visualize the total proteins (Fig. S2 Supplementary Data), and the other electroblotted onto nitrocellulose membrane at a costant current of 400 mA for 4 h (Vai et al., 1986) for Western analysis. Correct proteins transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters (Fig. S3 Supplementary Data). For each blot, the same amount of recombinant human Hsp60 (StressGen Bioreagents, British Columbia, Canada, ADI-SPP-540) was included. Filters were washed in TBS (0.01 M Tris, pH 7.4, 0.9% NaCl) followed by 1.30 h saturation in TBS containing 0.1% Tween 20 and 5% skimmed milk. Immunodetection was performed with anti-Hsp60 monoclonal antibody (IgG mouse clone LK-2, StressGen Bioreagents,

British Columbia, Canada, SPA-807) at 1:1000 dilution in TBS-Tween 20, 5% skimmed milk. After washing in TBS-Tween 20 (10 min, 3 times), membranes were incubated with secondary antibody (diluted 1:10000 in TBS-Tween 20, 5% skimmed milk) antimouse IgG conjugated with horseradish peroxidase (Thermo Scientific). Binding was visualized with the Pierce ECL Western Blotting Substrate followed by X-rays films.

2.2.4 Densitometric and Statistical analyses

Blot band intensities were compared by scanning the X-ray films and analyzing the scans with the Image J free software (http://rsb.info.nih.gov/ij/) of 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 standard Hsp60 protein band. Data were expressed as the mean ± standard error of the means (SEM). One-way analysis of variance (ANOVA) was performed for all the normalized Hsp60 intensity values obtained from the different groups of samples (C, H, D, WA and AO). Since the analysis revealed that the changes in the Hsp60 levels among the five considered groups were significant ($F(4,25) = 113.68$, $p < 0.0000$), the Tukey's HSD post hoc tests for pair-wise comparison of means was used to assess significant differences ($p < 0.000$).

2.3 Results

Colonies of *A. muricata* infected by SEB ciliates showed the typical dark band which separates the dead tissue from the healthy tissue. Moreover, the band of ciliates causing SEB might be confused with Black band disease (BBD) caused by bacteria, but microscopic analysis of the coral fragments

collected at the level of the dark band, revealed the presence only of the protozoa of the species *Halofolliculina corallasia* responsible for the SEB disease. *H. corallasia* is sessile in a lorica, sac-like with a rounded posterior and a cylindrical neck. The cell body is attached at its pointed posterior end to the base of the lorica. The cell is large and elongated with two conspicuous pericystomial wings (Fig. 2.1A and B). These protozoa appeared densely packed forming an indistinguishable black mass that cover the dead tissue below (Fig. 2.1C).

The monoclonal antibody anti-Hsp60 recognized a single specific 62-kDa band in all the coral fragments of all sampled colonies of *A. muricata* (Fig. 2.1D). No detectable and significant changes in the Hsp60 levels were detected in healthy fragments sampled far from the dark band (H) compared with the control (C). On the contrary, a strong induction of Hsp60 was observed nearby the infected site in fragments sampled just above the ciliate dark band (D), (Tukey's HSD post hoc tests for pair-wise comparison of means, $p < 0.0000$), where the Hsp60 level was almost twice compared to the control and samples H (Fig. 2.1E).

By contrast, no detectable and significant modulation of the Hsp60 expression was detected in coral overgrown by algae. As shown in Fig. 2.2B and C, in the fragments of living coral branch free of algae (WA) and in the fragments of living coral tissue sampled just next to the algal interface (AO), the level of Hsp60 was similar to the level present in the control. Thus, no modulation of the Hsp60 expression was detected in coral overgrown by algae. The seawater temperature measured at sampling time (October 2010) was 28.9°C and it appeared in line with the regular mean seasonal trend (± STD Dev) as shown in Fig. S4, Supplementary Data. Moreover, no anomalies regarding the salinity values (~ 35.5‰) were detected.

2.4 Discussion and conclusions

The present study investigated the effects of biotic stresses on Hsp expression in a scleractinian coral, comparing the modulation of the Hsp60 levels in response to two different types of biological factors: the coral disease Skeleton eroding band (SEB), whose first record in the Maldives has been recently reported (Montano et al., under review), and the algal-coral interaction causing the algal overgrowth on corals. Both these different types of biological interactions are recognized to be among the most important contributors to the worldwide decline of coral reefs (Harvell et al., 2002; Gardner et al., 2003; Hughes et al., 2003; Wilkinson, 2004).

To date, Hsp analyses have been predominantly performed in corals exposed to short-term, extreme stress regimes in the laboratory, confirming that corals possess temporally dynamic and responsive cellular machinery to counteract stresses (Van Oppen and Gates, 2006). In particular, Hsp60 is a molecular chaperone known to assist *de novo* folding, to refold misfolded proteins and to counteract protein aggregation under normal conditions. In response to environmental stresses, the deleterious increase of unfolded proteins triggers the induction of Hsp60 (Richter et al., 2010). In reef building corals the up-regulation of Hsp60 has already been observed under laboratory culture conditions testing stress induced by elevated temperature and light (Chow et al., 2009, 2012). Our analyses, performed on the scleractinian coral *A. muricata* in the natural environmental habitat of the lagoon of Magoodhoo Island, indicate that two different biological interactions trigger diverse responses on Hsp60 level. In fact, corals displayed a robust up-regulation of Hsp60 in response to the infection of the protozoa *H. corallasia* which causes the SEB disease, while for the algal overgrowth we did not detect any effect on the modulation of Hsp60

expression. With regard to the low level of Hsp60 present in the control sample of the healthy colonies this indicate that this chaperonine also has an important function under normal physiological conditions of the organism, in agreement with data reported in literature (Choresh et al., 2001; Chow et al., 2009, 2012).

In our experiments the whole holobiont was considered and the antibody used in this study, monoclonal clone LK-2, cross-reacts with a broad group of organisms that include bacteria, yeast, plants and animals. For this reason, the heat shock response could be produced by the coral polyps only, by the holobiont (microbial community, symbiotic zooxanthellae and cnidarians animal) or by the zooxanthellae only. It is important to emphasize that in our experiments all coral tissue samples were free of necrosis and morphologically undamaged.

SEB is one of the most common disease of corals widespread in the Indian and Pacific Ocean (Page and Willis, 2008). In the studied area about 3000 colonies belonging to 19 genera were analyzed and the percentage of colonies infected by SEB (about 2%) and other coral diseases were reported in Montano et al. (under review). SEB occurs in sheltered, lagoon-type environments showing the greatest abundance at depths between 0.5 m and 3 m (Antonius and Lipscomb, 2001). Sessile ciliates settled between living tissues and recently exposed coral skeletons and their presence alters the normal body functions of the host starting lysis of the coral tissue and delays and/or reduces tissue regeneration (Rodriguez et al., 2009). Coral mortality is thought to be caused by spinning and chemical secretions (organic acids) of the asexually produced motile swarming phase of *H. corallasia* (Antonius and Lipscomb, 2001). As a consequence of these processes, once infection has passed over an area the coral tissue dies and the bare coral skeleton loses all fine trabecular limestone structure. Progression rate of the band is very

rapid and it has been estimated to change between 1 mm/week and 1 mm/day (Antonius and Lipscomb, 2001) similar to other "band" diseases, such as Black band disease or White band disease (Antonius, 1999; Antonius and Lipscomb, 2001). A study in 2008 found that SEB spread at about 2 mm/day in colonies of *A. muricata*, eventually wiping out 95% of its victims (Page and Willis, 2008).

From the microscopic analyses of the dark band, the total absence of living polyp tissue has been observed, revealing that the host cells were already died. For this reason, in our field sampling we decided to collect fragments of living coral tissue placed at different distances along the advancing front of the SEB band to analyze Hsp60 level, which would be meaningless in the coral fragments collected at the dark band. Our results show that in *A. muricata* infected by SEB disease different levels of stress protein 60-kDa are found in different portions of the colonies. In fact, the coral fragments sampled just above the SEB dark band, on the interface of ciliates progression, displayed a remarkable up-regulation of Hsp60, whose level was twofold higher compared to the control, indicating that the aggressive behavior of *H. corallasia* can cause cellular damage also in coral portions neighboring the infection and suggesting that disease infection causes stress at the cellular level, even in cells not yet infected by ciliates.

In the aquatic organisms, many interactions also involve chemical communication (Brönmark and Hansson, 2000), such as the case of coral SEB disease. The chemicals associated with the unhardened lorica, combined with the mechanical disruption caused by the spinning larvae, appear to damage the coral skeleton and initiate the lysis of the coral tissue (Antonius, 1999). The chemical secretions produced by *H. corallasia* in the infected zone could trigger the induction of Hsp60 in the portions just above the band. In this context, the Hsp60 up-regulation might represent a defense

from underlying coral portions colonized by ciliates which excrete harmful substances. Otherwise the up-regulation of the Hsp could represent a strategy/mechanism to stop and circumscribe the infection, preventing it from spreading throughout the coral. However, corals have an immune system based on self/non-self recognition and cellular and humoral processes (Mydlarz et al., 2010). Recognition receptors such as Toll-like receptor (TLR) domain genes (Hemmrich et al., 2007; Miller et al., 2007) have been characterized in anthozoan corals (Dunn, 2009). Recently, it has been suggested that in mammalian, Hsp60 were implicated in autoimmune disease and antigen presentation since they are potent activators of the innate immune system (Tsan and Gao, 2009). In particular Hsp60 activation appears to be mediated by TLRs ligand (Ohashi et al., 2000). Although no morphological differences were detected in tissues next to SEB band compared to those of healthy colonies, in line with what reported by (Antonius and Lipscomb, 2001) who suggested that the coral polyps immediately ahead of an advancing front of SEB appear undisturbed, our results indicate that physiological processes aimed to counteract the damage caused by infection are active.

The coral fragments sampled distant to the dark band of ciliates had a Hsp60 level comparable to that observed in healthy colonies of *A. muricata*. This might suggest that the stress response appears confined in a restricted area near the infection even if in a coral colony polyps are linked together by a common tissue named coenosarc or coenenchyme.

The other biological factor analyzed for the Hsp60 expression in *A. muricata* is the algal overgrowth phenomenom. Different responses of corals to different species of algae or different impacts of algae on corals have been largely documented (McCook, 2001; McCook et al., 2001; Jompa and McCook, 2002; Smith et al., 2006; Diaz-Pulido et al., 2009), suggesting a

great variability in the processes and outcomes of coral–algal interactions, even within an algal functional group, algal family, and coral life-forms and genera (Jompa and McCook, 2003a). Also in this case we sampled fragments of living tissue of coral placed at different distances along the progression of the algal turf which caused the death of the backwards coral tissues. In particular, to test whether coral-algal competition may affect the modulation of Hsp60, portions of living coral tissue colonized by a few algal filaments were sampled. Analyzing the expression of Hsp60, no detectable differences have been observed between the healthy and the overgrown colonies and also between the different coral portions of the same colony subjected to algal overgrowth. Different explanations might be envisaged. It's conceivable that benthic algae and algal turf have light inhibitory effects on *A. muricata* colonies. In fact some studies have reported minor effects of algal turf on corals, or have even suggested that algal turfs are relatively poor competitors having little effect on corals (McCook et al., 2001) or that corals were competitively superior to the algal turfs (Van Woesik, 1998; McCook, 2001). Nevertheless, algae can actively overgrown on the live coral by exuding allelochemical or secondary substances, as reported in others studies (Littler and Littler, 1997; Jompa and McCook, 2003b). Presumably, in the fragments sampled next to algal interface, the coral cells have not yet been damaged by algal toxins, and hence have not up-regulated their levels of Hsp60. It is widely known that various species or genera of algae can negatively influence corals (McCook et al., 2001; Jompa and McCook, 2003a, 2003b; Smith et al., 2006) leading to reef degradation. Since further investigations performed five months after the sampling revealed that the same colonies were completely overgrown by algae and turf, this latter scenario appears to be the most likely.

In conclusion, with this study we propose Hsp60 expression might be a

useful tool and promising biomarker for the holobiont of scleractinian corals to evaluate physiological stress caused by coral diseases such as SEB, laying the basis for subsequent monitoring in the field of other diseases and other types of biological stresses. Further studies on the different groups of Hsps and their expression in each member of the holobiont association may also be important for the health assessment of scleractinian corals and for the conservation of coral reefs.

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2.6 Figures

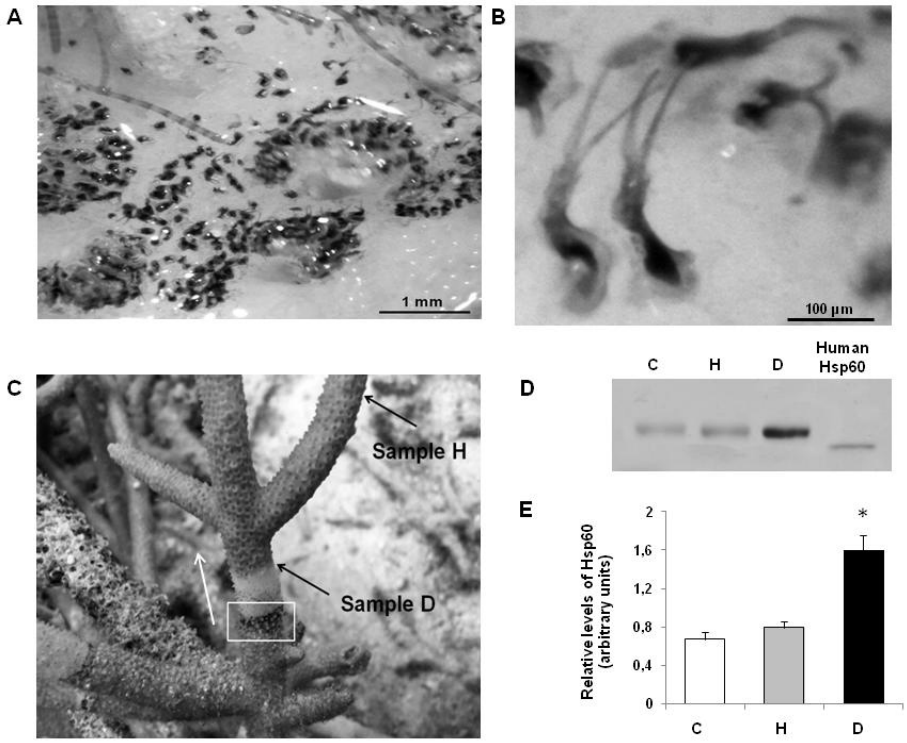

Fig. 2.1 - A. Microscope photo of the protozoan *Halofolliculina corallasia*, a species of folliculinid, heterotrich ciliate responsible of the SEB disease in *A. muricata*. **B.** *H. corallasia* in a lorica, sac-like. To note the two conspicuous pericytostomial wings. **C.** Colony of *A. muricata* affected by the Skeleton eroding band (SEB) disease. The infection appears as a dark band (surrounded by a rectangle) with skeleton recently devoid of tissue below. Just above the band, a white part of naked skeleton is visible. Loricae of *H. corallasia* are scattered loosely over this area occupying freely accessible terrain. Above this part, the coral tissue is still healthy and the sampling points are shown. Sample D: fragment of coral collected just above the ciliates dark band and eroded skeleton and tissue. Sample H: fragment of a healthy coral branch in the infected colony. The white arrow indicates the disease

progression direction **C.** Effect of SEB disease on induction of Hsp60 in the different portions (D and H) of the scleractinian coral *A. muricata*. Samples prepared from healthy colonies (C) were also analyzed. Western blot representative of seven experimental repeats is shown. For each blots, the same amount of recombinant human Hsp60 was included **D.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for seven different blots were analyzed. Data are expressed as arbitrary units and as mean±SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.00$).

