

**ALMA MATER STUDIORUM  
UNIVERSITA' DI BOLOGNA**

**SCUOLA DI SCIENZE**

**Corso di laurea magistrale in Biologia Marina**

**Gene expression responses to acute and  
chronic heat stress in the common  
reef-building coral *Pocillopora verrucosa***

Tesi di laurea in **Adattamenti degli animali all'ambiente marino**

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III sessione

Anno Accademico 2013/2014



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# 1 INTRODUCTION

## Summary

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- **The Greenhouse effect and the Global Warming**  
The greenhouse effect is a climatic and atmospheric effect that shows the capacity of a planet to keep part of the solar energy within the atmosphere; but how it works?
- **Climate change and ocean ecosystem**  
All the elements of the earth, land, oceans, and atmosphere, appear not only connected but interdependent. The Global Warming is rising the global average surface temperatures. Many measurement have shown upward trends in all regions, and have shown that rates of change are now greater than 2°C per century in many tropical seas.
- **Climate change on coral reefs: Bleaching event**  
What happen in places where the organism can't escape from the warming? Elevated sea temperatures as small as 1°C above long-term summer averages lead the loss of coral algal symbiont, and coral's dead.
- **Geographic Region description – Coral Triangle**  
The CT was delineated on the basis that it is an area that contains a high proportion of the species diversity of the Indo-Pacific and that this diversity occurs in an area small enough to permit meaningful conservation.
- **Pocillopora verrucosa**  
It have true verrucae which are evenly sized and spaced. This specie occurs between the surface to 40 m deep.
- **Genes description**  
The genes useful for this study were chosen from two different kind of possible genes: two basic life genes and one that intervene during the stress event to restore the constitutive cellular environment.



## INTRODUZION

Coral reefs are essential spawning, nursery, breeding, and feeding grounds for numerous organisms. In terms of biodiversity, the variety of species living on a coral reef is greater than in any other shallow-water marine ecosystems and is one of the most diverse on the planet, yet coral reefs cover less than one tenth of one percent of the ocean floor (Spalding, MD, Ravilious D, and Green EP 2001). During the last decade this habitats are threatened with worldwide decline from multiple factors, chief among them climate change (Hoegh-Guldberg O. et al. 2007; Hughes TP et al. 2003). The ability of corals to cope with this change, such as increased temperature, relies on the physiological mechanisms of acclimatization and long-term genetic adaptation. There is sample evidence that corals can be tolerant of and even thrive in temperature extremes (Coles SL, Brown BE 2003). During mass coral bleaching events, survival of scattered coral colonies suggests that some groups of corals may possess inherent physiological tolerance to environmental stress (Marshall PA, Baird AH 2000; West JM, Salm RV 2003). In addition, some high-temperature environments naturally retain healthy, growing coral populations (Coles S 1997; Oliver TA, Palumbi SR 2011), and these corals can show elevated bleaching tolerances (e.g. refs. Jokiel PL, Coles SL 1990; Oliver TA, Palumbi SR 2011). But what are the mechanisms that result in differences in thermal tolerance and physiological response? Are they due to genotypic adaptation where selection drives differences in susceptibility over evolutionary times scales or rather are they due to phenotypic acclimatization where organisms respond to extremes using their existing genomic repertoire within the lifetime of an individual? (Weis VM 2010). Under simulated bleaching stress, sensitive and resilient corals change expression of hundreds of genes, but the resilient corals had higher expression under control conditions across many genes. These “frontloaded” transcripts were less up-regulated in resilient corals during heat stress and included thermal tolerance genes such as heat shock proteins and antioxidant enzymes, as well as a broad array of genes involved in apoptosis regulation, tumor suppression, innate immune response, and cell adhesion (Barshis DJ et al. 2013). Thus represent an essential source of information about mechanisms underlying observed differences in coral physiological resilience. In this study we intend to deepen into the fundamental cellular processes responsible for enhanced stress tolerances that may enable some organisms to better persist into the future in an era of global climate change.

## 1.1 The Greenhouse effect and the Global Warming

The greenhouse effect is a climatic and atmospheric effect that shows the capacity of a planet to keep part of the solar energy within the atmosphere. It is part of the complex mechanisms for the thermic regulation of the planet and it operate through the presence in atmosphere of some gases called “Greenhouse gases” (Hansen J - Earth's Energy Imbalance). The atmosphere began about 4.5 BY ago, as a mixture of water vapor, hydrogen, hydrogen chloride, carbon monoxide, carbon dioxide and nitrogen (Gray, 2002). Through interaction with surface rocks and living organisms, it gradually reached its present composition, some 280 million years (MY) ago and has remained, more or less, unchanged (Khandekar 2005). During the past 4.5 BY to 280 MY, the most important transformation was the conversion of much of the carbon dioxide (CO<sub>2</sub>) into oxygen by abundant plant life, particularly during the carboniferous period, when most of our coal and oil deposits were formed (Gray, 2002). The gases nitrogen and oxygen that make up the bulk of the atmosphere neither absorb nor emit thermal radiation. If they were the only atmospheric constituents there would be no clouds and no greenhouse effect (Houghton 2005). The atmosphere is composed either of water vapor, carbon dioxide, ozone, methane and nitrous oxide: greenhouse gases. Sun’s radiation is mostly in short wavelengths and passes through the atmosphere without much absorption, except the ultraviolet part of the solar radiation which is absorbed by ozone in the stratosphere (Figure n.1.1). Solar radiation heats the earth and the oceans and they in turn emit radiation back to space in longer wavelengths, hence known as longwave radiation. The greenhouse gases in the atmosphere, including water vapor and CO<sub>2</sub> absorb this longwave radiation from the earth (and oceans) and in this process, maintain an annual global surface temperature of about 14 °C to 15 °C (Khandekar 2005). Without this effect the average Earth’s surface temperature would be about -6°C (Houghton 2005).



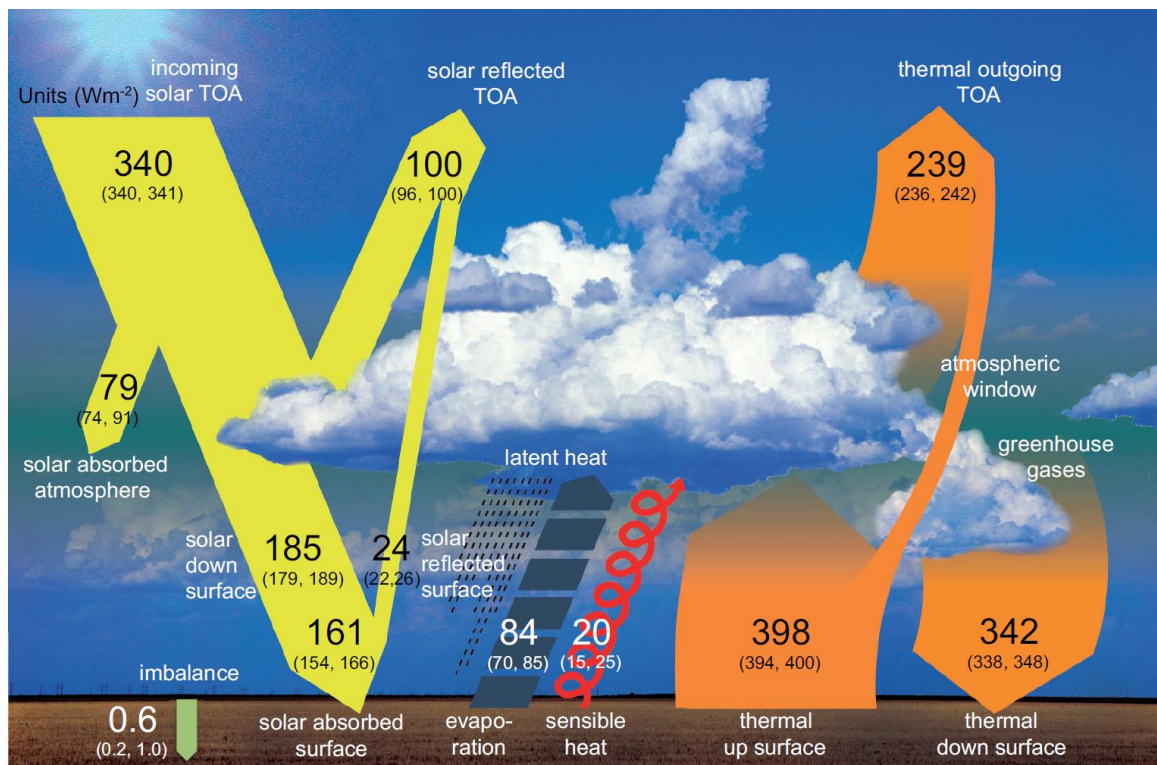


Figure n. 1.1 – Global mean energy budget under present-day climate conditions. Numbers state magnitudes of the individual energy fluxes in  $\text{Wm}^{-2}$ , adjusted within their uncertainty ranges to close the energy budgets. Numbers in parentheses attached to the energy fluxes cover the range of values in line with observational constraints. (TOA: radiation budget at the top of the atmosphere) (Wild et al., 2013)