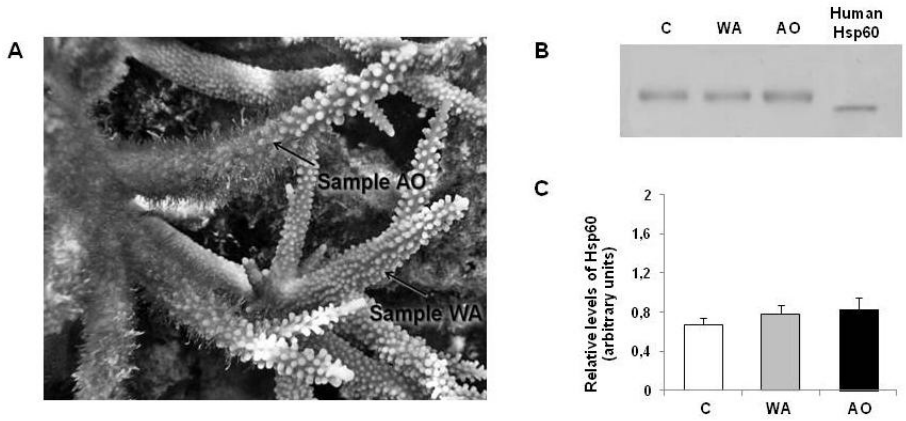

Fig. 2.2 - A. Colony of *A. muricata* overgrown by filamentous and mixed-species algal turf. Sample AO: fragment of live coral tissue sampled corresponding to the algal interface in a colony overgrown by algae. Sample WA: fragment of a coral branch free of algae in a coral colony overgrown by algae. **B.** Effect of algal overgrowth on induction of Hsp60 in the different portions (AO and WA) of the scleractinian coral *A. muricata*. Samples prepared from healthy colonies (C) were also analyzed. Western blot representative of seven experimental repeats is shown. For each blots, the same amount of recombinant human Hsp60 was included **C.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for seven different blots were analyzed. Data are expressed as arbitrary units and as mean±SEM.

2.7 Supplementary Data

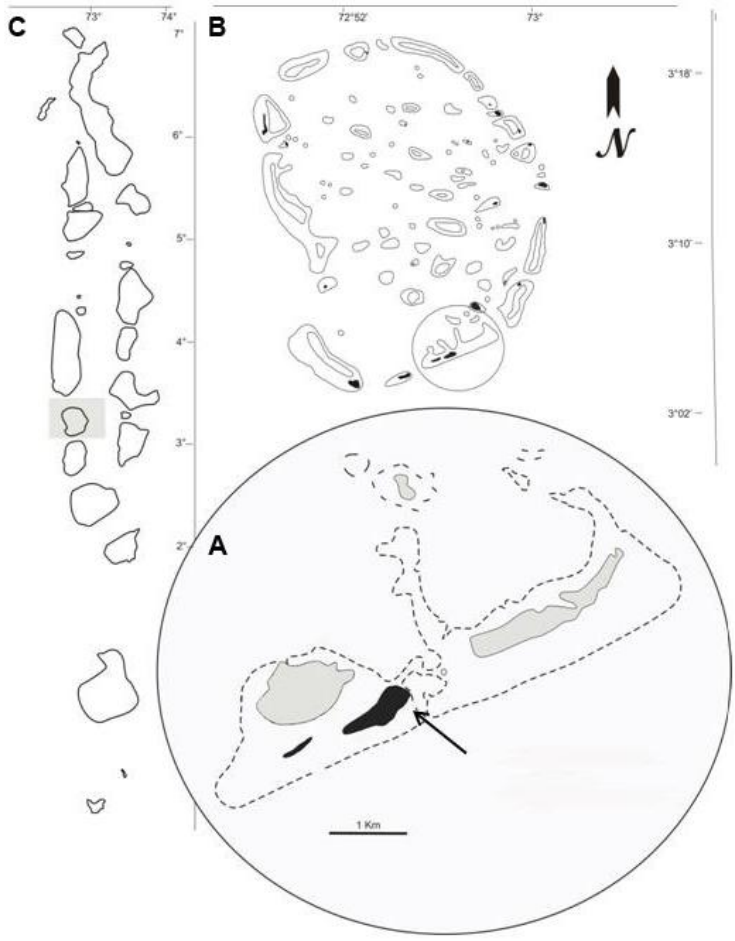

Fig. S1 - Map of Magoodhoo Island (A), located in Faafu Atoll (B), Republic of Maldives (C). The islands are indicated in black, the deeper part of the lagoons in grey and the dotted line indicates the reef edges. An area of approximately 150 m^2 and about 70 m distant from the shore in the Magoodhoo lagoon (3° 04' 45.60" N; 72° 58' 02.20" E), was chosen as sampling site to create an easy and short time access area (indicated by the arrow).

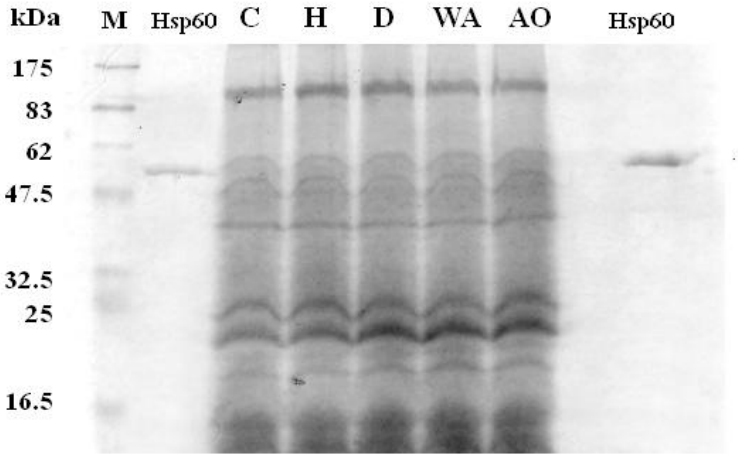

Fig. S2 - A representative SDS-PAGE on polyacrylamide gel (8%) stained with Coomassie Brilliant Blue to visualize total proteins extracted from different coral fragments is shown. Pre-stained protein markers (M) and two different amount of recombinant human Hsp60 (5 and 10 µg) were loaded on the gel. The abbreviations of the samples are described in the text.

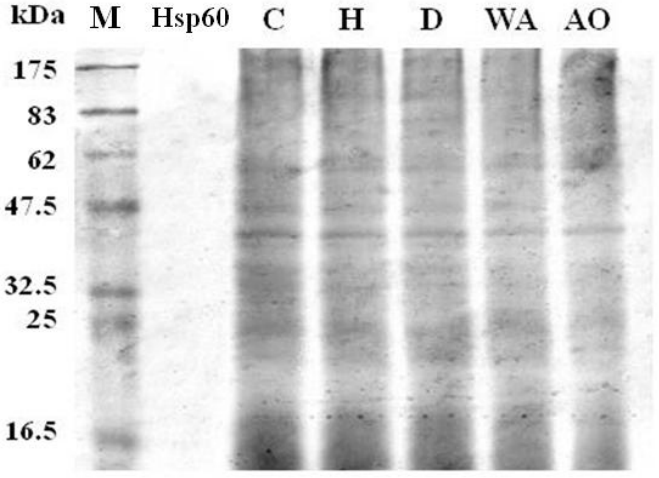

Fig. S3 - Representative filter stained with Ponceau used as control for equal protein loading and to ensure a correct protein transfer. Pre-stained protein markers (M) are also shown. The abbreviations of the samples are described in the text

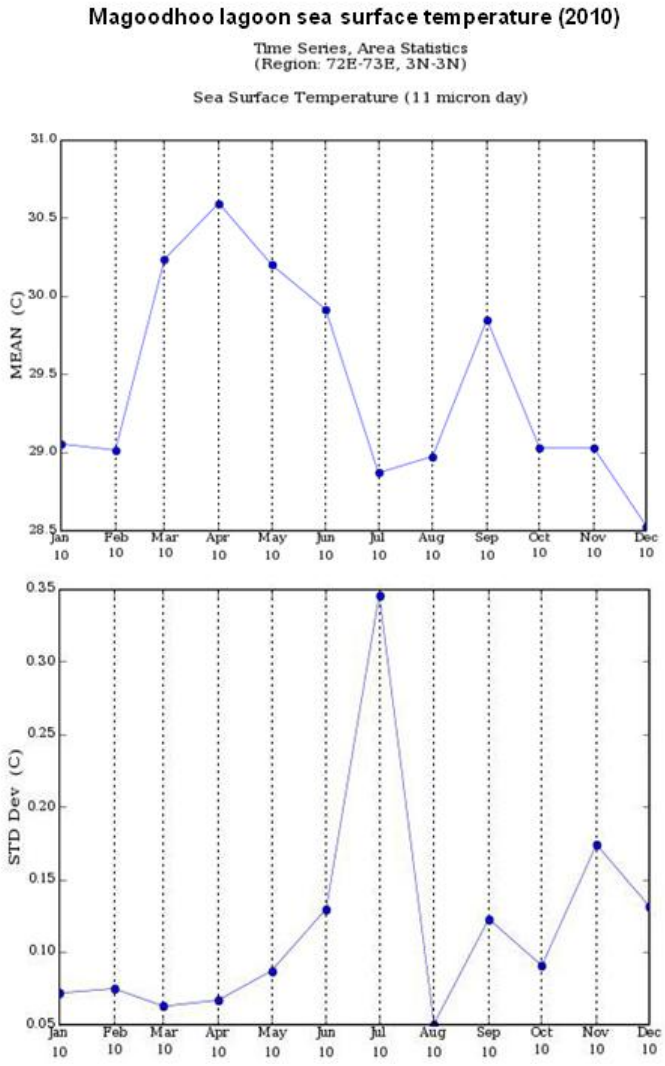

Fig. S4 – Graph showing the trend of sea surface mean temperature for the lagoon of Magoodhoo Island (Maldives) on 2010 (panel above). On panel below, graph reporting the values of standard deviation relative to the average monthly temperature (Source:http://disc.sci.gsfc.nasa.gov/techlab/giovanni/)

– Chapter 3 –

Exploring the tolerance of *Seriatopora caliendrum* to salinity change by the Hsp60 modulation

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3.1 Introduction

Although coral reefs commonly inhabit areas where salinity is generally permanent and stable over long timescales (Coles & Jokiel 1992), they can experience extreme changes in salinity levels of varying duration. Heavy rainy seasons which are common in tropical regions, hurricanes, storms and coastal freshwater runoff can significantly reduce surface and depth salinity in short term (min to h) or over longer periods (days to weeks), (Goreau 1964, Van Woesik et al. 1995, Moberg et al. 1997, Devlin et al. 1998, Berkelmans & Oliver 1999, Porter et al. 1999). At the other extreme, a high rate of evaporation in tide pools during low tides and prolonged drought may cause salinities to rise to stressful levels (Smith 1941, Wells 1957), and lead also in this case to the physiological damage of reef organisms.

Osmotic stress, which occurs when the cell experiences volume and osmolyte fluctuations that compromise macromolecular structures and metabolic function (Mayfield & Gates 2007), has long been recognized as a limiting physical parameter for marine organisms. Corals are generally considered stenohaline, with a limited ability to adapt to or survive salinity changes (Wells 1957, Coles & Jokiel 1992), even if some species are euryhaline and can withstand significant changes in external osmolarity (Coles 1992, Muthiga & Szmant 1987, Manzello & Lirman 2003, Mayfield & Gates 2007). In particular, reef-building corals, like the majority of marine

invertebrates, are osmoconformers. Since they lack the capability of osmoregulation, they do not possess a constant cellular osmolarity but respond to dynamic changes in their environment rapidly by absorbing water to become iso-osmotic with their surroundings (Rankin & Davenport 1981, Hoegh-Goldberg & Smith 1989, Tytlianov et al. 2000, Mayfield & Gates 2007). Sudden deviations in ambient salinity negatively affect the basal metabolic functions of the corals inducing changes in respiratory pathways and symbiont photosynthetic efficiency. In this context, the majority of the studies have analyzed the effects of reduced salinity on coral metabolism (Nystrom et al. 1997, Tytlianov et al. 2000, Downs et al. 2009). For example, following hyposaline treatments, a significant drop in net photosynthesis of *Porites furcata* has been reported (Manzello & Lirman 2003). Also decreased rates of gross photosynthesis due to cellular damage to algae have been observed in *Siderastrea siderea*, *Porites lutea* and *Pocillopora damicornis* (Muthiga & Szmant 1987, Moberg 1997). In the long term, the salinity stress condition can imply decreased growth potential (Coles 1992) and also give rise to higher-order physiological diseases, such as gamete abnormalities and reduced viabilities, affecting fecundity (Richmond 1993) or even leading to death (Coles & Jokiel 1978,1992, Hoegh-Guldberg & Smith 1989) due to their inability to initiate an osmotic stress response before significant changes in cell volume occur. However, despite the importance of salinity to metabolic functions request for reef coral survival, little information is presently available about the physiological responses and strategies of corals exposed to salinity stress and the majority of these studies have focused on the effects of hypo-salinity on coral photosynthesis and/or respiration.

Heat shock proteins (Hsps) represent one of the most important defense mechanisms of all organisms (Kumsta & Jakob 2009) working as molecular

chaperones and interacting with, stabilizing or helping another protein to acquire its functionally active conformation, without being present in its final structure (Hartl & Hayer-Hartl 2009, Hartl et al. 2011). In particular, the 60-kDa chaperonine (Hsp60) participates broadly in de novo protein folding and refolding and target improperly folded proteins to degradative pathways (Mayer 2010, Vabulas et al. 2010; Hartl et al. 2011) even under normal physiological conditions. Nevertheless, in response to environmental stresses their expressions appear to be up-regulated in order to increase cellular repair and cellular tolerance to adverse conditions preserving metabolic functions (Lanneau et al. 2008, Richter et al. 2010). Hsps are commonly used as biomarkers in coral health assessment and the expression of Hsp60 after abiotic stresses such as extreme temperatures (Brown et al. 2002, Kingsley et al. 2003, Chow et al. 2009, 2012), elevated light intensity (Downs et al. 2000, Chow et al. 2009, 2012) xenobiotics (Downs et al. 2005) and also after biotic stresses (Seveso et al. 2012) has been extensively reported.

On the other hand, little information is available on the expression of Hsps in scleractinian corals subjected to salinity stress (Downs et al. 2009). In the present paper, in order to study this issue further, we analyzed the Hsp60 expression profiles under three salinity scenarios (hypersalinity, hyposalinity and extreme hyposalinity) during the time course of a 2 days period, and also evaluated the gross morphological changes of the coral colonies. These analyses were performed in the branching scleractinian coral *Seriatopora caliendrum*, an important Indo-Pacific reef builder which exhibits low ability to survive stressful environmental conditions (Loya et al. 2001, Bhagooli & Yakovleva 2004).

3.2 Materials and methods

3.2.1 Maintenance and growth condition of corals

Experiments were performed at the Civic Aquarium of Milan, Italy, where colonies of *S. caliendrum* were maintained in a flow-through aquaria system consisting of four tanks (120 l, 80 x 30 x 50 cm each one) connected to a 330-l sump containing gravel-bed filter, protein skimmer and a 500 W Titanium Heater (Aqua Medic) connected to a temperature controller**.** In all the tanks, the light was provided by 400 W metal halide lamps (Powerstar HQI-T, Osram), which were turned on at 9 a.m. for the day cycle and turned off at 9 p.m. (12:12 light:dark cycle).

The colonies were acclimatized for 25 days before any experimental manipulations performed under controlled laboratory conditions. Normal water temperature was set at 27°C (±0.3 °C) and the normal oceanic salinity (35 ppt) was reproduced. Evaporation in aquaria was offset by the addition of deionized water. Calcium, magnesium, iodium and alkalinity were assessed and reintegrated every three days to ensure stable water chemistry. Salinity and pH of the seawater in the aquarium tanks were kept stable and constantly monitored using a Hach Lange HQ 30D flexi portable meter. Twice a week, all corals were fed with *Artemia salina* nauplii.

3.2.2 Experimental design and stress exposure

Colonies of *S. caliendrum* were divided into small fragments (6–7 cm in height) of similar size and immediately immersed (randomly distributed) into the three tanks. The coral fragments were left to recover from the handling procedures for 20 days before the salinity stress exposure. All the

chemical and physical parameters of the seawater were kept stable and constantly monitored. To minimize the variations due to light bulb diffusion and water movement, the position of nubbins within each tank was changed every four days. Tanks were cleaned on a weekly basis in order to minimize algal growth (Rodolfo-Metalpa et al. 2005).

After the acclimatization period to salinity of 35 ppt (control), coral fragments from each of the three tanks were first photographed for assessing the coral health through morphological analysis and then sampled for protein analysis. They represented the control samples (Time 0). After sampling, coral fragments were immediately frozen in liquid nitrogen and stored at −80°C until analysis. Only healthy coral fragments exhibiting healing at fragmentation lesions, growth, characteristic color, dark pigmentation and fully extended polyps were used for exposure experiments and were assigned randomly to each treatment.

S. caliendrum fragments (n = 7 per treatment) were subjected to three different salinity concentrations which may realistically occur on a reef. The three experimental exposures were: 45 ppt in the first tank (hypersalinity), 27 ppt in the second tank (hyposalinity) and 15 ppt in the third tank (extreme hyposalinity). Exposures were carried out for 48 h, and sampling was performed at 6, 12, 24 and 48 h. All the samples were quickly frozen in liquid nitrogen and stored at −80 °C until protein extraction. Each sampling was accompanied by photographic documentation in order to monitor gross morphological changes in coral. Coral health was assessed as tissue coloration and condition and polyp appearance (retracted or extended). Colony coloration was classified as normal (normal pigmentation throughout the colony), pale (lighter coloration in the coral theca and coenosteum), and bleaching (white coloration throughout the colony), (Lirman et al. 2002). At the end of the osmotic stresses, tissue condition and necrosis of coral polyps

was visually assessed using a stereomicroscope Leica EZ4 HD with Integrated LED Illumination and HD Camera.