The effect was first recognized by the French scientist Jean-Baptiste Fourier in 1827 (Mudge F.B. 1997). This climate system can respond to changes in external forcing: physical factors external to the climate system that force a net increase (positive forcing) or net decrease (negative forcing) of heat in the climate system as a whole (Hansen, Sato et al. 2005). Examples of external forcing include changes in atmospheric composition (e.g., increased concentrations of greenhouse gases), solar luminosity, volcanic eruptions, and variations in Earth's orbit around the Sun. However this response capacity can be overcome when the external forces had a synergic effect in a long term period of time (Gray, 2002). A British scientist, John Tyndall around 1860 measured the absorption of infrared radiation by carbon dioxide and water vapor and suggested that a cause of the ice ages might be a decrease in the greenhouse effect of carbon dioxide. It was a Swedish chemist, Svante Arrhenius in 1896 who first calculated the effect of increasing concentrations of greenhouse gases; he estimated that doubling the concentration of carbon dioxide would increase the global average temperature by 5–6°C (Houghton J. 2005).

## 1.2 Climate change and ocean ecosystem

A rise in global average surface temperatures is the best-known indicator of climate change. Although each year and even decade is not always warmer than the last, global surface temperatures have warmed substantially since 1900 (Hartmann D.L. et al. 2013). Warming land temperatures correspond closely with the observed warming trend over the oceans. Warming oceanic air temperatures, measured from aboard ships, and temperatures of the sea surface itself also coincide, as borne out by many independent analyses (Hartmann D.L. et al. 2013). A global perception started to emerge, recognizing that the earth was a single, interconnected unit, within which the biosphere is an active and essential component. All the elements of the earth, land, oceans, and atmosphere, appear not only connected but interdependent. Surely an impact on one of these components must reflect on the others and on the whole. However, the extent to which the earth behaves as a single, interlinked, self-regulated system was not put into focus until scientists reconstructed the chemical history of the earth's atmosphere from 420,000-year-old Antarctica ice cores (the Vostok record; Petit et al. 1999), and more recently from the 650,000-year-old cores from European Project for Ice Coring in Antarctica (EPICA) (Siegenthaler et al. 2005). These data indicated that the CO<sub>2</sub> levels in the atmosphere had been constrained between 180 and 290 ppm by volume for over 500,000 years, with minima and maxima corresponding to the glacial and interglacial periods, respectively. Equivalent cycles from atmospheric temperature proxies demonstrated a strong coupling between climate and the carbon cycle as well as the stability of this coupling over the recorded time. For some time scientists have suspected that the glacial/interglacial cycles that the earth has experienced every 100 kyr (Siegenthaler et al. 2005) are caused by smooth changes in the eccentricity of the earth's orbit. However, these changes are too smooth to generate such sharp, non-linear responses in the chemical composition of the atmosphere, unless these are strongly modulated by biological, chemical, and physical feedbacks (Barange M. et al. 2010). Furthermore we have to consider that the earth system responds to the physical forces in complex ways, often non-linearly. These responses involve interactions between land, atmosphere, water, ice, biosphere, societies, technologies, and economies.

### 1.2.1 The ocean in the earth system

The ocean is one of the major components of the earth system, providing 99% of the available living space on the planet. Water is essential to our existence, having secured life from the time of the primeval soup. It has been estimated that 80% of all life on earth depends on healthy oceans and coasts and more than a third of the world's population lives in coastal areas and small islands, even though they amount to less than 4% of the earth's land. The ocean's heat capacity is about 1,000 times larger than that of the atmosphere, and the oceans net heat uptake since 1960 has been around 20 times greater than that of the atmosphere (Bindoff et al . 2007). Long-term records, starting at various points in the 20th century, additional observations, including balloon-borne measurements and satellite measurements, and reanalysis products allow analyses of indicators such as atmospheric composition, radiation budgets, hydrological cycle changes, extreme event characterizations and circulation indices show how global trends in Greenhouse Gases (GHGs) are indicative of the imbalance between sources and sinks in GHGs budgets, and play an important role in emissions verification on a global scale (Wielicki et al., 1996, Kopp and Lawrence, 2005) . IPCC (2007) has demonstrated that increasing atmospheric burdens of well-mixed G in a 9% increase in their RF from 1998 to 2005. These trends resulted in a 7.5% increase in RF from GHGs from 2005 to 2011, with carbon dioxide (CO<sub>2</sub>) contributing 80%. Of note is an increase in the average growth rate of atmospheric methane (CH<sub>4</sub>) from ~0.5 ppb yr<sup>-1</sup> during 1999–2006 to ~6 ppb yr<sup>-1</sup> from 2007 through 2011 (Hartmann D.L. et al. 2013). 'Recent' warming (since the 1950s) is strongly evident at all latitudes in SST over each ocean. Prominent spatio-temporal structures including the ENSO and decadal variability patterns in the Pacific Ocean and a hemispheric asymmetry in the Atlantic Ocean were highlighted as contributors to the regional differences in surface warming rates, which in turn affect atmospheric circulation. There are direct evidence to conclude that the world oceans have warmed substantially (Figure n.1.2) over the last half century, and that the warming accounts for over 80% of the changes in the

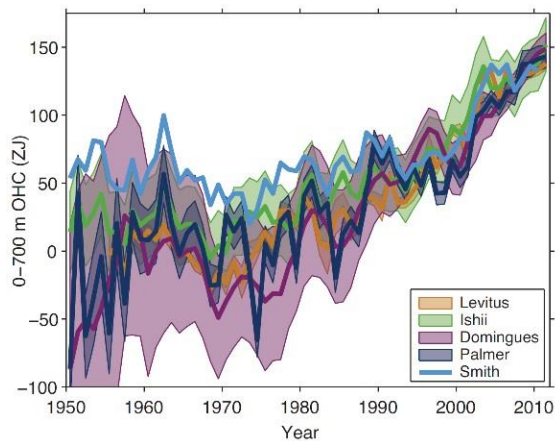


Figure n.1.2: Observation-based estimates of annual global mean upper (0 to 700 m) ocean heat content in ZJ (1 ZJ = 1021 Joules) updated from (see legend): Levitus et al. (2012), Ishii and Kimoto (2009), Domingues et al. (2008), Palmer et al. (2007) and Smith and Murphy (2007). Uncertainties are shaded and plotted as published (at the one standard error level, except one standard deviation for Levitus, with no uncertainties provided for Smith). (Rhein, M. 2013)

response to enhanced concentrations of atmospheric greenhouse gases (Bijlsma et al. 1995). Sea temperatures over the past 20 years, extensively measured and cross-compared by instruments on satellites, ships and buoys, have shown upward trends in all regions, and have shown that rates of change are now greater than  $2^{\circ}\text{C}$  per century in many tropical seas (O. Hoegh-Guldberg 1999). This global trend, however, has not been homogeneous or linear. Significant decadal variations have been observed in the sea surface temperature (SST) time series, and there are large regions where the oceans have been cooling (Bindoff et al. 2007; Harrison and Carson 2007). Although the oceans are interconnected, there are specific modes of response to forcing that show important differences as well (Barange M. et al. 2010, Drinkwater K.F. 2003). Alternating short-term and long-term event (e.g. *El Niño* –Southern Oscillation (ENSO) and Pacific Decadal Oscillation (PDO) in the Pacific Ocean) provides ways of moving heat from the tropical ocean to higher latitudes and out of the ocean into the atmosphere (Trenberth et al. 2002). The dynamic nature of the different oceans, exemplified by the varying heat exchange processes, is responsible for the diversity of marine environments and habitats. The properties of water generate different density layers and gradients. Tides, currents, and upwelling break this stratification and, by forcing the mixing of water layers, enhance primary production. This network of surface and deep water currents tightly connects marine ecosystems and defines its dynamics (Drinkwater K.F. 2009).

energy content of the earth's climate system during this period (Bindoff et al. 2007; Domingues et al. 2008). A large-scale coherent trend of salinity and observed changes in precipitation are also observed. More water is transported in the atmosphere from low latitudes to high latitudes producing a global regime shifts, as well expected poleward range shift and freshening in sub- polar latitudes and a salinification of shallower parts of the tropical and subtropical oceans (Barange M. et al. 2010). Increases of  $1\text{-}2^{\circ}\text{C}$  in sea temperature are expected by 2100 in

### 1.3 Climate change on coral reefs: Bleaching event

Decades of ecological and physiological research document that climatic variables are primary drivers of distributions and dynamics of marine plankton and fish (Hays et al. 2005, Roessig et al. 2004). Globally distributed planktonic records show strong shifts of phytoplankton and zooplankton communities in concert with regional oceanic climate as changes in timing of peak biomass (deYoung et al. 2004, Hays et al. 2005, Richardson & Schoeman 2004). Some copepod communities have shifted as much as 1000 km northward (Beaugrand et al. 2002). The data show a significant increase in southern-ranged species in that geographic regions had experienced a period of significant warming in SST and decrease of northern-ranged species. Likewise the regions (e.g. United Kingdom) that experienced a significant cooling in SST have seen the shifting of the community to a northern-ranged species (Sagarin et al. 1999, Southward et al. 2005). But what happen in places where the organism can't escape from the warming? What happen to the coral reefs? Elevated sea temperatures as small as 1°C above long-term summer averages lead the loss of coral algal symbiont, and global SST has risen an average of 0.1°–0.2°C since 1976 (Hoegh-Guldberg 1999, IPCC 2001). Coral bleaching, the paling of tissues resulting from the drastic decline in zooxanthella densities and/or the loss of photosynthetic pigments which occurs when the thermal tolerance of corals and their photosynthetic symbionts (zooxanthellae) is exceeded, is a classic stress response of corals and allied symbiotic marine animals to perturbations in environmental conditions (Glynn, 1993, Brown, 1997, Hoegh-Guldberg O. 1999).

The central feature of shallow-water coastal ecosystems, known since the middle of the last century (Odum and Odum 1955), is the predominance of symbioses between invertebrates and dinoflagellate microalgae commonly referred as "zooxanthellae". All the Reef-building corals, the heart of coral reefs, are obligate endosymbiotic with algae of the dinoflagellate genus *Symbiodinium* (Mieog et al. 2009). *Symbiodinium* is the most studied genus in this paraphyletic group and is commonly found in shallow water tropical and subtropical cnidarians. These algae are ubiquitous members of coral reef ecosystems (Rowan 1998, Taylor 1974, Trench 1993): Cnidarian species reported to contain *Symbiodinium* include many representatives from the class Anthozoa (including anemones, scleractinian corals, zoanthids, corallimorphs, blue corals, alcyonacean corals, and sea fans) and several representatives from the classes Scyphozoa (including rhizostome and coronate jellyfish)

and Hydrozoa (including milleporine fire corals) (Baker A.C. 2003). Members of six of the eight Symbiodinium clades (A–D, F, G) are known to associate with scleractinian corals, the reef builder corals (Baker A.C. 2003). It is not uncommon for the same coral colony to harbour multiple clades (reviewed by Baker A.C. 2003; Mieog et al. 2007) or types of symbionts simultaneously. Some species also undergo seasonal change in their endosymbiont community (Chen et al. 2005). These zooxanthellae are intracellular (Trench 1979) and are found within membrane-bound vacuoles in the cells of the host. Zooxanthellae photosynthesize while residing inside their hosts, and provide energy and nutrients for the invertebrate host by translocating up to 95% of their photosynthetic production to it (Figure n.1.3a) (Muscatine 1990; Woldridge S.A and Done T.J. 2009). Zooxanthellae selectively leak amino acids, sugars, complex carbohydrates and small peptides across the host-symbiont barrier. These compounds provide the host with a supply of energy and essential compounds (Muscatine 1973; Trench 1979; Swanson and Hoegh-Guldberg 1998). Corals and their zooxanthellae form a mutualistic symbiosis, as both partners appear to derive benefit from the association. Corals receive photosynthetic products (sugars and amino acids) in return for supplying zooxanthellae with crucial plant nutrients (ammonia and phosphate) from their waste metabolism (Trench 1979). The latter appear to be crucial for the survival of these primary producers in a water column that is normally devoid of these essential inorganic nutrients. Dubinsky Z. and Jokiel P.L. (1994) seen that when corals exposed to various light intensities, in corals exposed with Low-Light intensity, photosynthesis alone cannot account for all of the colony respiration; therefore such colonies have to supplement zooxanthellae autotrophy with heterotrophic animal predation on zooplankton (Falkowski et al. 1984). Indeed, under low light, most corals remain with their tentacles extended continuously, showing the needing to hunt more for zooplankton to supply to all the energy request (Dubinsky Z. and Jokiel P.L. 1994). This close association of primary producer and consumer makes possible the tight nutrient recycling that is thought to explain the high productivity of coral reefs (Muscatine and Porter 1977). Reef-building corals are greatly influenced by the biological and physical factors of their environment. Temperature, salinity and light have major effects on where reef-building corals grow. Environments in which coral reefs prosper are also typified by a high degree of stability (Hoegh-Guldberg O. 1999). Sea temperatures in many tropical regions have increased by almost 1°C over the past 100 years, and are currently increasing at ~1.2°C per century. When this changes occur too rapidly the thermal tolerance of corals

and their photosynthetic symbionts (zooxanthellae) is exceeded. This causes the breakdown of the symbiosis (coral bleaching) and a significant proportion of the zooxanthellae complement is expelled from the coral animal (Coles and Jokiel 1977, 1978; Glynn and D.Croz 1990; Lesser et al. 1990; Brown 1997).

The warm-water bleaching syndrome of the coral–algae endosymbiosis follows a sequence of algal photoinhibition, oxidative damage, and host cell membrane disruption (e.g., Gates et al. 1992, Lesser 1996, Jones et al. 1998, Warner et al. 1999). Wooldridge (2009) proposes that the onset of this sequence is linked with a disruption to the “dark” photosynthetic reactions of the algal endosymbionts, implicating limited availability of  $\text{CO}_2$  substrate around the Rubisco enzyme (Figure n.1.3b). In this way, biophysical factors that cause  $\text{CO}_2$  demand to exceed  $\text{CO}_2$  supply within the coral’s intracellular milieu are identified as bleaching risk factors.

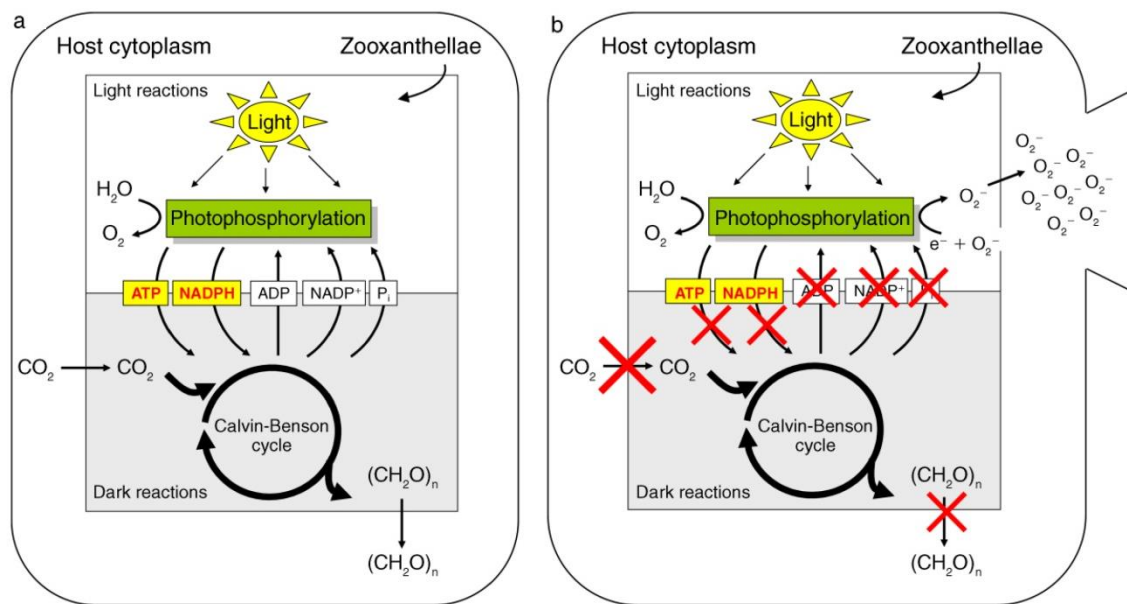


Figure.n 1.3 – (a) Schematic overview of the internal carbon cycling that is maintained by the coral–zooxanthellae symbiosis. (b) Schematic representation of the breakdown of the symbiosis ( $\frac{1}{4}$  zooxanthellae expulsion), as triggered by a limitation of  $\text{CO}_2$  substrate for the “dark” reactions of zooxanthellae photosynthesis. “Pi” indicates orthophosphate. (Wooldridge S.A and Done T.J. 2009)