Elevated salinity (45 ppt) was achieved by the addition of Insant Ocean salts to seawater whereas low salinity (27 and 15 ppt) was obtained by diluting the seawater (35 ppt) with distilled water (MilliQ) whose pH was the same as that of the seawater. Fresh solutions were made immediately before each experiment. During stress exposures each aquarium tank was a closed system (static flow) but contained Koralia immersed pumps (1200 l/h) in order to continuously mix and aerate the seawater.

Salinities were checked at regular intervals (i.e., every 2 h) and the temperature was maintained always constant using a temperature controller (Eliwell, PC 902T) connected to submersible Acqua Medic Titanium Aquarium Heater (model TH-100, 240 V).

3.2.3 Protein extracts and Western analysis

In the laboratory, 1g of each frozen coral fragment was powdered using mortar and pestle. Proteins of the holobiont were extracted by homogenizing the tissue powder in 400 µl of SDS-buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol) containing 1mM phenylmethylsulfonyl fluoride (Sigma) and Complete EDTA-free cocktail of protease inhibitors (Roche Diagnostic). Samples were boiled for 10 min, the skeleton fragments were removed by a single step of centrifugation (15 min at 12000 rpm, 4°C) and algal symbionts were separated from the coral tissue by another centrifugation (5 min at 4500 rpm, 4°C) as reported (Middlebrook et al. 2010). Supernatants were clarified (5 min at 12000 rpm) and then frozen at -20°C until used. Aliquots of the supernatants were used for protein concentration determinations using the Bio-Rad protein assay kit

(Bio-Rad Laboratories). Protein samples were separated by SDS-PAGE on 8% polyacrylamide gels (Seveso et al. 2012). The same amount of proteins (80 µg) was loaded on each lane of the gel. 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 as previously described (Seveso et al. 2012). Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters. For each blot, the same amount of recombinant human Hsp60 (Stressgen Bioreagents) was included. Anti-Hsp60 monoclonal antibody (IgG mouse clone LK-2, SPA-807, Stressgen Bioreagents) at 1:1000 dilution was used as previously reported (Seveso et. al. 2012). Filters were also probed with mouse anti-Actin monoclonal antibody (clone C4, MAB1501, Millipore) at 1:1000 dilution in TBS-Tween 20, 5% skimmed milk. Secondary antibody (diluited 1:10000 for Hsp60 and 1:7500 for Actin in TBS-Tween 20, 5% skimmed milk) anti-mouse IgG conjugated with horseradish peroxidase (Thermo Scientific) was used.

To evaluate the absence of contamination of *Symbiodinium* proteins in our samples, filters were probed with anti-Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (AS03037, Agrisera) at 1:2000 dilution in TBS-Tween 20, 5% skimmed milk, followed by incubation with secondary antibody (diluted 1:7500 in TBS-Tween 20, 5% skimmed milk) anti-rabbit IgG conjugated with horseradish peroxidase (Thermo Scientific). Different amounts of Rubisco protein standard (AS01017S, Agrisera) were loaded as control. Binding was visualized with the Pierce ECL Western Blotting Substrate followed by X-ray films.

3.2.4 Densitometric and statistical analyses

Following ECL detection, films were scanned on a Bio-Rad GS-800 calibrated imaging densitometer and quantified with the Image J free software (http://rsb.info.nih.gov/ij/) of 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 standard Hsp60 protein band. Data were expressed as the mean ± standard error of the means (SEM). One-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc tests for pair-wise comparision of means was performed for all the normalized Hsp60 intensity values obtained from the different groups of samples to assess significant differences ($p < 0.0000$) in Hsp60 protein levels in response to the different exposure times for each salinity level.

3.3 Results

3.3.1 Absence of *Symbiodinium* contamination

The coral-*Symbiodinium* association is an endosymbiosis in which the dinoflagellate reside within the anthozoan host's gastrodermal cells and contribute to the latter's nutrition. However, several studies revealed that, under stressful conditions causing bleaching events, the zooxanthellae showed a specific and modulation of their own specific Hsps, regardless of the host (Brown et al. 2002, Downs et al. 2005). Nevertheless, in this study we are interested in analyzing the expression of Hsp solely by the animal tissue (polyp tissue).

For this reasons, the symbiotic algae were separated from the animal tissue

during the extraction process through centrifugation step which produce a bottom phase with insoluble debris (coral skeleton) and algae and a supernatant phase representing the animal protein extract. To check that the *Symbiodinium* proteins were not included in the final extraction, Western blot analysis were performed for all the coral samples subjected to the different salinity conditions using the antibody anti-Rubisco, specific to photosynthetic organisms, such as *Symbiodinium.*

The anti-Rubisco recognized a ~55-kDa band in the bottom phases, revealing the presence of the zooxanthellae (data not shown). On the contrary, the Western blot analysis confirm the total absence of *Symbiodinium* contamination in all the collected fractions, supporting that our final extracts included only coral polyps proteins (Fig. 3.1).

3.3.2 Salinity exposures

The expression of Hsp60 was analyzed in *S. caliendrum* subjected to three different salinity treatments and at different exposure time. The monoclonal antibody anti-Hsp60 recognized a single specific 62-kDa band in all *S. caliendrum* fragments subjected to any level of salinity.

At the same time, the host Actin (molecular weight of ~43 kDa) were selected as additional host tissue-specific marker proteins being highly-expressed in the host cells (Peng et al. 2010). The Western blot analysis revealed the presence of host Actin in all *S. caliendrum* fragments subjected to any level of salinity.

At control salinity of 35 ppt the corals (samples Time 0) appeared with extended polyps and intact tissue with normal coloration, proving that they are healthy (Tab. 3.1 and Fig. 3.2).

3.3.2.1 Hypersalinity condition (45 ppt)

In a hypersaline seawater of 45 ppt a transient induction of Hsp60 expression was detected (Fig. 3.3A) with a robust up-regulation of Hsp60 already after the first 6 h of stress respect the control Time 0. After this sudden exposure, *S. caliendrum* fragments displayed a strong decrease of the expression of Hsp60 at the twelfth hour of stress. Until the end of treatment (48 h), the signal of hsp60 remains weak and almost unchanged.

As expected, no modulation of the expression of Actin was observed between control colonies and corals subject to hypersaline stress, and also between the different sampling times (Fig. 3.3A).

The densitometric analysis (Fig. 3.3B) confirmed the transient expression of Hsp60. A significative increase of the level after 6 h (ANOVA, Tukey's HSD post hoc tests for pair-wise comparision of means; $p < 0.000$ compared to Time 0) was detected, followed by a strong down-regulation of Hsp60 after 12 h of hypersaline stress when the level fell becoming even less than half compared to those found in the control samples ($p < 0.000$ compared to Time 6 and Time 0). From 12 h until the end of exposure (48 h) no detectable and significant changes in the modulation of Hsp60 were observed.

About the morphological analysis of the coral colonies, after 48 h of hypersaline treatment the colonies showed similar morphological features of control colonies. Therefore, they displayed not fully extended polyps but normal tissue coloration and complete absence of necrosis as revealed by the photos of the colonies and by the microscopic analysis of the tissues (Tab. 3.1 and Fig. 3.3C).

3.3.2.2 Hyposalinity condition (27 ppt)

The initial response (after 6 h) of *S. caliendrum* fragments subjected to a moderate hyposalinity condition of 27 ppt was an important rise in Hsp60 amount, as happened for the hypersalinity condition (Fig. 3.4A). Subsequently, the signal slightly lowered and after 24 h returned comparable to the those relative to 6 h of stress. The signal remained constant up to the end of experiment. Also for this salinity condition no modulation of the expression of Actin was observed (Fig. 3.4A).

The densitometric analysis revealed a significance up-regulation of Hsp60 after 6 h ($p < 0.000$ compared to Time 0), (Fig. 3.4B). The expression reverted to pre-stress level after 12 h ($p > 0.000$ compared to Time 0; $p < 0.000$ compared to Time 6). From 12 to 24 h a slight up-regulation of Hsp60 was observed and the expression remained high over time until the end of the exposure and comparable to that found after 6 h of low salinity stress ($p > 0.000$ compared to Time 6).

After 48 h of exposure to salinity of 27 ppt, colonies of *S. caliendrum* displayed retracted polyps and a pale tissue coloration, indicating a beginning of bleaching. However, no evident sign of tissue degradation was observed by microscopic analysis (Tab. 3.1 and Fig 3.4C).

3.3.2.3 Extreme hyposalinity condition (15 ppt)

The Western blot analysis showed a severe increase in Hsp60 signal intensity in coral fragments subjected to an extreme hyposalinity condition of 15 ppt after 6 h. However, exceeded the 12 h of exposure, a strong and progressive reduction in Hsp60 expression was observed over the time. At the end of the exposure the signal was almost disappeared (Fig. 3.5A). No detectable and

significant changes in the Actin level was detected during the experiment (Fig. 3.5A).

After 6 h of hyposaline stress the Hsp60 level was almost twice compared to the control samples Time 0 ($p < 0.000$) as showed by the densitometric analysis (Fig. 3.5B). After 12 h it returned similar to that found in the control ($p > 0.000$ compared to Time 0; $p < 0.000$ compared to Time 6). This represented only the first step of a gradual and progressive down-regulation of the Hsp60 expression. After 24 h the Hsp60 level was significantly lowered even below the basal level ($p < 0.000$ compared to Time 0 and Time 6) and finally, at the end of exposure (48 h), the Hsp60 level approached values near zero.

The morphological analysis of the *S. caliendrum* colonies after 48 h in extreme hyposaline ambient showed clear signs of stress: full retraction of polyps, copious production of mucus and bleaching of the coral fragments which appeared unhealthy (Tab. 3.1). Moreover, the microscopic analysis revealed that the coral bleaching of the colonies was due to the appearance of the first events of tissue necrosis and degradation (Fig. 3.5C).

3.4 Discussion and conclusion

Osmotic stress, resulting from significant changes in salinity, are known to cause a considerable metabolic drain on marine organisms, having the potential to dramatically influence the biological functions of the cell (Lang et al. 1998, Mayfield & Gates 2007).

Nevertheless, little attention has been given to understanding how osmotic shifts under hyper and hypo-osmotic conditions can affect the host physiology. However, since sessile marine invertebrates lack any developed physiological regulatory systems (Brown 1997), they are expected to possess

well-developed cellular adaptation abilities.

Protein chaperoning is the cellular status of protein synthesis, protein maturation, and protein degradation and changes in any of these processes are indicative of a significant change in cellular metabolism and homeostasis (Downs 2005).

In particular, Hsp60 is the major mitochondrial chaperonin that is typically held responsible for the transportation and refolding of proteins from the cytoplasm into the mitochondrial matrix (Hood et al. 2003). Hsp60 in coral may have enhanced protein folding ability to cater for changes in environmental conditions (Chow et al. 2009). Elevation of this protein signifies a general shift in the protein chaperoning and degradation within the mitochondria and implies a change in the balance of mitochondrial-associated metabolic pathways (Papp et al. 2003).

Only recently, the expression of Hsps in scleractinian coral subjected to salinity stress has been investigated, however focusing only on hyposalinity condition (Downs et al. 2009).

In this work the different modulation of the Hsp60 expression in the coral *S. caliendrum* subjected to both hypo- and hypersalinity stress was studied, increasing knowledge on the kinetics and persistence of the stress response. The study was also accompanied by the assessment of the health condition through the morphological analysis of the coral tissues at the end of the treatments. Our results show that exposure of *S. caliendrum* to salinity stress induced significant and different physiological and morphological changes, both in the hypersalinity and in the hyposalinity condition. Moreover, the changes in salinity were found to influence the Hsp60 expression differently based on salinity values and exposure length, confirming that Hsp60 is involved in the mechanisms of cellular response to stress caused by exposure to adverse salinity.

As shown in Figure 3.6, the Western blot analysis showed for each salinity a similar modulation of Hsp60 after the first 6 h of stress. In fact, the presence of a basal constitutive level of Hsp60 in all control samples Time 0, followed by a significant up-regulation already after 6 h of exposure were detected, since that for all the colonies subjected to any salinity the trend of expression of Hsp60 was almost equal. Induction of Hsp60 response has proceeded rapidly confirming that, among the chaperones, the first proteins to respond to the change in salinity were Hsp60 (Downs et al. 2009).

With regard to the level of Hsp60 present in the control samples, this indicate that this chaperonine also has an important function under normal physiological conditions of the organism, in agreement with data reported in literature (Choresh et al. 2001, Chow et al. 2012, Seveso et al. 2012).

From 6 hours to the end of the exposure, Hsp60 exhibited for each salinity treatment specific patterns of expression, as evidenced by the gradual separation and spacing of the Hsp60 levels for each salinity with increasing the exposure time (Fig. 3.6). In hypersalinity condition (45 ppt) a negative trend of Hsp60 expression was observed after the 6 h of exposure. The Hsp60 level fell below that found in control. This might suggest a progressive death of coral, but at the end of the exposure the colonies showed a morphological appearance similar to healthy control colonies, with no bleaching and necrosis events and polyps not fully retracted. Polyp retraction constitute an immediate reaction of hermatypic corals to salinity stress, as decreases the amount of tissue surface area in contact with the surrounding environment, to limit water and gas exchange with the external medium (Muthiga & Szmant 1987). For these reasons the down-regulation of the expression of Hsp60 could be interpreted as a possible metabolic acclimation to the stress of the treatment.

However, the impact of salinity stress appears to be highly species-specific.

Patterns of high tolerance extreme salinity were recorded for *Siderastrea siderea* and *Porites furcata*, which were able to withstand sudden and extended changes in salinity without lethal impacts (Muthiga & Szmant 1987; Manzello & Lirman 2003). *Montastraea annularis* was able to sustain autotrophy and showed no tissue mortality after exposure to 40 ppt for 36 h (Porter et al. 1999), and *Porites* spp showed mortality only after 5 day of exposure to 45 ppt (Marcus & Thouraug 1981).

In *S. caliendrum* exposed to moderate hyposalinity (27 ppt), Hsp60 exhibited marked oscillation, but the level of Hsp60 generally remained high over time and comparable to that found after 6 hour. This trend may represent transient acclimatization patterns, except that the morphological analysis revealed the presence of unhealthy colonies with pale tissues and polyps retracted. Polyp retraction was also observed for many other species of coral subject to hyposalinity stress (Muthiga & Szmant 1987, Lirman 2001, Manzello & Lirman 2003). Also changes in coloration (i.e., paling) are commonly reported as a signal of stress for corals subjected to low salinity events and the progression from polyp retraction to paling to bleaching has been previously reported (Coles & Jokiel 1992, Stafford-Smith 1993, Wessling et al. 1999). Despite this, there is no consensus on the mechanism associated with reduced salinity bleaching. Certain studies report impairment of algae (Ferrier-Pages et al. 1999, Alutoin et al. 2001; Kerswell & Jones 2003), while others have highlighted that the animal host is primarily damaged by reductions in salinity, resulting in morphological changes, tissue swelling and necrosis (Van Woesik et al. 1995, Hoegh-Guldberg 1999, Downs et al. 2009). In this study, at the end of the hyposalinity treatment, the significant accumulation of Hsp60 could indicate a serious alteration of metabolic structures and processes in the animal host, confirming that cellular damages were in progress. Probably if the stress was prolonged, this could mean a

progressive depression of the Hsp60 expression.

Finally, in extreme hyposalinity condition (15 ppt), a considerable gradual down-regulation of Hsp60 was detected after the 6 h. The time course of this heat shock response appeared to be short and time-limited as the Hsp60 signal was almost completely disappeared at the end of the exposure. Hypotonicity appeared to inhibit the expression of Hsp60 over the time. For example, hyposalinity has been shown to block induction of the heat-shock protein response in hepatocytes, thus reducing their ability to deal with stress (Kurz et al. 1998). We speculate that probably the Hsp60 was unable to counteract the strong cellular stress for a long time exposure, bypassing the defense mechanisms as occurred in other studies (Hayes & King 1995; Carpenter et al. 2010). Furthermore, Hsp60 synthesis could be depressed due to energy constraints. The synthesis of Hsps represent one of the most ATP-costly processes occurring in cells (Hochachka & Somero 2002). Extreme conditions severe enough to cause bleaching effects, such as those demonstrated by this study, may result in increased damage and/or death of the organism, due to energy expenditure to neutralize or dissipate the effects of stress and restore cellular or tissue damage (Gates & Edmunds 1999, Morgan & Snell 2002).