Figure.n1.4 – Healthy, bleached and dead organisms of the species *Plerogyra sp.* in Bangka island, North Sulawesi, Indonesia (Photo credit: Davide Poli 2014)



## 1.4 Geographic Region description – Coral Triangle

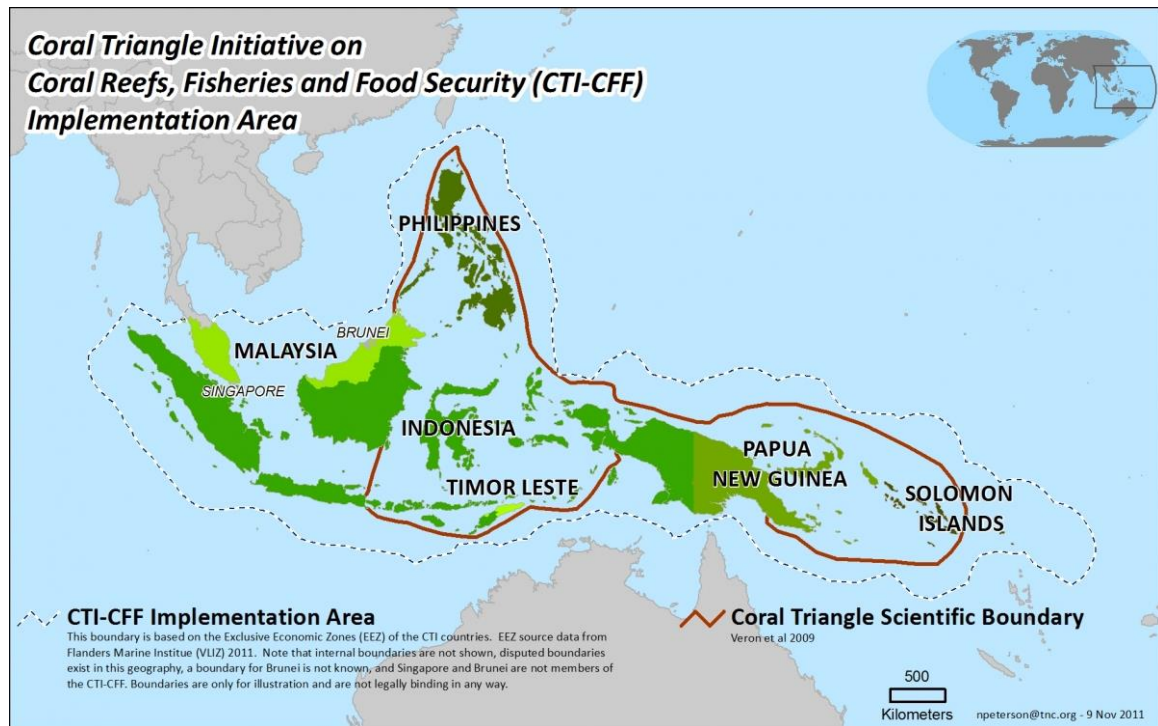


Figure.n 1.5 – Extension of the Coral Triangle. official CTI-CFF regional map (Peterson N. 2011)

*“If we look at a globe or a map of the Eastern Hemisphere, we shall perceive between Asia and Australia a number of large and small islands, forming a connected group distinct from those great masses of land and having little connection with either of them. Situated upon the equator, and bathed in the tepid water of the great tropical oceans, this region enjoys a climate more uniformly hot and moist than almost any other part of the globe, and teems with natural productions which are elsewhere unknown.”* So begins Alfred Russel Wallace’s seminal book *“The Malay Archipelago”*, first published in 1869 (Wallace, 1869). That was the born of the biogeography and MacArthur and Wilson escorted the field into the modern age in 1963 with their theory of island biogeography (Barber PH. 2009). These as well as other authors of the time have almost completely ignored the marine reign although there were occasional attempts to map marine life, notably by the American geologist James Dana (1853), the British naturalist Edward Forbes (1856) as well as Charles Darwin himself (1859). There were a few scattered publications on the distribution of some marine taxa during the latter part of the 19th century (George 1981), but it was not until the publication of Bartholomew’s Atlas in 1911 and Ekman’s historic compendium in 1935 that marine biogeography became established as a science in its own right (Veron JEN. et al. 2009) During the following decades biogeographers have proposed centers of

marine biodiversity of varying shapes, all centered on the Indonesian/Philippines Archipelago. Some stem from biogeographic theory or geological history, others from coral and reef fish distributions. These centers have been given a variety of names: Wallacea, East Indies Triangle, Indo-Malayan Triangle, Western Pacific Diversity Triangle, Indo-Australian Archipelago, South-east Asian center of diversity, Central Indo-Pacific biodiversity hotspot, Marine East Indies, among others (Hoeksema 2007).

For various reasons it is important to know where the highest concentration of species can be found: coastal areas offer a great potential of natural resources for local human populations, especially in countries rich in marine species; for the designation of a network of Marine Protected Areas (MPAs) and for other conservation efforts; the marine tourism industry benefits from the high variety of marine life; the position and boundaries of the centre of marine diversity are relevant for evolutionary and ecological problems (Hoeksema 2007). Delineation of the CT (Veron JEN et al. 2009) was established by the spatial database *Coral Geographic*, a major update of the original species maps of Veron JEN et al. (2000). This database contains comprehensive global species maps of zooxanthellate coral distributions in GIS format, allowing them to be interrogated to compare geographic regions or to elucidate patterns of diversity and endemism (Veron JEN. et al. 2009). This coral ecoregions so identified provide a blueprint for establishing a globally representative network of coral reef MPAs. The world's highest diversity occurs in the CT, an area where more than 500 coral species are found in each ecoregion (Veron JEN. et al. 2011). The CT was delineated on the basis that it is an area that contains a high proportion of the species diversity of the Indo-Pacific and that this diversity occurs in an area small enough to permit meaningful conservation. With this delineation, the 16 ecoregions of the CT each host >500 reef coral species (Veron JEN. et al. 2009). The CT defined by *Coral Geographic* – that adopted by the six-nation Coral Triangle Initiative (CTI) – is an area of  $5.5 \times 10^6$  km<sup>2</sup> of ocean territory of Indonesia, the Philippines, Malaysia (Sabah), Timor Leste, Papua New Guinea, and the Solomon Islands (Figure n.1.5) less than 1.6% of the world's total ocean area. The CT is not a distinct biogeographic unit, but comprises portions of two biogeographic regions (Indonesian-Philippines Region, and Far Southwestern Pacific Region, Veron 1995). In total, the CT has 605 zooxanthellate coral species of which 66% are common to all ecoregions. This diversity amounts to 76% of the world's total species complement (Figure n.1.6). Within the CT, highest richness resides in the Bird's Head Peninsula of Indonesian Papua, which hosts 574 species.

Individual reefs there have up to 280 species  $\text{ha}^{-1}$ , over four times the total zooxanthellate scleractinian species richness of the entire Atlantic Ocean (Turak and DeVantier in press). Within the Bird's Head, The Raja Ampat Islands ecoregion has the world's coral highest biodiversity, with 553 species (Turak and Souhoka 2003). It contains 52% of Indo-Pacific reef fishes (37% of reef fishes of the world) (Allen 2007). Other major faunal groups, notably molluscs (Wells 2002) and crustaceans (Grave 2001), have very high numbers of undescribed or cryptic species and thus are relatively little-known at species level (Meyer et al. 2005). Even azooxanthellate corals, which have none of the physiological restrictions of zooxanthellate species, have a centre of global diversity within the CT (Cairns 2007).

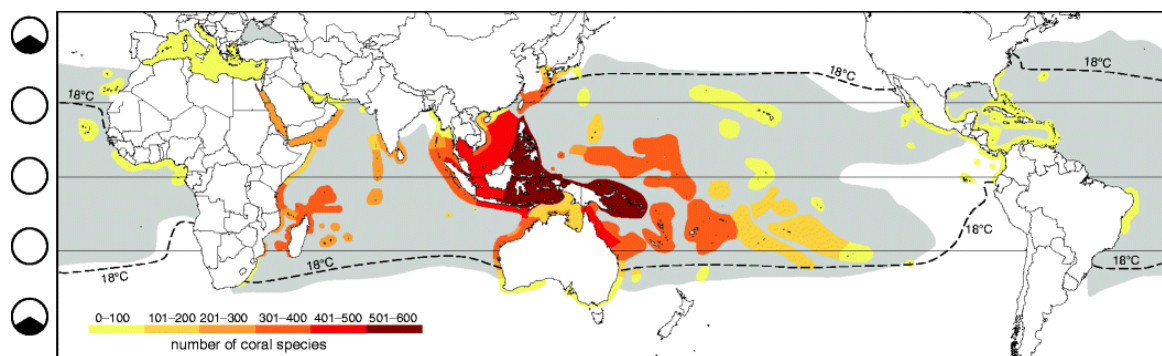


Figure n.1.6 - Global biodiversity of zooxanthellate corals. Colours indicate total species richness of the world's 141 coral biogeographic 'ecoregions' (From the spatial database Coral Geographic, Veron JEN. et al. 2000)

So much interest from so many points of view begs the question: why does the CT exist? There are several interacting factors operating over different temporal and spatial scales: geological history; larval dispersal and genetic mixing; Biogeographic patterns; Evolutionary processes (Veron JEN. et al. 2009). The diversity of the CT has no single explanation. Plate tectonics created the biogeographic template, one of complex island coastlines and extreme habitat heterogeneity. Patterns of dispersion, mediated by ocean currents, formed sequences of attenuation away from the equator leaving the CT with the region's highest biodiversity. Many environmental parameters, especially ocean currents and temperature, underpin this pattern. Evolutionary patterns, the genetic outcomes of environmental drivers, show why the CT is a centre of biodiversity (Veron JEN. et al. 2009). Whatever the drivers of diversity that created the CT, it is the future that matters as far as conservation is concerned. Anthropogenic increase in tropical ocean temperature has been less than  $0.8^{\circ}\text{C}$  to date but could be as much as  $2\text{--}3^{\circ}\text{C}$  this century (IPCC 2007a, b), further magnified  $1\text{--}2^{\circ}\text{C}$  above normal by El Niño events. Ocean acidification is another

threat. In a business-as-usual regime of anthropogenic carbon dioxide increase, calcification of equatorial corals will be marginal by the year 2030 (Guinotte et al. 2003). From a conservation perspective, all corals are threatened by the dual process of mass bleaching and ocean acidification. Furthermore, approximately 225 million people live within the CT, of which 95% live adjacent to the 131,254 km of coast. This population is largely dependent on the productivity of coral reefs and adjacent waters and a larger population is dependent on protein from these sources. There are no estimations of the value of the reefs of the CT; however, as Indonesia's reefs were valued at \$1.6 billion annually in 2002 (Burke et al. 2002), the current value of the CT is at least \$2.3 billion annually. Although correlations between ecological and economic impacts on reefs stemming from environmental decline cannot be estimated with certainty, it has been estimated that Indo-Pacific reefs are declining at >2% per year (Bruno and Selig 2007 and Burke et al. 2002) and this is becoming increasingly difficult to reverse (Hoegh-Guldberg et al. 2009).

### 1.5 *Pocillopora verrucosa*

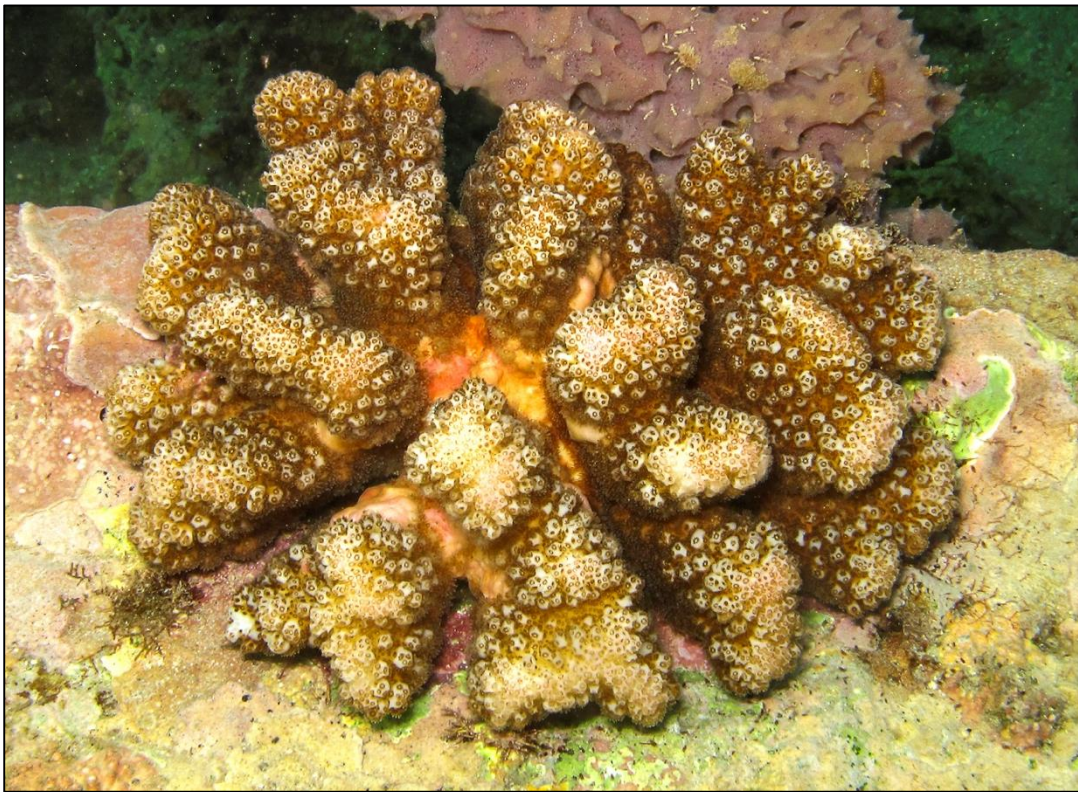


Figure n.1.7 – *Pocillopora verrucosa* (Photo credit: Davide Poli 2014)

#### Taxonomy

Kingdom	Phylum	Class	Order	Family
ANIMALIA	CNIDARIA	ANTHOZOA	SCLERACTINIA	POCILLOPORIDAE

Specific Name	<i>Pocillopora verrucosa</i> (Ellis and Solander 1786)								
Previous name	<i>Madrepora verrucosa</i> (Ellis & Solander, 1786 - original combination, basionym)								
	<i>Pocillopora danae</i> (Verrill, 1864 )								
	<i>Pocillopora hemprichi</i> (Ehrenberg, 1834 )								
Common name	Rasp Coral								
Red List Category	Not Evaluated	Data Deficient	Least Concern	Near Threatened	Vulnerable	Endangered	Critically Endangered	Extinct in the Wild	Extinct
	Least Concern								
Distribution	This species is common								

Hoeksema B.W. et al. 2014

*Pocillopora verrucosa* have broad, club shaped or more flattened branches (Figure n.1.7). It have true verrucae which are evenly sized and spaced. This species occurs between the surface to 40 m deep. On fore reef slopes of moderate but not extreme exposure, it is extremely common, and helps to define clear coral communities in many sites. It is not well tolerant to sedimentation, so is much less abundant in lagoon areas (Sheppard, 1998). Colonies are composed of uniform upright branches clearly distinct from the verrucae, but the latter are irregular in size. Colonies usually form hemispheres and grow in isolation. The stout branches radiate from a central holdfast and tend to be clubbed at their tips. Large verrucae are up to 6 mm high, and corallites 1 mm across. They have permanently coloured red-brown stalks. This colour can varies from pale lime-green or pink to dark brown or bluish-brown (Veron JEN. et al. 2000).