The down-regulation of Hsp60 was accompanied by extreme degradation and necrosis of coral tissues leaving the bare, white coral skeleton. Necrosis and sloughing of the tissues from the skeleton was observed in other scleractinian corals and and anemones exposed to reduced salinity (Engebretson & Martin 1994, Van Woesik et al. 1995, Kerswell & Jones 2003). Nevertheless, *S. caliendrum* colonies were not death as demonstrated by the expression of actin in coral host tissue. Coles & Jokiel (1992) state that salinities around 15 sustained for more than 2 days will lead to coral mortality.

The results suggest that the negative impacts on Hsp60 modulation were more severe under low salinity (15 ppt) than under high salinity (45 ppt). Hypotonic conditions damage a number of different organelles such as mitochondria, which are among of the most vulnerable cell structures to osmotic damage (Jahnke & White 2003). Changes in osmolarity can disrupt mitochondrial electron transport and alter NADH redox capacity, producing an increase in reactive oxygen species (Martinez et al. 1995). Mitochondrial enzymes that play key roles in amino acid metabolism in invertebrates are depressed in response to hypo-saline conditions (Devin et al. 1997).

In conclusion, with this study we have demonstrated that *S. caliendrum* responds differently to hyper- and hyposaline conditions at morphological and cellular levels and the response of corals to osmotic stress reflects the severity and duration of the disturbance. We propose Hsp60 expression might be a useful biomarker of coral health prior to bleaching, as changes at the cellular and biochemical levels are usually the first detectable response to environmental stress and can be observed before the morphological signs of stress are evident. So, the integration of morphological and cellular aspects could allow to understand the physiological tolerance of corals exposed to environmental stresses, such as salinity stress, and their ability to withstand these stresses in an ecosystem increasingly affected by global changes.

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3.6 Tables and Figures

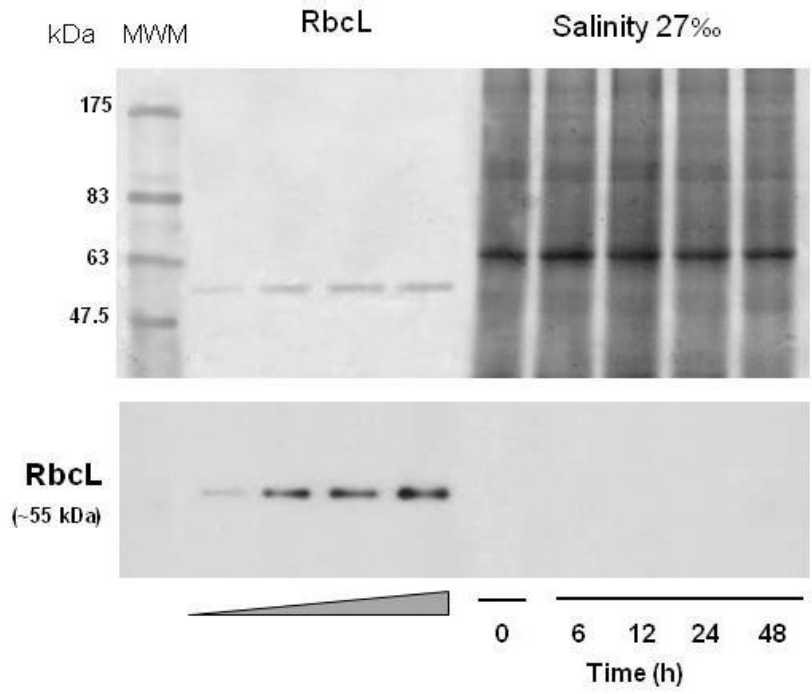

Fig. 3.1 – Absence of *Symbiodinium* contamination in protein extracts of *S. caliendrum* coral fragments **Panel Above.** Different increasing amounts of Rubisco protein standard (RbcL) and the collected fractions obtained from coral fragments subjected to 6,12, 24 and 48 h of salinity stress were loaded on a 8% polyacrylamide gel. Samples prepared from healthy control colonies (Time 0) were also analyzed. Pre-stained protein markers (MWM) are also shown. The gel which was subsequently electroblotted onto nitrocellulose membrane and stained with Ponceau S Red. **Panel Below.** The purity of the collected fractions was further assessed by Western blot analyses with anti-Rubisco (55 kDa) confirming that the isolated protein fractions was devoid of marker proteins of the *Symbiodinium* (Rubisco) and included only coral polyps proteins. Representative filter and Western blot is shown. The same results were obtained for each salinity condition

Tab. 3.1 - Analysis of the polyp appearance, colony coloration and tissue necrosis in *S. caliendrum* fragments before the stress exposure (35 ppt) and after 48 h of hypersaline (45 ppt), hyposaline (27 ppt) and extreme hyposaline (15 ppt) treatment

	Control	**Stress treatments**		
Coral health	**35 ppt**	**45 ppt**	**27 ppt**	**15 ppt**
Polyp appearence	Extended	Not fully extended	Retracted	Retracted
Colony coloration	Normal	Normal	Pale	Bleached
Tissue necrosis	No	No	No	Yes

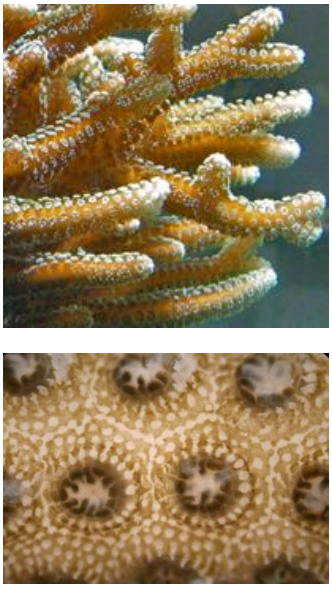

Fig. 3.2 - Representative photographic documentation of the morphological characteristics of corals before salinity stresses (control, Time 0). In the panel below, a photo of the coral tissue under the stereomicroscope is reported (see also Tab 3.1).

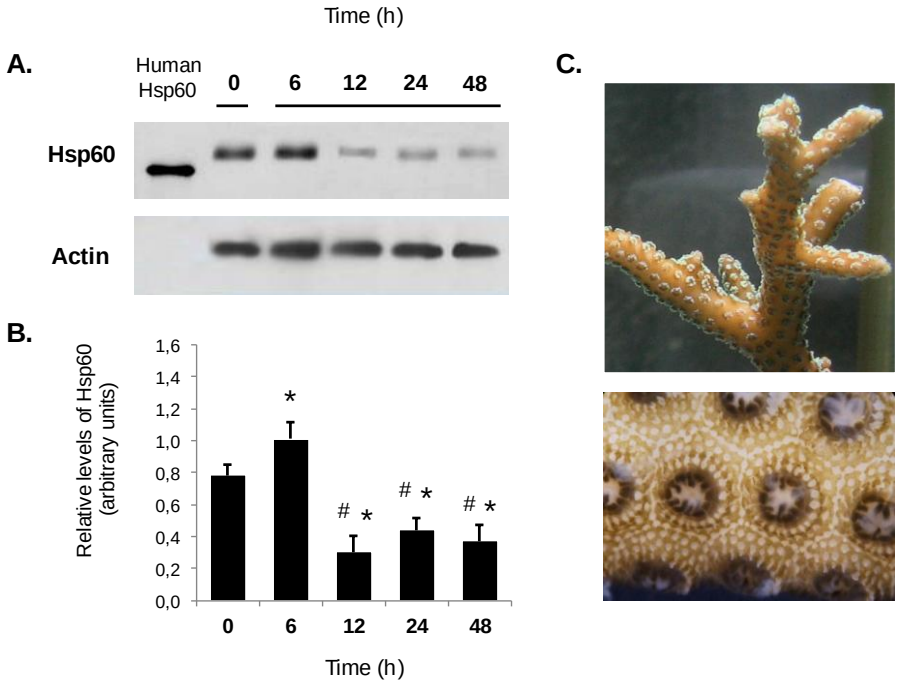

Fig. 3.3 - A. Effect of hypersaline treatment (45 ppt) on induction of Hsp60 and Actin in *Seriatopora caliendrum* colonies. Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 and Actin was observed after 6, 12, 24 and 48 h of exposure. Samples prepared from healthy control colonies (Time 0) were also analyzed. For each blot, the same amount of recombinant human Hsp60 was included. Western blot representative of 7 experimental repeats is shown **B.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for 7 different blots were analyzed. Data are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.000$ compared to 0, # $p < 0.000$ compared to 6). **C.** Representative photographic documentation of the morphological characteristics of corals after 48 h of hypersalinity stress. In the panel below, a photo of the coral tissue under the stereomicroscope is reported (see also Tab 3.1)

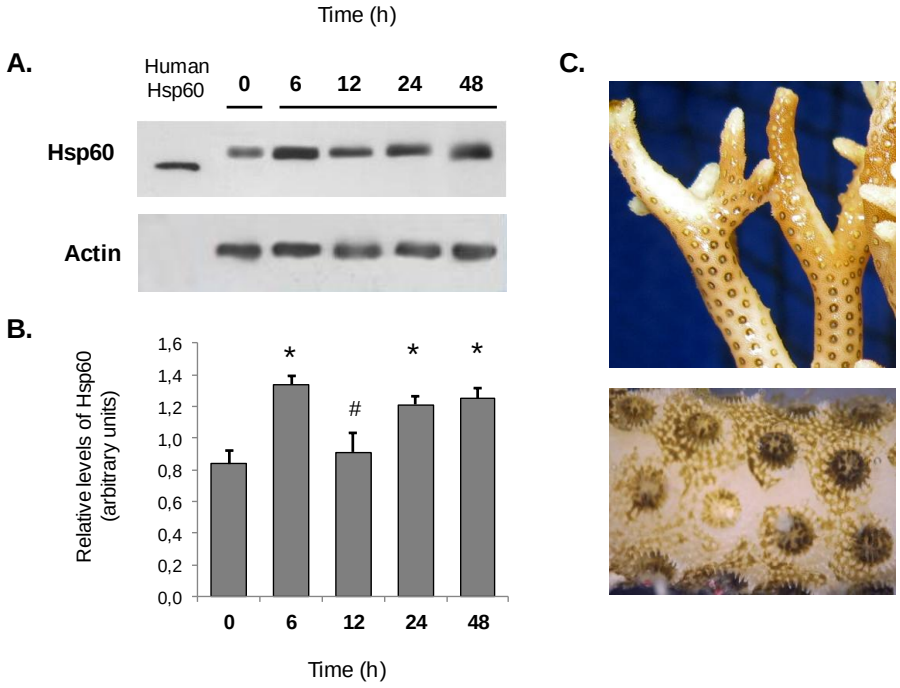

Fig. 3.4 - A. Effect of hyposaline treatment (27 ppt) on induction of Hsp60 and Actin in *Seriatopora caliendrum* colonies. Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 and Actin was observed after 6, 12, 24 and 48 h of exposure. Samples prepared from healthy control colonies (Time 0) were also analyzed. For each blot, the same amount of recombinant human Hsp60 was included. Western blot representative of 7 experimental repeats is shown **B.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for 7 different blots were analyzed. Data are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.000$ compared to 0, # $p < 0.000$ compared to 6). **C.** Representative photographic documentation of the morphological characteristics of corals after 48 h of hyposalinity stress. In the panel below, a photo of the coral tissue under the stereomicroscope is reported (see also Tab 3.1)

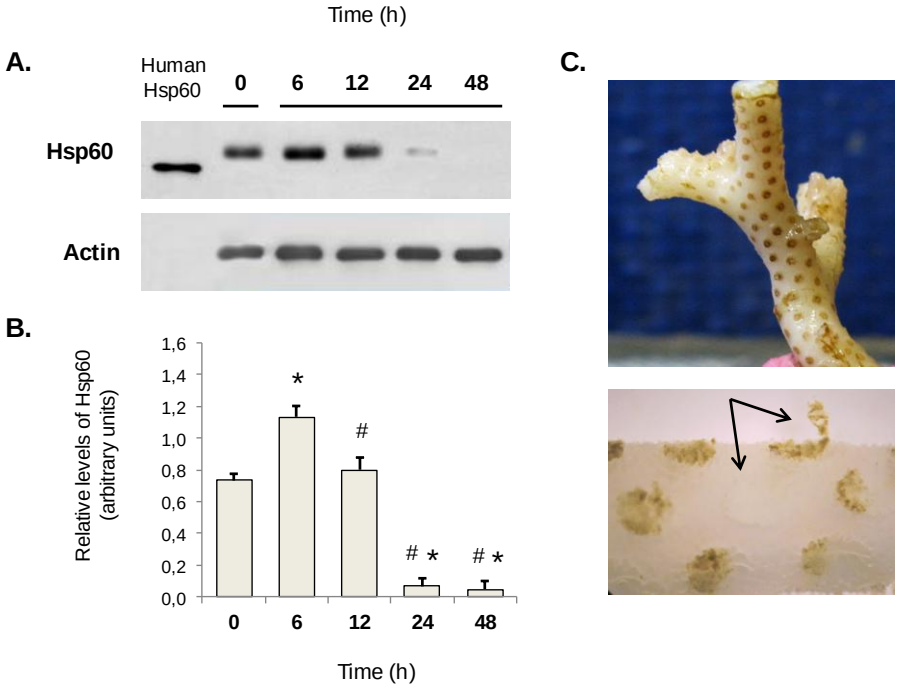

Fig. 3.5 - A. Effect of extreme hyposaline treatment (15 ppt) on induction of Hsp60 and Actin in *Seriatopora caliendrum* colonies. Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 and Actin was observed after 6, 12, 24 and 48 h of exposure. Samples prepared from healthy control colonies (Time 0) were also analyzed. For each blot, the same amount of recombinant human Hsp60 was included. Western blot representative of 7 experimental repeats is shown **B.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for 7 different blots were analyzed. Data are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.000$ compared to 0, # $p < 0.000$ compared to 6). **C.** Representative photographic documentation of the morphological characteristics of corals after 48 h of extreme hyposalinity stress. In the panel below, a photo of the coral tissue under the stereomicroscope is reported. (see also Tab 3.1). The arrows indicate the beginning of events of tissue necrosis

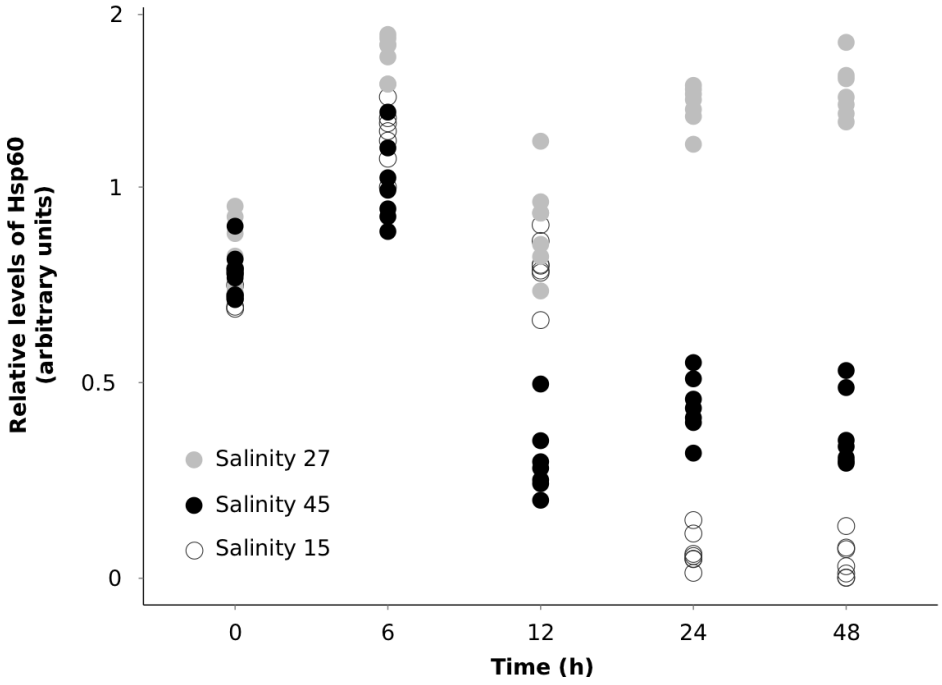

Fig. 3.6 – The graph shows the general trend of the expression of Hsp60 at different exposure times, for each condition of salinity (27, 45 and 15 ppt). The level of Hsp60 found in each of the seven replicates per salinity treatment is displayed

– *Chapter 4* –

The expression of Hsp60 as tool for detect the susceptibility of scleractinian corals to thermal stresses

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4.1 Introduction

The susceptibility of corals to temperature stress causing coral bleaching has taken on particular significance in the context of global climate change and global warming. Coral bleaching events have become more frequent and widespread through the last 20 years, largely due to elevated sea surface temperatures (Hughes et al. 2003). 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), 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. As a result of these increases, bleaching events are set to amplify in frequency and intensity (Hoegh-Guldberg 1999, IPCC 2007).

Climate change, however, may also lead to local decreases in temperature or to greater variability in seasonal conditions (Hoegh-Guldberg & Fine 2004). While average temperatures are warming in tropical areas due to greenhouse warming (IPCC 2000), the influence of climate change on the overall variability of weather systems like the El Niño Southern Oscillation suggest that there may be periods in which colder than normal temperatures may be

experienced (e.g. during La Niña periods), (Saxby et al. 2003). Cold water bleaching is therefore an additional component to be considered for coral reefs and some bleaching episodes has also been correlated with decreases in sea surface temperatures (Coles & Jokiel 1978, Gates et al. 1992, Hoegh-Guldberg & Fine 2004).

However, in many coral bleaching reports, there is noticeable variation, both intra- and inter-species, in the level of susceptibility and extent of bleaching on the reef (Brown & Suharsono 1990, Marshall & Baird 2000, Loya et al. 2001). The bleaching response is highly species/genus specific (McClanahan 2004, McClanahan et al. 2007, Montano et al. 2010) and differences among species in their susceptibility represent a critical aspect of community dynamics and species diversity (Hughes & Connell 1999). In particular among the scleractinian corals, acroporids and pocilloporids appear to be taxa most sensitive to bleaching stresses, since these faster growing species typically suffered high mortality during bleaching events (Glynn 1990, Gleason 1993, Marshall & Baird 2000, Edwards et al. 2001, Loya et al. 2001, McClanahan 2004).

For these reasons in this study we examined and compared the responses and tolerance to heat stress in three different coral genera belonging to acroporids (genera *Montipora* and *Acropora*) and to pocilloporids (genus *Seriatopora*), to investigate and understand which of them showed an higher susceptibility to sudden and extreme temperature increases. As a biomarker of thermal resistance, the expression and modulation of Heat shock proteins (Hsps) was analyzed, and the gross morphological changes of the coral colonies subjected to such heat shock were also observed. Hsps play important roles in cellular repair and protective mechanisms (Lanneau et al. 2008). Synthesis of these proteins during stress confers increased cellular tolerance to adverse conditions (Branton et al. 1999) and representing a

commonly used biomarker in coral health assessment (Sharp et al. 1997, Downs et al. 2000). In particular, the 60-kDa Hsp (Hsp60) is known to play a significant role, as molecular chaperone, in resistance to adverse temperatures (Cheng et al. 1989, Choresh et al. 2001, Chow et al. 2009, 2012). Moreover, we decided to deeply investigate the effect of the thermal stresses on *Seriatopora* which appeared to be the most susceptible genus. To do this *Seriatopora caliendrum* was chosen and the responses of this coral to a cold shock was also analyzed.

4.2 Materials and methods

4.2.1 Experimental setup

All the experiments were performed at the Civic Aquarium of Milan, Italy, using a flow-through aquaria system consisting of four tanks (120 l, 80 x 30 x 50 cm each one) connected to a 330-l sump containing gravel-bed filter, protein skimmer and a 500 W Titanium Heater (Aqua Medic) connected to temperature controller. In all the tanks, the light was provided by 400 W metal halide lamps (Powerstar HQI-T, Osram), which were turned on at 9 a.m. for the day cycle and turned off at 9 p.m. (12:12 light:dark cycle).

4.2.2 Experiment I: preliminary test of susceptibility to heat stress

For the preliminary test, colonies of the genera *Montipora, Acropora* and *Seriatopora* were acclimatized under controlled laboratory conditions for 15 days before any experimental manipulations. Normal water temperature was set at 27°C (±0.3°C). Calcium, magnesium, iodium and alkalinity were assessed and reintegrated every three days to ensure stable water chemistry.

Salinity (35 ppt) and pH (8.2) of the seawater in the aquarium tanks were kept stable and constantly monitored using a Hach Lange HQ 30D flexi portable meter.

After the acclimation period, all the colonies belonging to all 3 genera were divided into small fragments of similar size and placed in one of the three treatment tanks. The coral nubbins were left to recover from the handling procedures for 15 days before the stress exposure. All the chemical and physical parameters of the seawater were kept stable and constantly monitored. Subsequently, coral fragments of *Montipora, Acropora* and *Seriatopora* were photographed for the morphological analysis and after they were sampled. They represented the control samples (Time 0). They were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Only healthy coral nubbins exhibiting healing at fragmentation lesions, growth, characteristic color, dark pigmentation and extended polyps were used for exposure experiments. Fragments of *Montipora, Acropora* and *Seriatopora* (n = 6 for each genus) were subjected to a severe and extreme heat shock of 36°C that, nevertheless, represents a temperature value realistically registered in a reef. Exposures were carried out for 12 h and sampling was performed every 2 hours. All the samples were frozen in liquid nitrogen and were stored at −80 °C until protein extraction. After 6 h and 12 h of treatment a photographic documentation of coral colonies was performed in order to monitor the coral health as tissue coloration and morphological aspects. Elevated temperature was achieved using submersible Acqua Medic Titanium Aquarium Heaters (model TH-100, 240 V) connected to a temperature controller (Eliwell, PC 902T). Salinities and pH were checked at regular intervals. During stress exposures the aquarium tank was a closed system (static flow) but contained Koralia immersed pumps (1200 l/h) in order to continuously mix and aerate the seawater.

4.2.3 Experiment II: thermal stress on *Seriatopora caliendrum*

The colonies of *S. caliendrum* were acclimatized for 20 days before any experimental manipulations at the same parameters used for the preliminary test (control water temperature: 27°C). All the parameter were constantly controlled as previously reported.

The colonies were divided into small fragments of similar size and randomly distributed into the three exposure tanks. Nubbins were left to recover from the handling procedures for 10 days before the salinity stress exposure. The chemical and physical parameters of the water were constantly monitored. To minimize the variations due to light bulb diffusion and water movement, the position of nubbins within each tank was changed every four days.

After the acclimation period to temperature of 27°C (control), coral fragments from each of the three tanks were first morphologically analyzed and then sampled for protein analysis. They represented the control samples (Time 0). After sampling, coral fragments were immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Also in this case, only healthy coral fragments exhibiting healing at fragmentation lesions, growth, normal color, dark pigmentation and fully extended polyps were used for exposure experiments and were assigned randomly to each treatment.

S. caliendrum fragments (n = 7 per treatment) were subjected to three different temperature regimes which may realistically occur on a reef. In the first tank the coral fragments were exposed to a cold shock treatment of 21°C for 48 h, while in the second tank another group of nubbins were subjected to a moderate heat shock of 29°C for 48 h. The sampling was performed at 6, 12, 24 and 48 h. In the third tank a severe heat shock of 34°C for 24 h was reproduced and sampling was performed at 4, 8, 12 and 24 h. All the samples were quickly frozen in liquid nitrogen and were stored at

−80 °C until protein extraction. At the end of the treatments, to assessing the health status of the corals a morphological analysis was performed in order to monitor gross morphological changes. Coral health was assessed as tissue coloration and condition and polyp appearance (retracted or extended). Colony coloration was classified as normal (normal pigmentation throughout the colony), pale (lighter coloration in the coral theca and coenosteum), and bleaching (white coloration throughout the colony), (Lirman et al. 2002).
During stress exposures each aquarium tank was a closed system (static flow) but contained Koralia immersed pumps (1200 l/h) in order to continuously mix and aerate the seawater. The temperature was checked using a temperature controller (Eliwell, PC 902T) connected to submersible Acqua Medic Titanium Aquarium Heater (model TH-100, 240 V).

4.2.4 Protein extracts and Western analysis

For both experiments, in the laboratory 1 g of each frozen coral fragment was powdered using mortar and pestle. Proteins were extracted by homogenizing the tissue powder in 400 µl of SDS-buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 5% 2-mercaptoethanol) containing 1mM phenylmethylsulfonyl fluoride (Sigma) and Complete EDTA-free cocktail of protease inhibitors (Roche Diagnostic). Samples were boiled for 10 min, the skeleton fragments were removed by a single step of centrifugation (15 min at 12000 rpm, 4°C) and algal symbionts were separated from the coral tissue by another centrifugation (5 min at 4500 rpm, 4°C). Supernatants were clarified (5 min at 12000 rpm) and then frozen at -20°C until used. Aliquots of the supernatants were used for protein concentration determinations using the Bio-Rad protein assay kit (Bio-Rad Laboratories). Protein samples were separated by SDS-PAGE on 8%

polyacrylamide gels (Seveso et al., 2012). The same amount of proteins (80 µg) was loaded on each lane of the gel. 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 as previously described (Seveso et al. 2012). Correct protein transfer was confirmed by Ponceau S Red (Sigma-Aldrich) staining of filters. Anti-Hsp60 monoclonal antibody (IgG mouse clone LK-2, SPA-807, StressGen Bioreagents) at 1:1000 dilution was used as previously reported (Seveso et al. 2012). To evaluate the absence of contamination of *Symbiodinium* proteins in the *S. caliendrum* samples, filters were probed with anti-Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit (AS03037, Agrisera) at 1:2000 dilution in TBS-Tween 20, 5% skimmed milk, followed by incubation with secondary antibody (diluted 1:7500 in TBS-Tween 20, 5% skimmed milk) anti-rabbit IgG conjugated with horseradish peroxidase (Thermo Scientific). Different amounts of Rubisco protein standard (AS01017S, Agrisera) were loaded as control.

Binding was visualized with the Pierce ECL Western Blotting Substrate followed by X-rays films.

In addition, for the experiment II on *S. caliendrum*, the same amount of recombinant human Hsp60 (StressGen Bioreagents) was also included as standard control for each blot. Moreover, the filters were also probed with mouse anti-Actin monoclonal antibody (clone C4, MAB1501, Millipore) at 1:1000 dilution in TBS-Tween 20, 5% skimmed milk. Secondary antibody (diluited 1:10000 for Hsp60 and 1:7500 for Actin in TBS-Tween 20, 5% skimmed milk) anti-mouse IgG conjugated with horseradish peroxidase (Thermo Scientific) was used. Binding was visualized with the Pierce ECL Western Blotting Substrate followed by X-rays films.

4.2.5 Densitometric and statistical analyses

Only for the second experiment, afterwards ECL detection, the films were scanned on a Bio-Rad GS-800 calibrated imaging densitometer and quantified with the Image J free software (http://rsb.info.nih.gov/ij/) of 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 standard Hsp60 protein band. Data were expressed as the mean ± standard error of the means (SEM). One-way analysis of variance (ANOVA) followed by Tukey's HSD post hoc tests for pair-wise comparision of means was performed for all the normalized Hsp60 intensity values obtained from the different groups of samples, to assess significant differences ($p < 0.000$) in Hsp60 protein levels in response to the different exposure times for each temperature regime.

4.3 Results and discussion

4.3.1 Different susceptibility of coral genera to heat stress

Before the exposure of the coral fragments of *Montipora*, *Acropora* and *Seriatopora* to heat shock, the absence of *Symbiodinium* contamination in the samples was verified, using the antibody anti-Rubisco, specific to photosynthetic organisms. Western blot analysis confirmed that isolated protein fractions was devoid of marker proteins of the *Symbiodinium* (Rubisco) and included only coral polyps proteins (data not shown).

To test and compare the susceptibility to extreme heat shock, colonies of *Montipora*, *Acropora* and *Seriatopora* were subjected to a temperature of 36°C for 12 hours. The analysis of Hsps for the evaluation of the sensitivity

of coral species to thermal stresses has been widely studied in the literature (Black et al. 1995, Hayes & King 1995, Chow et al. 2009, Fitt et al. 2009).

As regards the morphological appearance of the corals, all the colonies belonging to the three genera suffered intense bleaching episodes (Fig. 4.1). Already after 6 h of heat shock the colonies showed pale tissues which became completely bleached at the end of the exposure, confirming that acroporids and pocilloporids possess an high/severe susceptibilities to bleaching (Marshall & Baird 2000). In literature, variation among coral genera has been associated with coral growth and metabolic rates (Gates & Edmunds 1999). Corals with high growth rates and low metabolic rates (branching species) would have a lower capacity to acclimatize than corals with low growth and high metabolic rate (massive species). Moreover, the taxa most susceptible to bleaching tend to be those corals that are quick to colonize free space and often short-lived and those corals that have low tissue retractability and thickness and so high degree of tissue exposure to water column stressor (Hueerkamp et al. 2001, Loya et al. 2001). Our results regarding the appearance of the tissues showed no differences among coral genera in resistance and tolerance to stress.

Nevertheless, the Western analysis revealed that the coral genera displayed a different modulation of Hsp60 (Fig. 4.2). The monoclonal antibody anti-Hsp60 recognized a single specific 62-kDa band both in all stressed coral samples and in all control samples (Time 0). Sampling every 2 h during the stress experiment, it was possible to investigate the kinetic and the short-term fluctuations of the Hsp60 response to extremely high temperature. So, a transient induction of Hsp60 was detected in *Montipora*, *Acropora* and *Seriatopora* after temperature elevation from 27°C to 36°C, with an up-regulation of Hsp60 already after the first 2 h of stress followed by a further increase of Hsp60 level after 4 h of exposure. This confirms that Hsps are

primarily involved in the mechanisms of cellular response to stress caused by exposure to adverse temperature (Choresh et al. 2001) and their expression is rapidly modulated. After the 4 h of thermal stress each coral genus showed a different pattern of Hsp60 expression.

Montipora displayed after 6 h a progressive decrease of the level which, after 10 h of severe heat stress, appeared similar to that found in control (Fig. 4.2A). At the end of the experiment the colonies showed a slight level of Hsp60. However, *Montipora* displayed the most prolonged expression of Hsp60 in time (until the end of exposure) that might suggest a greater defensive capability to thermal stress.

The genus *Acropora* displayed after 6 h a further increase of Hsp60 level, but after 8 h of heat shock a severe down-regulation of the expression was observed (Fig. 4.2B). After 10 h of exposure the Hsp60 level appeared very weak and significantly lower than the control. This signal remained similar until the end of the treatment.

Finally, the genus *Seriatopora* showed a rapid and strong down-regulation of the expression of the Hsp60 at the sixth hour of stress (Fig. 4.2C). The Hsp60 signal was completely disappeared after 10 h of heat shock at 36°C, when probably the Hsp60 was unable to counteract the strong cellular stress, bypassing the defense mechanisms.

Hsps represent a temporally dynamic and responsive cellular machinery to combat the impacts of stress (van Oppen & Gates 2006). The heat shock response to heat stress appeared to be time-limited, with a gradual decrease with increasing the time of exposure. Thus, despite the Hsp60 modulation appeared similar among the different coral genera subjected to the same thermal shock, each genus displayed a different persistence of the Hsp60 signal. This could indicate a different threshold of tolerance and resistance and so a different resilience capability. In this context the Western analysis

showed that at the cellular level *Seriatopora* could probably be the most susceptible genus to heat stress, as its defense mechanism represented by the Hsp60 was immediately suppressed. Instead *Montipora* could be the genus most resistant and *Acropora* displayed intermediate features. For this reasons we decided to better analyze the *Seriatopora* genus, focusing on *S. caliendrum* which, as is well known from literature, exhibits low ability to survive stressful environmental conditions (Muscatine et al. 1991, Loya et al. 2001, Bhagooli & Yakovleva 2004).

4.3.2 Response of *S. caliendrum* to thermal stresses

The control colonies of *S. caliendrum* showed an healthy appearance before the treatments, with normal tissue coloration and extended polyps (Tab. 4.1). Also in this situation, Before the exposure of the coral fragments to thermal stresses, the absence of *Symbiodinium* contamination in the samples was verified, using the antibody anti-Rubisco, specific to photosynthetic organisms (data not shown).

S. caliendrum was exposed to 3 different thermal treatments (21, 29 and 34°C) analyzing the modulation of Hsp60 and the morphological changes. Moreover, the host Actin with a molecular weight of ~43 kDa were selected as additional host tissue-specific marker proteins being highly-expressed in the host cells (Peng et al. 2010). Exposures to elevated and depressed temperature were conducted in parallel in order to provide a consistent framework for the comparison of the effects of temperature on the physiological state of the coral.