### Ecology and Distribution:

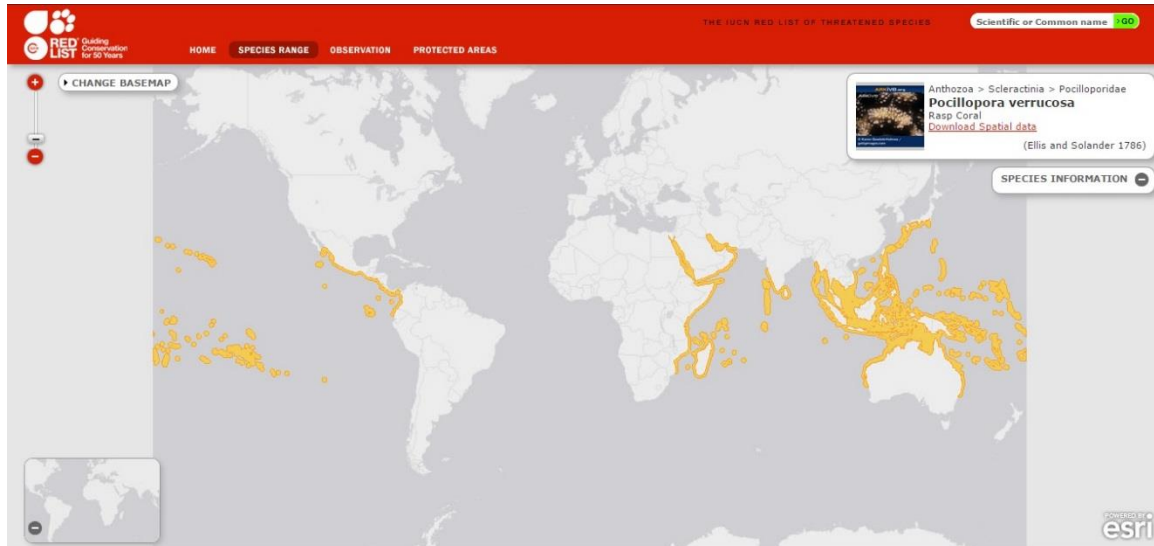


Figure. n. 1.8 – Geographic distribution of *Pocillopora verrucosa* (Hoeksema B.W. et al. 2014)

Pocilloporid corals usually occurs in shallow reef environments (1-15m depth) but is been found till 54m depth (Reyes-Bonilla et al. 2005). This species lives in exposed reef fronts as in to protected fringing reefs in coral communities on rocky substrata. Pocilloporid, including *P. verrucosa*, it is amongst the strongest coral competitors (Glynn 2001) and represent one of the principal framework builders of many reefs (Glynn 2001). This species has a widespread distribution (Figure n.1.8) within the Indo-West Pacific and Eastern Tropical Pacific regions. In the Indo-West Pacific, this species is found in the Red Sea and the Gulf of Aden, the Southwest and Northwest Indian Ocean and the Arabian/Iranian Gulf, the Central Indian Ocean, the Central Indo-Pacific, Tropical Australia, Southern Japan and

the South China Sea, the Oceanic West Pacific, the central Pacific, Hawaiian Islands and Johnston Atoll, and the Far Eastern Pacific. In the Eastern Tropical Pacific, this species is found in Mexico: Baja California Sur, Nayarit, Jalisco, Colima, Michoacán, Guerrero and Oaxaca; Costa Rica: mainland Costa Rica including Cocos Island; Panama; Colombia: mainland Colombia including Malpelo Island; Ecuador: mainland Ecuador (Hoeksema B.W. et al. 2014).

### **Threats**

The major threat is global climate change, in particular, temperature extremes leading to bleaching and increased susceptibility to disease, increased severity of ENSO events and storms, and ocean acidification. There are evidence that overall coral reef habitat has declined, and this is used as a proxy for population decline for this species. This species is more resilient to some of the threats faced by corals and therefore population decline is estimated using the percentage of destroyed reefs only (Wilkinson 2004). As reported by Glynn *et al.* (1988) Pocilloporid species as well as other major reef building corals catastrophically declined after 1983. This situation is exasperated, in the affected region, during the ENSO event (Glynn 2000). In addition to this threat there is the overfishing that is probably responsible for some ecological imbalance on coral reefs that could prolong recovery from other disturbances.

In addition “*This species is targeted for the aquarium trade. Indonesia is the largest exporter with an annual quota of 5,600 live pieces in 2005*” (Hoeksema B.W. et al. 2014). Coral disease are emerged as a serious threat to coral population worldwide. The increasing number of disease and population affected is related to the deterioration of the quality of the water and to the increasing of the sea surface temperature (Willis *et al.* 2007).

Localized threats to corals include invasive species; changes in native species dynamics; fisheries; dynamite and chemical fishing; human development; domestic, agricultural and industrial pollution; human recreation and tourism activities and sedimentation.

*Pocillopora* species are preyed on by some different type of consumers including species that scrape skeletal surface (Trizopagurus, etc), species that bite off colony branch-tips (Scaridae, etc.), Species that abrade tissues and skeleton (*Eucidaris galapagensis*), Species that remove tissues but leave the skeleton intact (*Stegastes acapulcoensis*, *Acanthaster planci*, etc.) (Glynn 2001, Hoeksema B.W. et al. 2014).

## 1.6 Genes description

The genes useful for this study were chosen from two different kind of possible genes. The first type is the basic life genes. It includes all those fundamental genes needed for the normal cellular metabolism. From this pool, ATP Synthase's and NADH Dehydrogenase's genes were chosen. The second genes type is that one that intervene during the stress event to restore the constitutive cellular environment.

The first two genes encode for two fundamental protein complexes for the process of ATP production: the Oxidative Phosphorylation (Figure n.1.9). This process represents the final stage of the energetic metabolism of the aerobic organisms. All the enzymatic step of the oxidative degradation of the carbohydrates, of the fat acids and of the aminoacids within the aerobic cells converge to the final step of the cellular respiration (Nelson D.L. and Cox M.M.). In this stage the oxidative energy is used for the production of ATP. In Eukaryotic cells the oxidative phosphorylation runs in the mitochondria where through the electron provided by NADH and FADH<sub>2</sub> the oxygen is reduced to water. All these processes are based on the Chemiosmotic Theory (Nelson D.L. and Cox M.M.), a Peter Mitchell hypothesis where the energetic transduction is made throw the creation of trans-membrane gradient. The mitochondria have two membranes. The outer membrane is easily permeable from the ion moving throw a transmembrane channels. These channels are part of a family of integral membrane proteins called porins. The inner membrane is almost completely impermeable to all the small molecules and ions, including the protons (H<sup>+</sup>). The only chemical species that can go through it are the ones that have a specific transmembrane transporter. In this structure are included the component of the respiration chain and the ATP synthesis enzymatic complex. ADP and P<sub>i</sub> are transported to the inner mitochondria and at the same time the ATP is transported out of the organelle. The electrons carriers of the respiratory chain are organized in membrane-embedded supramolecular complex. Each of this complex represents part of the respiratory chain and has the capacity to catalyze the electrons transfer thorough the chain (Nelson D.L. and Cox M.M.). The complex I and II transfer the electrons to the ubiquinone from two different donors: NADH (complex I) and succinate (complex II). The Complex III transport the electrons from the ubiquinone to the cytochrome *c* and the Complex IV close the sequence carrying the electrons to the oxygen (Djafarzadeh R., 2000).



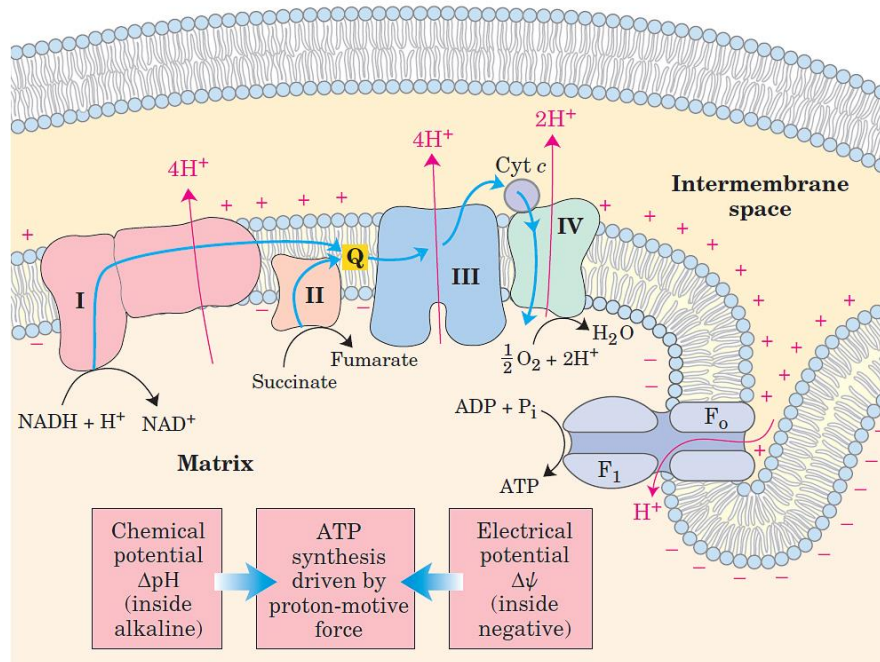
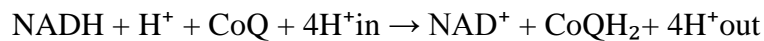


Figure n.1.9 – Chemiosmotic model (Nelson D.L. and Cox M.M.)

### 1.6.1 NADH dehydrogenase (NDH: Bay, L. K., 2013)

The oxidative phosphorylation starts in the respiratory chain with the electrons input. The majority of these electrons are from the action of dehydrogenase enzymes working in the catabolic processes. These electrons are transported to the universal acceptor: the nicotinamide nucleotides (NAD<sup>+</sup> or NADP<sup>+</sup>) or flavin nucleotides (FMN or FAD) (Fearnley I. M. & Walker J. E. 1992). The complex I is a large dimension enzyme with more than 42 different polypeptide chains including a FMN-containing flavoprotein and at least six iron-sulfur centers. The oxidized flavin nucleotide (FMN) can accept either one electron or two (Djafarzadeh R., 2000). Electron transfer occurs because the flavoprotein has a higher reduction potential than the compound oxidized. In the iron-sulfur center the iron is associated with inorganic sulfur atoms or with the sulfur atoms of Cys residues in the protein, or both. All iron-sulfur proteins are involved in redox reactions with one-electron transfer in which one iron atom of the iron-sulfur cluster is oxidized or reduced. The Complex I it is a L-shaped protein with one arm of the L in the membrane and the other extending into the matrix (Djafarzadeh R., 2000). This NAD<sup>+</sup>-linked dehydrogenases remove two Hydrogen atoms from their substrates. One of these is transferred as a Hydride ion (:H<sup>-</sup>) to the NAD<sup>+</sup>; the other ion is released to the medium as a H<sup>+</sup>.



(Nelson D.L. and Cox M.M.)

Simultaneously it catalyzes the endergonic transfer of four protons from the matrix to the intermembrane space (Figure n.1.10).

The Complex I is a proton pump driven by the energy of electron transfer. The reaction catalyzed is vectorial because it moves the protons in one specific direction from one location (the matrix, which becomes negatively charged with the departure of protons) to another (the intermembrane space, which becomes positively charged).

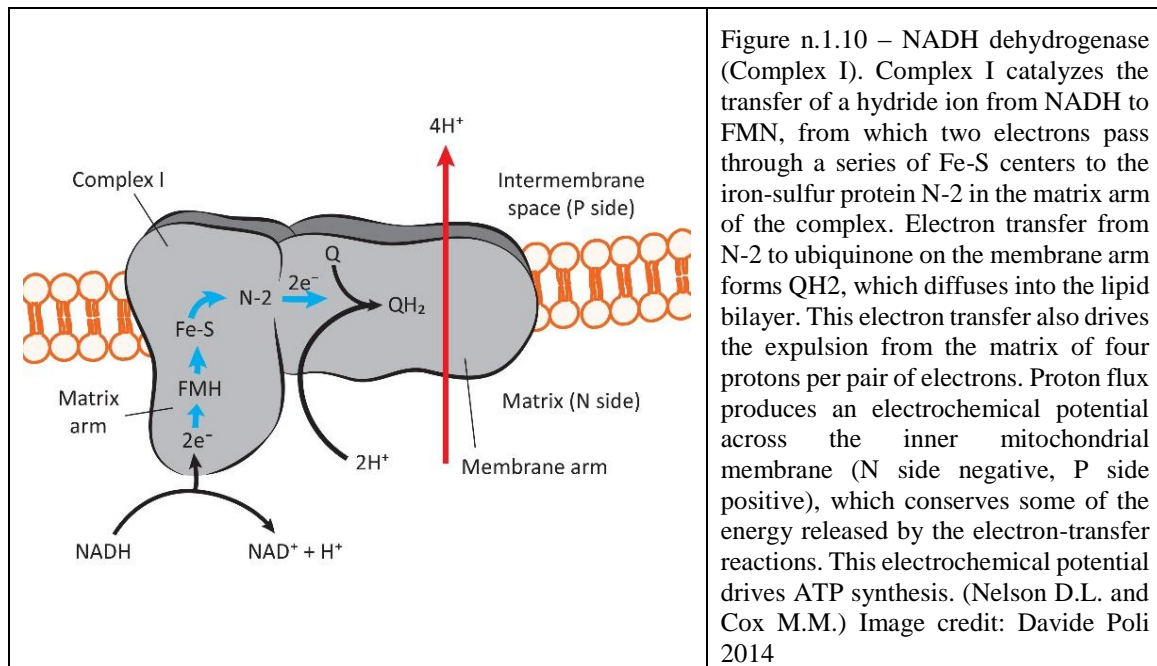


Figure n.1.10 – NADH dehydrogenase (Complex I). Complex I catalyzes the transfer of a hydride ion from NADH to FMN, from which two electrons pass through a series of Fe-S centers to the iron-sulfur protein N-2 in the matrix arm of the complex. Electron transfer from N-2 to ubiquinone on the membrane arm forms QH<sub>2</sub>, which diffuses into the lipid bilayer. This electron transfer also drives the expulsion from the matrix of four protons per pair of electrons. Proton flux produces an electrochemical potential across the inner mitochondrial membrane (N side negative, P side positive), which conserves some of the energy released by the electron-transfer reactions. This electrochemical potential drives ATP synthesis. (Nelson D.L. and Cox M.M.) Image credit: Davide Poli 2014

### 1.6.2 ATP synthase

The F-type ATPase active transporters play a central role in energy-conserving reactions in mitochondria, bacteria, and chloroplasts. The F-type ATPases catalyze the uphill transmembrane passage of protons driven by ATP hydrolysis (Boyer P.D. 1997). The mitochondrial ATP-synthase is one of these ATPase and it catalyzes the formation of ATP from ADP and P<sub>i</sub> as starting substrates, coupled with the proton flux from the P side to the N side of the membrane. The ATP synthase, also known as Complex V, has two distinct components: F<sub>1</sub>, a peripheral membrane protein, and F<sub>o</sub> (“o” denoting oligomycin-sensitive), which is integral to the membrane. In the laboratory it has been demonstrated that the F<sub>1</sub> subunit can catalyze electron transfer from NADH to O<sub>2</sub> even if separated from the F<sub>o</sub> subunit but cannot produce a proton gradient: F<sub>o</sub> has a proton pore through which protons leak as fast as they are pumped by electron transfer, and without a proton gradient the F<sub>1</sub>-depleted vesicles cannot make ATP (Nelson D.L. and Cox M.M.). Every F<sub>1</sub> complex has nine subunits of five different types, with the composition  $\alpha_3\beta_3\gamma\delta\epsilon$  (Figure n.1.11).

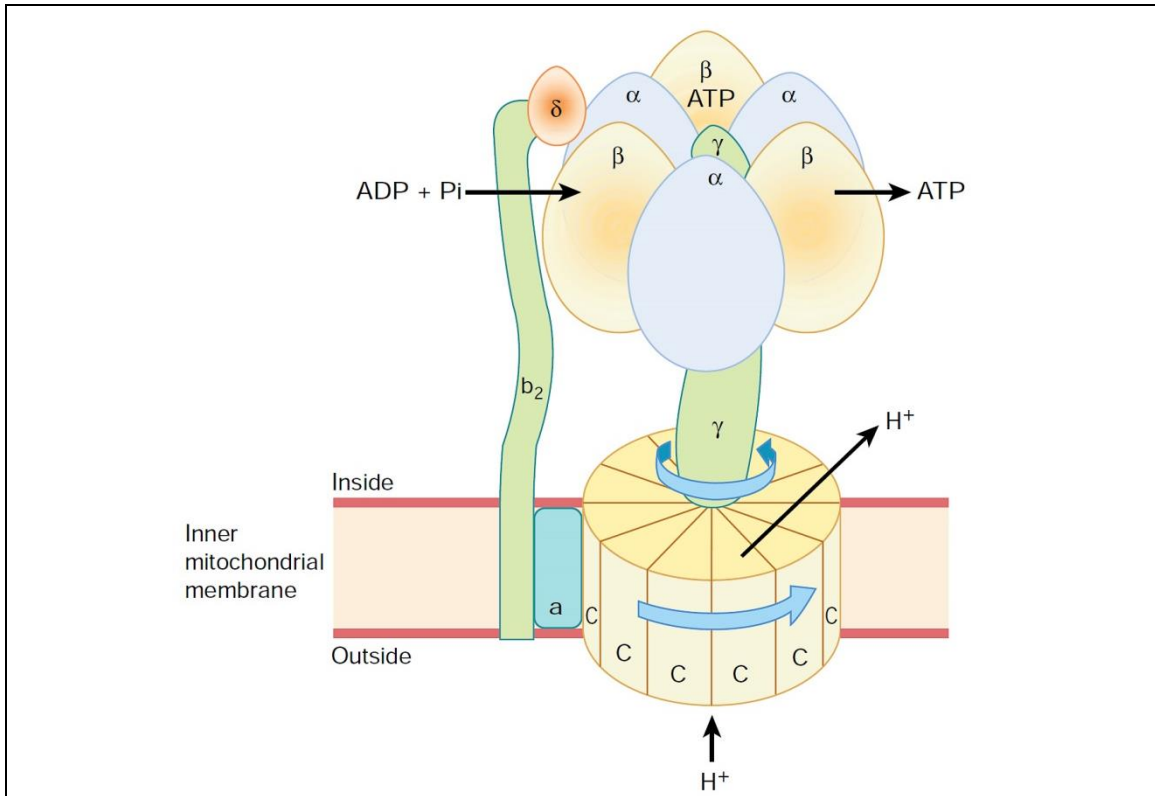


Figure n.1.11 - Mechanism of ATP production by ATP synthase. (Murray R.K. 2009)