4.3.2.1 Cold shock

There is little knowledge or understanding of how the colder excursions in sea temperature will affect marine organisms especially at cellular level. The work proposed by Kingsley et al. (2003) analyzed the expression of Hsp60 after cold shock, but on gorgonian *Leptogorgia virgulata.*

At low temperature of 21°C, Western blot analysis showed the presence of a basal constitutive level of Hsp60 in the control samples Time 0, followed by a severe up-regulation already after 6 h (Fig. 4.3A). After the twelfth hour of exposure the Hsp60 signal slightly decreased and remained similar up to 24 h of stress. At the end of the exposure the Hsp60 level appeared very low and the signal appeared almost disappeared. As expected, no modulation of the expression of Actin was observed between control colonies and corals subject to cold stress and also between the different sampling times (Fig. 4.3A). The densitometric analysis confirmed the transient induction of Hsp60 with the significative up-regulation after 6 h (ANOVA, Tukey's HSD post hoc tests for pair-wise comparision of means; $p < 0.000$ compared to Time 0), as shown in Fig 4.3B. The Hsp60 level after 12 and 24 h decreased not significantly respect the sixth hour ($p > 0.000$ compared to Time 6). Finally, after 48 h of stress a severe down-regulation was detected probably due to the prolonged cold shock and the Hsp60 level became even less than half compared to those found in the control samples ($p < 0.000$ compared to Time 0 and Time 6).

The results of the present study clearly show that exposure to cold-water stress has a negative impact on the physiology of the coral *S. caliendrum*. This is also indicated by the morphological analysis which showed nubbins with retracted polyps and pale tissue coloration (Tab. 4.1). Thus, the modulation of the Hsp60 at lowered temperatures are similar to those

involved in elevated temperature stress. It can therefore be suggested that cold-water bleaching events are analogous to warm-water bleaching events in that severe coral bleaching preceding mortality only occurs once the thermal and temporal limits of a particular coral are exceeded. Bleaching has also been correlated with decreases in sea surface temperatures (Coles & Jokiel 1978, Gates et al. 1992). The passage of polar continental air masses have been shown to have rapid cooling effects on shallow water with chilling and mixing of water bodies augmented by associated strong winds (Roberts et al. 1982). Upwelling may also affect open ocean reefs, with temperatures dropping several degrees with the changing of tides. Previous observations have shown that lowered temperature limit the survival and development of several coral species, included *S. caliendrum* (Muscatine et al. 1991) with 18°C accepted as the lower temperature threshold.

A number of other organisms have been shown to have a similar cold shock response including other bacteria, slime molds, yeast, unicellular algae, and higher plants (Jones & Inouye 1994). Reduced temperature is known to increase membrane permeability and change the relative rates of reactions (via effects on the kinetic energy of enzymes and substrates), leading to metabolic disorder and decrease and greatly reduced rates of cell division and large reductions in coral growth (Hoegh-Guldberg & Salvat 1995, Roth et al. 2012).

4.3.2.2 Heat shock

As a consequence of a moderate heat shock of 29°C, the Western analysis exhibited a marked oscillatory pattern of Hsp60 with the level that initially increased and then decreased to return, at the end of treatment, comparable to that found in the control (Fig. 4.4A). Nevertheless, no modulation of Actin

was observed after the heat stress (Fig. 4.4A). The densitometric analysis confirmed the oscillatory pattern characterized by biphasic peaks (Fig. 4.4B). A significative up-regulation after 6 h of stress was observed ($p < 0.000$ compared to Time 0). The Hsp60 level decreased significantly after 12 h returning similar to Time 0 ($p > 0.000$ compared to Time 0; $p < 0.000$ compared to Time 6), but after 24 h of treatment a further down-regulation of the expression was detected ($p < 0.000$ compared to Time 0 and Time 6). Finally at 48 h a slight up-regulation of Hsp60 was observed and the level appeared significantly comparable to that found after 12 h of exposure and in control sample ($p > 0.000$ compared to Time 0). The nubbins showed morphological features similar to those of healthy colonies (normal tissue coloration, but retracted polyps), (Tab. 4.1). These results might suggest two different interpretations. First, as suggested by Chow et al. (2012) the Hsp60 exhibits oscillation when the light and temperature conditions remain normal and constant. This oscillatory pattern is altered when coral is exposed to a severe temperature elevation. Probably, in our experiment the slight increase of the temperature (from 27 to 29°C) did not disrupt and affect the oscillatory trend of Hsp60 as the thermal stress appeared minor and confirmed that Hsp60 protein levels may be related to an oscillatory mechanism (Chow et al. 2012). Second, considering that at the end of the treatment the level of Hsp60 is returned as in the control, it could mean an attempt of the coral to acclimatize to the new temperature.

During the severe heat shock of 34°C for 24 h the nubbins showed a transient induction of Hsp60 (Fig. 4.5A). The level of Hsp increased progressively up to 8 h of stress when the change in the level of Hsp60 became significant ($p < 0.000$ compared to Time 0) as confirmed by the densitometric analysis (Fig. 4.5B). At 12 h of exposure the up-regulation of Hsp60 reached the highest level. This significant accumulation of Hsp60

could indicate a serious alteration of metabolic structures and processes, confirming that cellular damages were in progress. A drastic decrease of Hsp60 was finally observed at 24 h, when probably the cellular damage become irreparable. Thus, the signal was almost disappeared and the level of Hsp60 was significantly lower than the control ($p < 0.000$ compared to Time 0). So, the time course of this strong heat shock response appeared to be short and time-limited. Nevertheless, also in this case no modulation of Actin was observed (Fig. 4.5A). At the end of the heat treatment the colonies of *S. caliendrum* displayed an unhealthy morphological condition characterized by completely bleached tissue and fully retracted polyps (Tab. 4.1), suggesting that *S. caliendrum* is not able to tolerate this elevated temperature for a long time exposure.

4.4 Conclusion

Both experiments conclude that both heat and cold conditions can be deleterious to corals, inducing stress through divergent physiological mechanisms over time. Furthermore, the different treatments triggered distinct responses of Hsp60 at different time scales.

Because of climate change, corals will experience more temperature anomalies that will not only cause physiological stress, but also have long-term repercussions on growth and fitness, ultimately affecting stability of coral reefs. So, the worldwide degradation of coral reefs due to global climate change requires the development of an early warning system for ambient assessment of the health of marine benthic communities.

4.5 References

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4.6 Tables and Figures

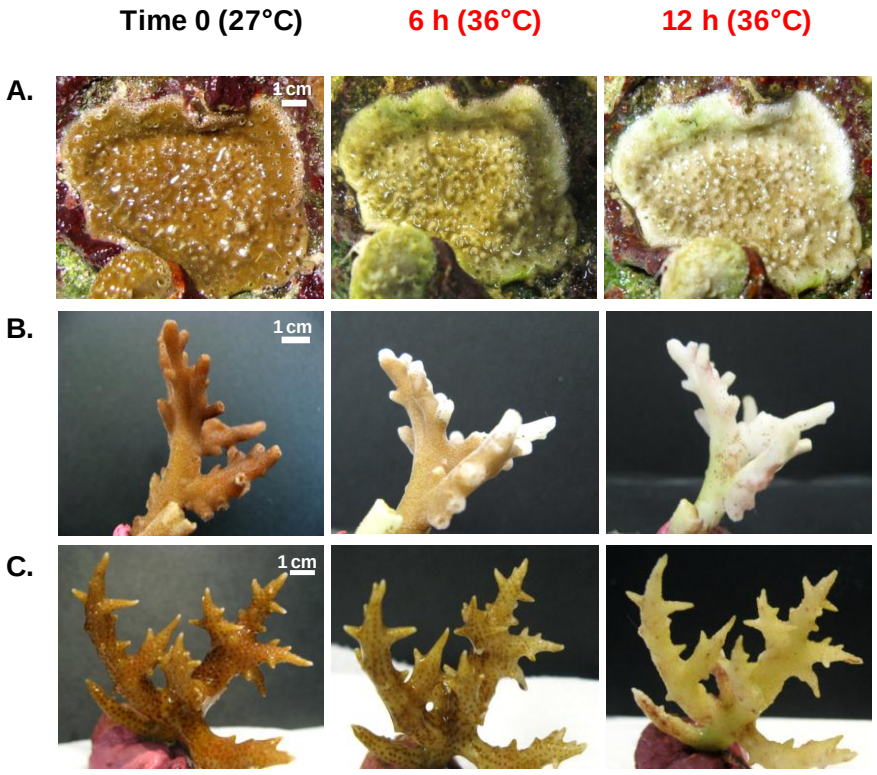

Fig. 4.1 - Colonies of the genera *Montipora* (A), *Acropora* (B) and *Seriatopora* (C) photographed before thermal stress at 27°C (control, Time 0), after 6 hours of heat stress at 36°C and at the end of the heat treatment (after 12 h). Bleaching episodes were visible. The transition from normal tissue pigmentation to paling and then bleaching was clearly visible for all the three genera

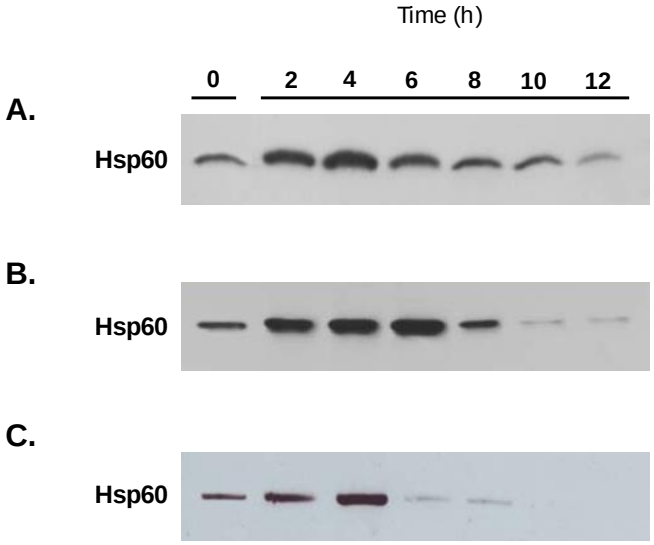

Fig. 4.2 – Effect of heat shock of 36°C on induction of Hsp60 in coral colonies of the genera *Montipora* (A), *Acropora* (B) and *Seriatopora* (C). Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 was observed every 2 hours until the end of the exposure (12 hours). Samples prepared from healthy control colonies (Time 0) were also analyzed. Western blots representative of six experimental repeats for each genus are shown.

Tab. 4.1 – Analysis of the polyp appearance and tissue coloration in *S. caliendrum* fragments before the stress exposure (27°C), after 48 h of cold shock (21°C) and moderate heat shock (29°C), and after 24 h of severe heat shock (34°C)

	Control	**Stress treatments**		
Coral health	**27°C**	**21°C**	**29°C**	**34°C**
Polyp appearance	Extended	Retracted	Retracted	Retracted
Colony coloration	Normal	Pale	Normal	Bleached

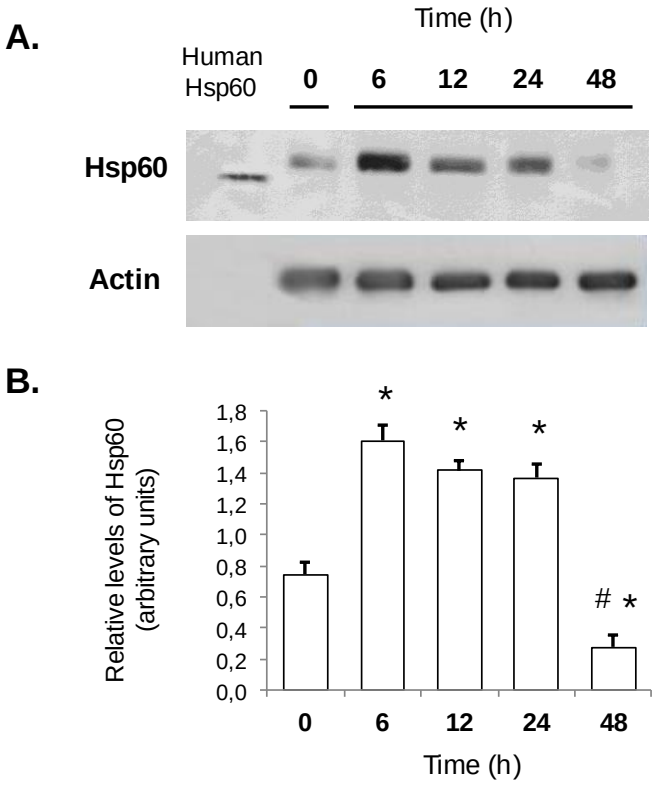

Fig. 4.3 – A. Effect of cold shock of 21°C on induction of Hsp60 and Actin in *Seriatopora caliendrum* colonies. Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 and Actin was observed after 6, 12, 24 and 48 hours of treatment. Samples prepared from healthy control colonies (Time 0) were also analyzed. For each blot, the same amount of recombinant human Hsp60 was included. Western blot representative of 7 experimental repeats is shown **B.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for 7 different blots were analyzed. Data are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.000$ compared to 0, # $p < 0.000$ compared to 6).

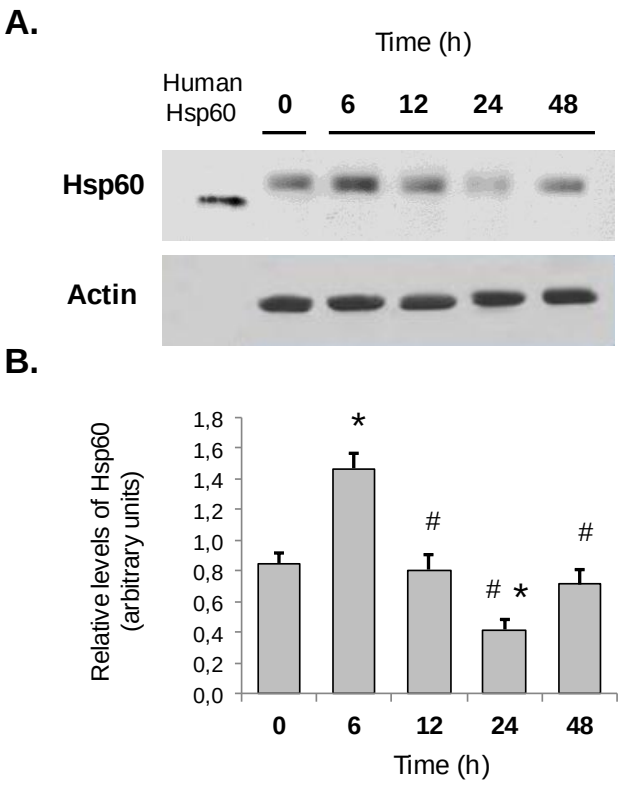

Fig. 4.4 – A. Effect of a moderate heat shock of 29°C on induction of Hsp60 and Actin in *S. caliendrum* colonies. Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 and Actin was observed after 6, 12, 24 and 48 hours of treatment. Samples prepared from healthy control colonies (Time 0) were also analyzed. For each blot, the same amount of recombinant human Hsp60 was included. Western blot representative of 7 experimental repeats is shown **B.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for 7 different blots were analyzed. Data are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.000$ compared to 0, # $p < 0.000$ compared to 6).

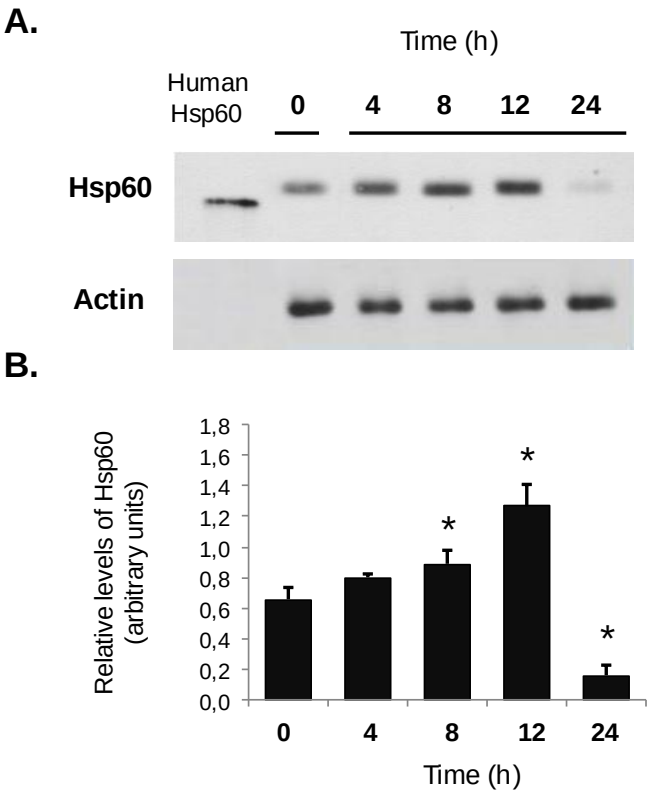

Fig. 4.5 – A. Effect of a severe heat shock of 34°C on induction of Hsp60 and Actin in *S. caliendrum* colonies. Equal amounts of total protein for each coral colony were loaded in each lane. The modulation of Hsp60 and Actin was observed after 4, 8, 12 and 24 hours of treatment. Samples prepared from healthy control colonies (Time 0) were also analyzed. For each blot, the same amount of recombinant human Hsp60 was included. Western blot representative of 7 experimental repeats is shown **B.** Hsp60 levels were determined by densitometric analysis as described under Methods. Signals for 7 different blots were analyzed. Data are expressed as arbitrary units and as mean ± SEM (one-way ANOVA followed by Tukey's HSD multiple pair-wise comparisons, * $p < 0.000$ compared to 0)

– *Chapter 5* –

General discussion and conclusion

The coral reefs not only possess an intrinsic value of natural beauty analogous to the tropical rainforests, but also provide an invaluable array of human and ecosystem services. Nevertheless, the current state and prognosis for corals require immediate and continued action. Understanding the mechanisms by which environmental variation impacts organisms and by which animal respond to their highly fluctuating environment is becoming increasingly important as the effects of climate change cascade through the biosphere, especially for organisms that occur in stressful habitats (Dahlhoff 2004). It is also intriguing to study whether responses to environmental stress might serve as a basis for the establishment of biomarkers.

All organisms react to stress, which causes damage to cellular proteins, by inducing the synthesis of Heat shock proteins. Since mitochondria are essential for the vitality of the cell, the mitochondrial Hsps play an important role in the function of the organism and the induction of their mitochondrial Hsps represent one of the first defense mechanism against stresses (Choresh et al. 2004). However, until now few research has been carried out about the mt-Hsps in cnidarians and especially in scleractinian corals. So, my PhD research was principally aimed at improving current knowledge about the heat shock response especially in the light of the coral bleaching.

In this study we proposed the application of the mitochondrial Hsp60 and the analysis of its modulation as an useful and accurate biomarker, to assess the effect of several types of stress in scleractinian corals, and to diagnose coral health prior that the coral bleaching occurred. Furthermore, in order to

develop a system based on Hsp60 expression it is necessary to investigate the relation between the specific Hsp60 modulation, the kinetic of the Hsp60 response, the type of stress and behavioral responses to stress by the corals (Choresh et al. 2001). For this reason, the analysis of heat-shock response has been accompanied by the morphological changes occurred to the organisms under stressful conditions, in order to establish a more complete outline of the coral health situation and better understand the pattern of expression of the protein and the meaning of a particular-specific modulation.

First of all, we demonstrated that Hsp60 is always expressed at low constitutive levels in different coral species, such as *Acropora muricata, Seriatopora caliendrum, Montipora sp.* and other species belonging to *Acropora* and *Seriatopora* genera, even when they are exposed to normal physiological conditions. The overall results support the importance of Hsps as an adaptive mechanism of cnidarians to stressful environmental conditions and evidence that Hsp60 play a significant role in the resistance and tolerance to perturbations generated by both physical factors (e.g. temperature and salinity) and biological interactions (e.g. diseases). In this thesis, I presented results from the first study of the response of Hsps to biotic stresses in scleractinian corals. With this research I proposed the Hsp60 expression as useful tool and promising biomarker for the holobiont of corals to evaluate physiological stress caused by biological stresses. It has been noted that different biological interactions trigger diverse responses on Hsp60 level. In particular, the fact that the Hsp60 appeared up-regulated in diseased colonies might suggest new evidences related to the immune response in corals. In fact, recent studies has indicated that Hsp60 in mammalian plays a role in a danger signal cascade immune response and in autoimmune disease (Hansen et al. 2003, Pockley 2003). The chaperonin

Hsp60 can be be released in the extracellular environment as intercellular signalling molecule. It can elicit cytokine production by, and adhesion molecule expression of, a range of cell types, and it can deliver maturation signals and peptides to antigen presenting cells through receptor-mediated interactions (Pockley 2003). Hsp60 could posses the capability of activating monocytes, macrophages and dendritic cells (Hansen et al. 2003). These functions suggest that Hsp60 could be immunoregulatory agents in mammalian. However, corals have an immune system based on self/non-self recognition and cellular and humoral processes (Mydlarz et al. 2010), so the present study could opens up a path for further investigation about the role of Hsp60 in the immune system of the scleractinian corals.

Furthermore, the fact that the Hsp60 modulation appeared different in the different portions of the same coral colony might suggest that coral polyps, although genetically identical and linked together by a common tissue named coenosarc or coenenchyme, act as indipendent individuals each of which shows its specific stress response.

The second part of my dissertation is focused on the responses of corals to abiotic stresses due to changes in temperature and salinity. In both cases, we have noticed two common characteristics as regards the expression of Hsp60:

1. a strong and significant up-regulation of Hsp60 was always detected shortly after the stress induction (after 6 hours). This confirm as Hsp60 in sessile cnidarian marine animals may exhibit extreme adaptability and great susceptibility to changing environmental conditions (Chow et al 2009). Not surprisingly, the Hsps are especially useful biomarkers because their induction is much more sensitive to stress than traditional indices (Feder & Hofmann, 1999).

2. After the initial up-regulation, the modulation of Hsp60 acquired specific

patterns based on the severity and duration of the stress. In particular, if the stress appeared extreme a progressive down-regulation of the expression was always detected to increasing the exposure time. Thus, the Hsps represent only a temporally, time-limited, cellular machinery to combat the impact of the stresses (van Oppen & Gates 2006). It is also hypothesized that there is a trade-off between the energetic demands of cell cycle progression and the synthesis of proteins that repair macromolecular damage, essentially leading to cell cycle arrest during acute stress. Severe stress can lead to apoptosis or controlled cell death (Tomanek 2011). For this reason the investigation of the kinetics of the Hsp60 proves very useful. It was also observed that the speed of the process of down-regulation may vary between different species subjected to the same stress, confirming that genetic and phenotypic differences among species or populations may lead to differences in Hsp expression among different organisms experiencing identical environmental conditions (Rank & Dahlhoff 2002). Analyze the persistence of the expression of Hsp in time could serve as a foundation for acclimatizing mechanisms that bestow a measure of resistance to environmental conditions that induce a bleaching event and could provide important information on the resilience capability of coral species. Furthermore, understand how some species survive and recover from stress, while others do not, may help to provide valuable information for the management and conservation of coral reefs.

Such data help to explain the consequences of stress on coral physiology and they are expected to disclose the significance of mt-Hsp60 expression for future local and global changes, which may have implications for organism abundance and distribution in various habitats. The results of the current research also lays the basis for further studies of the essential group of mitochondrial heat shock proteins in others marine invertebrates.

Some aspects of the stress response, however, present problems for the use of Hsps as biomarkers for environmental stress. Since that many stressors induce Hsps, investigators may be unable to attribute changes in Hsps modulation to any one particular stress. In fact, organisms in the field often undergo multiple stresses simultaneously, the interaction of which can yield significant Hsps expression even when no single monitored factor is at harmful levels. Only laboratory studies can support the difficulty of teasing apart environmental factors and attributing HSPs induction to a single stressor (Choresh et al. 2004, 2007).

The potential application of the present study lies in the use of Hsp60 expression as a biomarker or an 'early warning system' for stress in corals (Smith & Buddemeier 1992, Sanders 1993, Choresh et al. 2007). Considering that seawater temperature in many tropical regions is currently increasing by 1-2°C per century (Hoegh-Guldberg 1999), together with the growing concern over the worldwide degradation of coral reefs due to global climate change, human perturbations and coral diseases, the development of an ‘early warning system’ for assessment of the health of these marine benthic communities is of great importance (Choresh et al. 2001, 2007). The integration of biochemical and morphological features into a diagnostic profile allows for an in-depth understanding of the response of coral cells to environmental stress and their ability to withstand these stresses. Nevertheless, for the future research, the use of additional cellular biomarkers indicative of specific damage (others Hsps classes, MnSOD, Cn/ZnSOD, Ubiquitin, Heme-oxygenase, Protein carbonyl) along with targeted functional studies (e.g. histology), might provide definitive evidence that corals are in a diseased or incurable condition.

5.1 References

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APPENDIX I

First report of coral diseases in the Republic of Maldives

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Introduction

Coral disease epizootics have become a major threat to reef ecosystems globally, and an increasing number of newly emerging syndromes has been reported over the past 20 years (Harvell et al. 1999, Raymundo et al. 2005, Sokolow 2009). Because climate change is predicted to amplify host susceptibility, host range, pathogen survival and disease transmission (Ritchie et al. 2001, Myers & Raymundo 2009), outbreaks are expected to increase worldwide in the future (Willis et al. 2004, Bruno et al. 2007).

The coral reefs of the Maldivian archipelago are among the most diverse in the Indian Ocean, and are known to host more than 180 zooxanthellate coral species belonging to 51 genera (Pichon & Benzoni 2007). The major reef structures occupy an area of about 21,000 km^2, 21,1% of which can be categorized as marine productive reef habitats (Naseer & Hutcher 2004).

Around the world researchers are growing alarmed about the potential negative effects of infectious diseases on reef communities (Bruno et al. 2007). Temporary shifts from acroporid- to agaricid-dominated reefs caused by disease-induced mortality have already occurred in Belize (Aronson et al. 2002), and outbreaks of coral diseases caused a significant loss of coral cover in the Caribbean Sea and on the Great Barrier Reef (Willis et al. 2004, Weil et al. 2009).

The Maldivian archipelago has been heavily affected by 1998 coral bleaching event which led to a coral mortality of up to 100%, with varying effects depending on species and locality (Bianchi et al. 2003). The living coral cover was of 2-8% immediately after the mass mortality event, and have increased up to 12-37% in the following eight years (Lasagna 2008). The mass mortality event associated to coral bleaching induced also a qualitative change in coral communities, with a shift from *Acropora*

dominated reefs to *Porites* dominated reefs (Goreau 2000).

The ecological impact of bleaching on coral communities, and particularly its ability to increase coral susceptibility towards infectious disease, is well known (McClanahan 2009). However, there is no available information about the presence of coral diseases in Maldives. Here, we try to fill this gap, by reporting the results of a survey conducted to investigate the occurrence and prevalence of diseases affecting reef-building corals in an island of the Republic of Maldives.

Materials and Methods

Underwater surveys were conducted during October and November 2010 in order to investigate the presence and prevalence of infectious diseases affecting scleractinian corals in the waters around the inhabited island of Magoodhoo, Faafu Atoll, Republic of Maldives (3°04' N; 72°57' E) (Fig. 1). The island measures 900 x 450 m and it is located on the south-east part of the atoll rim, about 140 km South of the capital Malè. Magoodhoo reef is approximately 2.9 km long and 1.55 km wide, and exhibits the features of a typical low-energy reef with a luxuriant growth of coral and gently slope to all sides. Sites were selected haphazardly from those accessible.

During sampling period, which fell within the wet season (mid-May to November), the local monthly mean sea surface temperature (SST) was of 29,1 ± 0,12 °C, and temperature variation among seasons did not exceed 1 °C (http://disc.sci.gsfc.nasa.gov/techlab/giovanni/). Analyses were conducted by snorkeling at shallow sites (n= 4, 0-5m) and by scuba diving at deep ones (n= 4, 10-20 m) (Fig. 1c). For each site we performed an exploratory qualitative analysis aimed to compile a complete list of the hard coral diseases occurring in the area. Additionally, quantitative information about

coral disease prevalence was obtained by performing, at each site, three randomly placed 25 × 1 m belt transects (total = 24 transects) spaced 10 to 20 m apart. Colonies on the belt margin were counted only when 50% or more of the colony lay within the belt. The selected transect size was chosen as the most suitable in relation to field logistics and the size of the surveyed area.

In both qualitative and quantitative analyses, all corals were identified in situ at genus level (according to Veron 2000). Diseases were identified in situ as well (according to Rosenberg 2004). Small samples of both healthy and infected coral tissue were collected for further laboratory identification. Visible symptoms of disease or stress not ascribable to those reported from available literature were described and photographed when encountered. Micro photographs (32x) of infected colonies were obtained using a StemiDIV4 stereomicroscope paired with a Canon G11 camera.

All diseased colonies within each belt transect were noted, and the number of diseased and healthy colonies was counted in order to compute disease prevalence, which was calculated as the number of infected colonies quoted by the total number of colonies. Taxon specific prevalence was calculated as the number of cases of a specific disease or syndrome divided by the number of appropriate host encountered. The average total disease prevalence for each site was calculated by averaging the prevalence of all belt-transects.

Results

Occurrence and prevalence of observed diseases

Our survey of the reefs of Magoodhoo Island revealed the presence of 5 syndromes affecting different genera of reef-building corals, namely black

band disease, ulcerative white spot disease, white syndrome, skeleton eroding band, and brown band disease. Additionally, we observed a dark discoloration response on *Porites* spp. loosely similar to that typical of dark spot disease, to which we will refer as *Porites* dark discoloration response, in order to distinguish it from any common disease and tissue pigmentation response known for this genus (Raymundo et al. 2005).

In total we counted 2,761 colonies belonging to 19 genera. Among them, disease-induced lesions were observed on 64 colonies (belonging to 8 genera), 50 of which (belonging to 6 genera) were found within transects. Thus, the resulting overall prevalence of coral diseases on the reef was lower than 2 %. Individual prevalence of each investigated coral disease was lower than 1 %, ranging from 0.7 % (skeleton eroding band) to 0.18 % (*Porites* dark discoloration response). Although qualitative surveys revealed the presence of brown band disease and ulcerative white spot, the two diseases were not found within transects. For the other diseases (black band disease, skeleton eroding band and white syndromes) as well as for *Porites* dark discoloration response we report the number of occurrences across all the found coral genera (Table 1). All the diseases observed were found in the shallow site, while in the deeper sites Porites dark discoloration response was not found. A preliminary assessment of disease prevalence among affected coral genera is reported in Table 2.

Description of observed diseases

We found evidence of coral disease in five scleractinian families, namely Acroporidae, Poritidae, Faviidae, Pocilloporidae and Agariciidae. More than half (54.7 %) of the diseased colonies belonged to the Acroporidae, which resulted the most affected family. The other diseased colonies belonged to

the Poritidae (17.3 %), the Siderasteridae (12.5 %), the Faviidae (6.2 %), the Pocilloporidae (6.2 %) and the Agariicidae (3.1 %). Among affected genera, *Acropora* hosted the highest number of coral diseases (n=5), while the remaining genera were affected by a maximum of two diseases.

Black band disease (Figure 2a), that is constituted by a mat of microbes dominated by the cianobacterium *Phormidium corallitycum* (Rützler & Santavy 1983)(Figure 2b), showed the largest host range, affecting 5 different coral genera and particularly *Psammocora* and *Goniopora* genera. Ulcerative white spot disease (Figure 2c), which was found mainly on *Porites* genus, was characterized by discrete, bleached round foci of 3–5 mm in diameter, coherently to the description given by Raymundo (2003) (Figure 2d). Lesions that resembled ulcerative white spot were also observed on some colonies of *Acropora* spp. (Figure 2e). These lesions were characterized by discrete, multifocal round foci that revealed an underlying intact skeleton. These injured areas were mainly located in the basal portion of the colonies. Similarly to the description given by Work and Aeby (2006), the lesions due to white syndrome (Figure 2f) consisted of large, diffuse bands of tissue loss that revealed a bare, white, intact skeleton. The lesions were mildly to severely extended and the tissue loss involved the coenosarc and the polyps of the colonies belonging to the *Acropora* genus. We closely examined all the colonies affected by white syndrome and ulcerative white spot, and we found no evidence suggesting an involvement of coral predators in tissue death.

Skeletal eroding band (Figure 2g), which is associated with the ciliate *Halofolliculina corallasia* (Fig. 2h) (Antonius 1999, Willis et al. 2004) was found on genera *Acropora*, *Pocillopora* and *Goniastrea*, while brown band disease (Figure 2i), that is caused by a mobile ciliate (Figure 2j) such as the recently described *Porpostoma guamense* (Lobban 2011) was found only on

branching *Acropora* spp.. Members of the genus *Porites*, affected by dark discoloration response, were characterized by small to large areas of brown discoloration with indistinct undulating borders (Figure 3); in addition, diseased colonies showed lesions both focally and diffusely distributed at peripheral areas.

Discussion

The present study provides baseline information on the status of diseases affecting scleractinian corals in a previously unsampled region. Our surveys documented the presence of five different coral diseases and one anomalous pigmentation response in the reefs of Magoodhoo Island. All the pathologies observed in this study have been previously reported from the Indo-Pacific region. Nonetheless, the data presented here constitute the first records of coral diseases in the Maldives Archipelago. All observed coral diseases were present apparently with low prevalence, which is in contrast with several other studies on reef systems (Myers & Raymundo 2009, Weil et al. 2012). The most commonly observed diseases were skeleton eroding band, black band disease (coherent with the assumptions of their circumglobal distribution, see Croquer et al. 2006 and Myers & Raymundo 2009) and white syndrome. However, white syndrome showed a much lower prevalence than that already observed in similar ecosystems (see, for example, Willis et al. 2004 and Hobbs & Frish 2010 for the Indo-Pacific, and Sutherland et al. 2004 for the Caribbean).Our surveys revealed also the presence of one anomalous pigmentation response which we defined *Porites* dark discoloration response and that resulted relatively widespread in the area of study. We observed no skeleton or tissue damage by scars, tumors or other known lesions associated to the condition. This suggests that the

response may be triggered by unidentified chemical and/or microbial agents, or may be interpreted as a hypermelanization response to a pathogen (Petes et al. 2003). Further investigation would improve our knowledge of this response or disease. We recorded a few cases of brown band disease and ulcerative white spot which extend the documented geographic range for the two diseases (Weil et al. 2012). In particular we extend the range of brown band disease westward from Philippines and Great Barrier Reef (Raymundo et al. 2003, Willis et al. 2004). Interestingly, we noticed signs of infections similar to those due to ulcerative white spot on several *Acropora* colonies. Although still to be confirmed by more detailed analyses, this finding may suggest a possible increase in host range for the disease, that, up to date, has been reported mainly from *Porites* spp. (Raymundo et al. 2003; Kaczmarsky 2006). Among coral genera *Acropora* hosted the highest number of coral diseases. This supports the hypothesis that fast-growing corals might have a weaker disease resistance than slow growing corals (Willis et al. 2004; Palmer et al. 2008, 2010; Mydlarz et al. 2010). Overall, less than half of the sampled genera resulted affected by at least one coral disease. This could suggest a low spread of host- specific coral diseases (Raymundo et al. 2005). Despite Maldives and hence the area of study have been affected by a mass coral bleaching in 1998 (Longo et al. 2000, Zahir 2000), the currently available data do not make possible to individuate a relationship between the past thermal stress and the current diffusion of coral diseases. However the expected future increase in sea surface temperatures (Kleypas et al. 1999) could lead not only to new bleaching events, but also promote the spread of coral pathogens by increasing their growth rate and virulence (Ben-Haim et al. 2003b) and by reducing immune response in coral hosts (Alker et al. 2001; Mydlarz et al. 2009; Palmer et al. 2011). Considering the current state of regression of many reefs in the Maldives (Lasagna 2010), a better

understanding of the actual and potential impact of infectious diseases on coral ecosystem dynamics is fundamental to conservation planning. Although we cannot exclude that our results may be affected by sample size, number of replicates and local variation, and that they may be not representative of large scale patterns valid for the whole Magoodhoo reef, we hope that our preliminary study could stimulate the interest of coral pathologists and promote future in-depth investigations focusing on coral diseases in Maldivian reefs.

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Tables and Figures

Tab. 1 - Frequency of occurrence of the coral diseases at the genus level. BBD= black band disease; SEB= skeleton eroding band; WS= white syndromes; PDDr= *Porites* dark discoloration response; BrBD= brown band disease; UWS= ulcerative white spot; ?= to be confirmed; ( ) = not found within transect.

Genera	**n°**	**BBD**	**SEB**	**WS**	**PDDr**	**BrBD**	**UWS**
Acropora	1162	**1**	**14**	**15**		**(2)**	**(3)?**
Cyphastrea	10						
Favia	14						
Favites	28						
Fungia	45						
Echinopora	10			**(1)**			
Gardineroseris	6						
Goniastrea	22	**(2)**	**(1)**				
Goniopora	7	**2**					
Hydnophora	7						
Isopora	122						
Leptastrea	23						
Leptoria	19						
Montipora	56						
Pavona	742	**2**					
Platygyra	15						
Pocillopora	138		**4**				
Porites	252				**6**		**(3)**
Psammocora	83	**6**		**(2)**			
Total	**2761**	**11**	**18**	**15**	**6**	**-**	**-**

Tab. 2 - Total prevalence and depth distribution of observed coral diseases. LL 95% and UL 95% bootstrap lower and upper confidence limits.

		Diseases prevalence			Station	
		mean	**LL 95%**	**UL 95%**	**shallow**	**deep**
Black band disease		**0.34**	**0.13**	**0.74**		
	Psammocora	4.4	0.83	7.73	*y*	*n*
	Goniopora	5	0	18.75	*y*	*n*
	Pavona	0.5	0	1.38	*y*	*n*
	Acropora	0.1	0	0.27	*n*	*y*
Skeleton eroding band		**0.7**	**0.41**	**0.99**		
	Acropora	1.3	0.78	2.63	*y*	*y*
	Pocillopora	3.6	1.19	11.42	*y*	*y*
***Porites* dark discoloration response**		**0.18**	**0.07**	**0.31**		
	Porites	2.9	1.01	6.07	*y*	*n*
White syndrome		**0.64**	**0.41**	**0.92**		
	Acropora	1.4	0.78	2.41	*y*	*y*

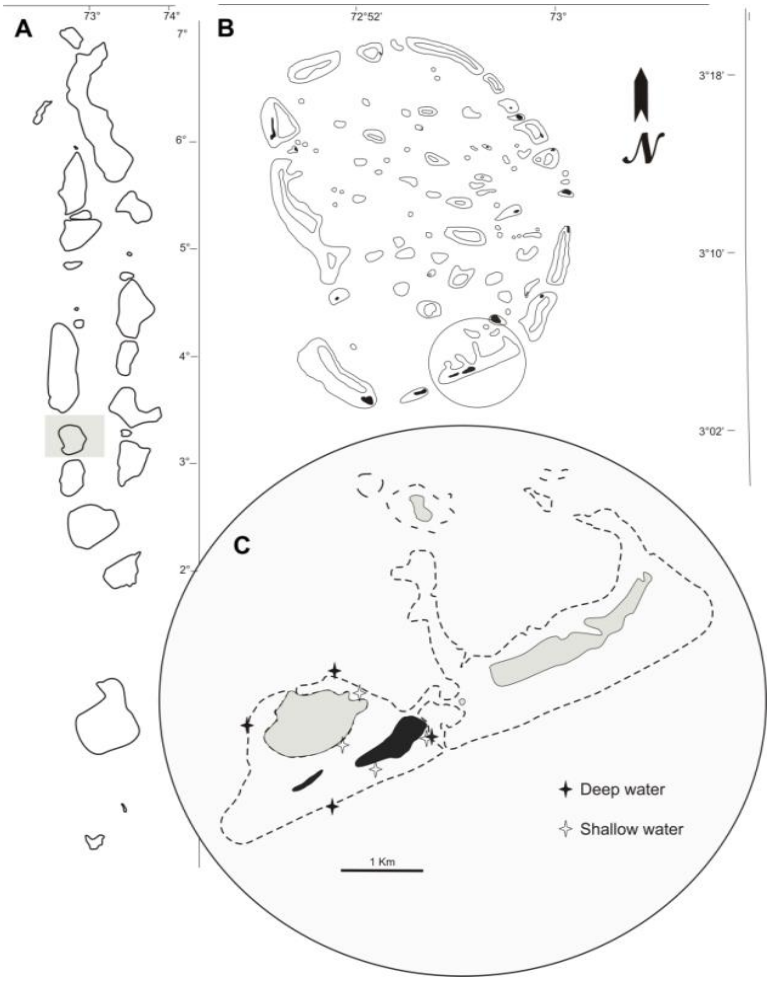

Fig. 1 - Map of the study area including the 8 sampling sites. A: Republic of Maldives; B: Faafu Atoll; C: Magoodhoo Island

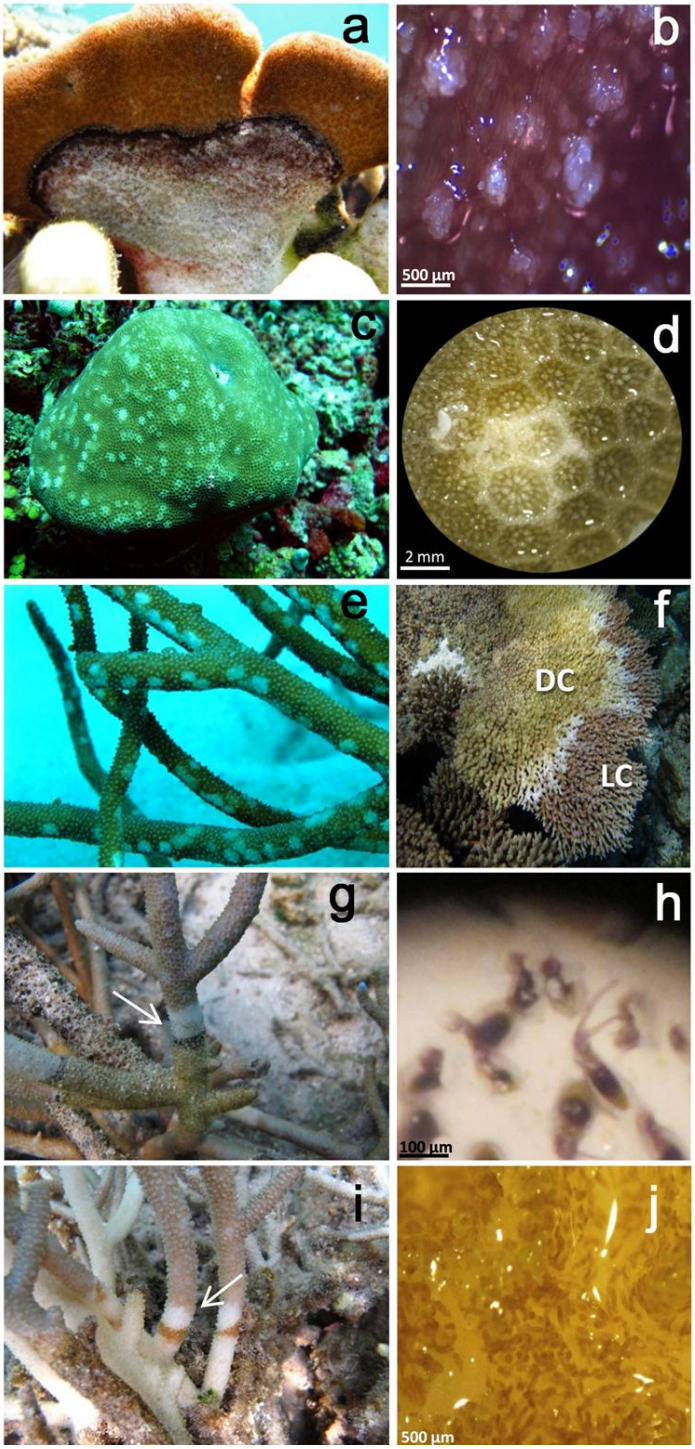
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Fig. 2 - Photographs illustrating the coral diseases found in Magoodhoo reef. **a** Black band disease on *Psammocora digitata* colony; **b** close up of the mat forming the black band; **c** ulcerative white spot on a massive *Porites* colony; **d** areas of white tissue discoloration with discrete margins; **e** signs similar to ulcerative white spot on an *Acropora* spp. colony; **f** *Acropora* plate coral with white syndrome; the white band is the lesion area, with live coral on the right (LC) and dead coral (colonized by filamentous algae) on the left (DC); **g** skeleton eroding band (pointed by the arrow) on a branching *Acropora muricata* colony; **h** *Halofolliculina corallasia*: note the large peristomial wings coming out of the lorica; **i** brown band disease (pointed by the arrow) on a branching *Acropora muricata* colony; **j** details of ciliate clustering constituting the band. Photos: S. Montano

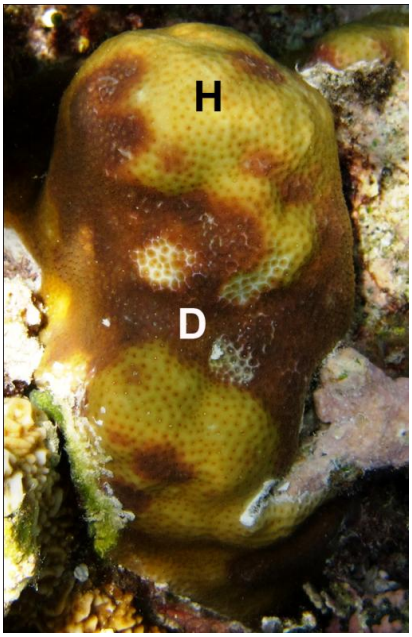

Fig. 3 - *Porites* dark discoloration response. (H) shows the regular color of the healthy part of the colony. (D) shows the anomalous pigmentation response founded in Magoodhoo island. It consists in an abnormal dark-like purple discoloration of the coral tissue. Photos: S. Montano

APPENDIX II

Acropora muricata mortality associated with extensive growth of *Caulerpa racemosa* in Magoodhoo island, Republic of Maldives

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Caulerpa racemosa, a common and opportunistic species widely distributed in tropical and warm-temperate regions, is known to form monospecific stands outside its native range (Verlaque et al. 2003). In October 2011 we observed an alteration in benthic community due to a widespread overgrowth of *C. racemosa* around the inhabited island of Magoodhoo (3°04'N; 72°57' E, Republic of Maldives). The mats formed a continuous dense meadow (Fig 1a) that occupied an area of 95 x 120 m (~11,000 m^2) previously dominated by the branching coral *Acropora muricata.* Partial and total mortality (Fig 1 b-c) were recorded on 45% and 30% of *A. muricata* colonies, respectively. The total area of influence of *C. racemosa* was however much larger

(~25,000 m^2) including smaller coral patches near to the meadow, where mortality in contact with the algae was also observed on colonies of *Isopora palifera, Lobophyllia corymbosa, Pavona varians*, *Pocillopora damicornis*, and *Porites solida*. Although species of the genus *Caulerpa* are not usually abundant on oligotrophic coral reefs, nutrient enrichment from natural and/or anthropogenic sources are known to promote green algal blooms (Lapointe and Bedford 2009). Considering the current state of regression of many reefs in the Maldives (Lasagna 2010) we report an unusual phenomenon which could possibly become more common

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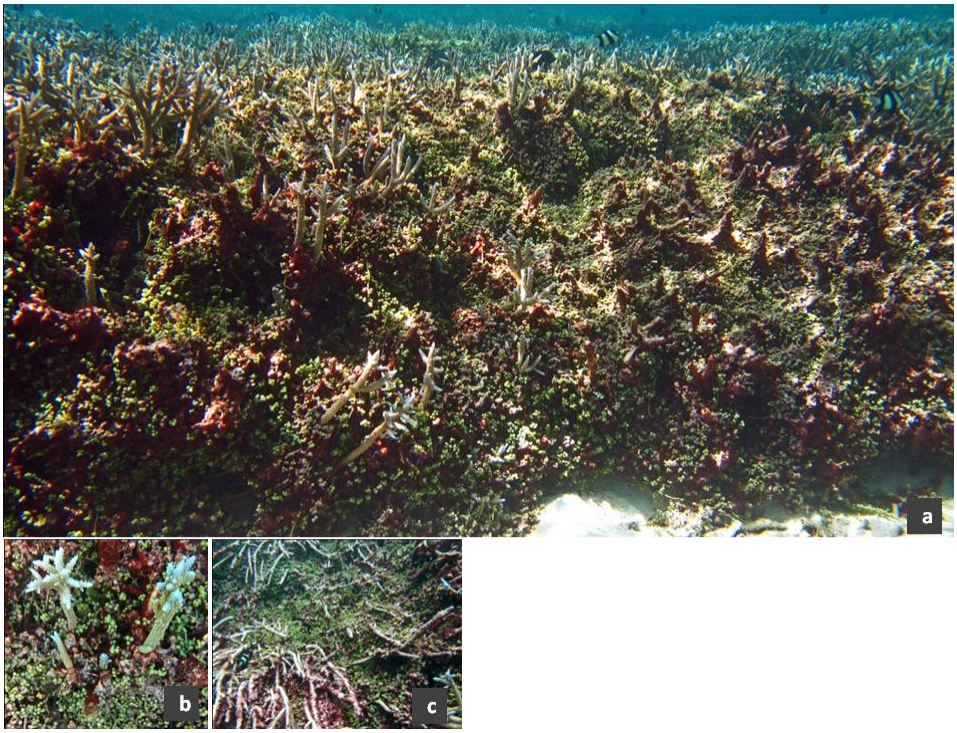

Fig 1. (a) Extensive meadows of *Caulerpa racemosa* overgrowing colonies of the dominant coral *Acropora muricata.* **(b-c)** Partial and total mortality of *A. muricata* following algal overgrowth.

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