The F<sub>1</sub> portion is a flattened sphere, 8 nm high and 10 nm across, consisting of alternating  $\alpha$  and  $\beta$  subunits arranged like the sections of an orange. The amino acid sequences of the three  $\beta$  subunits are identical but they have different conformation. These conformational differences among the  $\beta$  subunit extend to differences in their ATP/ADP-binding sites. One of the three subunits binds ATP, one ADP and one is empty because it is bound with the  $\gamma$  subunit and it is called  $\beta$ -empty. The other two are called respectively  $\beta$ -ATP and  $\beta$ -ADP (Capaldi R.A. and Aggeler R. 2002). On the basis of kinetic studies, Paul Boyer proposed the rotational catalysis mechanism in which the three  $\beta$  active sites of the F<sub>1</sub> subunit take turns catalyzing ATP synthesis (Boyer P.D. 1997). The F<sub>0</sub> complex making up the proton pore is composed of three subunits, a, b, and c, in the proportion Ab<sub>2</sub>c<sub>10-12</sub>. Subunit c is a small very hydrophobic polypeptide, consisting almost entirely of two transmembrane helices, with a small loop extending from the matrix side of the membrane. The flow of electrons through Complexes I, III, and IV results in pumping of protons across the inner mitochondrial membrane, making the matrix alkaline relative to the intermembrane space (Senior A.E. et al. 2002). This proton gradient provides the energy (in the form of the proton-motive force) for ATP synthesis from ADP and P<sub>i</sub> by ATP synthase (F<sub>0</sub>F<sub>1</sub> complex) in the inner membrane. ATP synthase carries out “rotational catalysis,” in which the flow

of protons through  $F_o$  causes each of three nucleotide-binding sites in  $F_1$  to cycle from (ADP + Pi)-bound to ATP-bound to empty conformations (Capaldi R.A. and Aggeler R. 2002). ATP formation on the enzyme requires little energy; the role of the proton-motive force is to push ATP from its binding site on the synthase.

### 1.6.3 HSP70

With the HSP70 term (Heat Shock Proteins 70 kilodaltons) is indicated a protein family both important for the normal proteins folding processes and involved in the heat shock response assisting the stress-denatured proteins. This family was discovered accidentally in the 60s during a study on *Drosophila* often called "fruit flies". Involuntarily one sample of this species was exposed to a higher temperature of the normal study case and during the microscopic observation of the chromosomes was noticed an elevated transcription of these unknown proteins (Yoshimune K et al., 2002). This unpredicted response has been called "Heat Shock Response" and these proteins have been called "Heat Shock Proteins" (HSPs). As central components of the cellular protein surveillance network these ATP-dependent chaperones are involved in a large variety of protein-folding processes and they are highly conserved in evolution (Mayer MP. 2013). HSP70s demonstrate a 60–78% base identity among eukaryotic cells and a 40–60% identity between eukaryotic HSP-70 and *Escherichia coli* DnaK (Caplan et al., 1993). These chaperones are central players in protein folding and proteostasis control and they regulate a large number of protein folding such as de novo folding of polypeptides, refolding of misfolded proteins and the regulation of stability and activity of certain natively folded proteins that were regulated in their activity, stability, and/or oligomeric state (Mayer MP. and Bukau B. 2005). This Chaperone machine is composed of an N-terminal 40-kDa ATP-binding domain and a C-terminal 30-kDa substrate-binding domain (SBD). The wide applicability of HSP70 is because they don't enclose entirely their substrate but they bind just a short part of it in the SBD domain. In this way their application is not limited by the dimension of the substrate. In this way, the HSP group is very versatile assisting in a large amount of protein-folding processes (Richter et al. 2010) from de novo polypeptide folding to translocation of proteins across the membrane. This features, linked to the fact that genes encoding HSP70 lack introns allowing a more rapid response to stress (Boutet et al. 2003), reflect the frequency of up-regulation and concentration during stress situation (Lewis et al. 1999). The interaction with substrates is regulated by the nucleotide status of the N-terminal nucleotide-binding

domain (NBD) (Figure n.1.12) (Mayer M.P. 2010). The ATP-dependent reactions are regulated by co-chaperones of the HSP40 (also known as DnaJ) (Hartl F.U et al. 2011). This because Hsp70s do not work alone but with a team of co-chaperones because the intrinsic ATP hydrolysis rates of Hsp70s are generally low, with one molecule of ATP being hydrolyzed per Hsp70 molecule per 20–30 min at 30°C (e.g., *Escherichia coli* DnaK, human Hsp70)(Mayer M.P. Et al. 2000; Gässler C.S. et al. 2001 Mayer M.P. 2013). In the ATP-bound state, association of peptides to the SBD and peptide dissociation from the SBD occurs at high rates, but affinity for polypeptides is low (Mayer M.P. 2010; Mayer M.P. 2013). In the ADP-bound and nucleotide-free states, after ATP hydrolysis by the NBD, peptide association and dissociation rates are decreased by several orders of magnitude, leading to an increase in affinity for substrate peptides and a low exchange rates (Schmid D. et al. 1994).

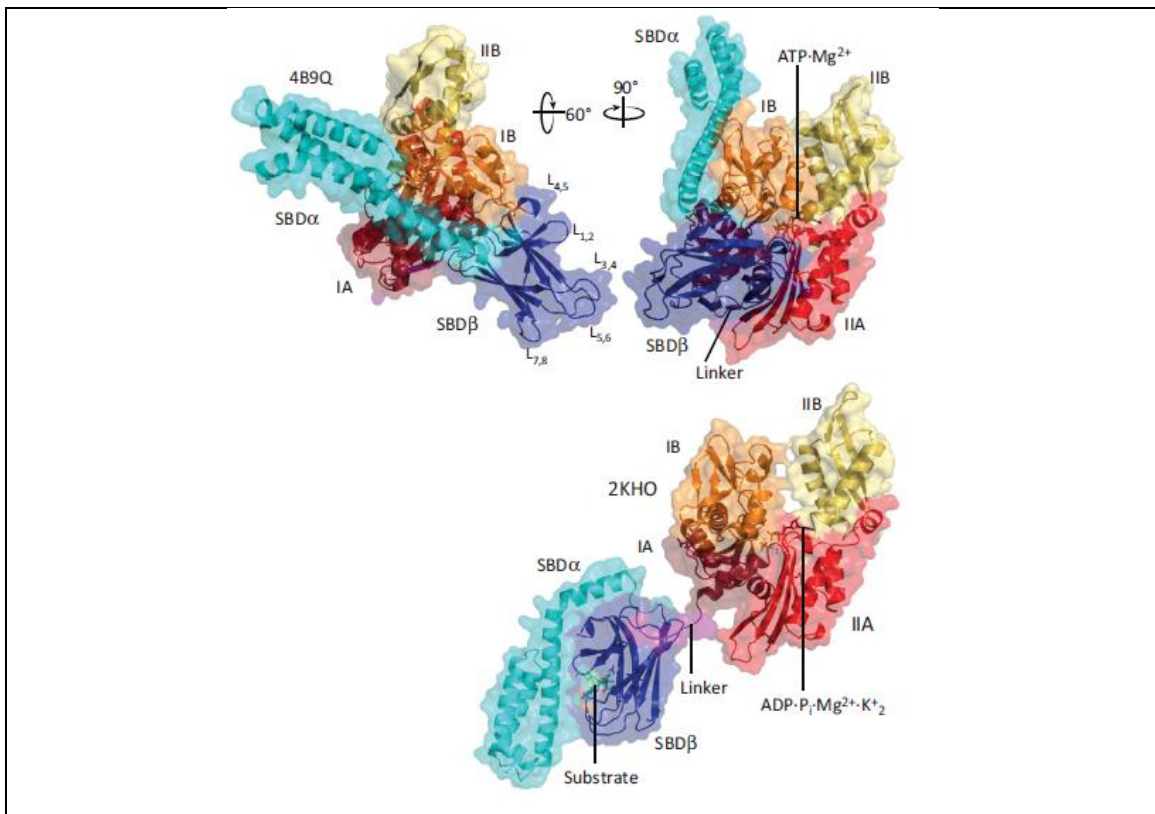


Figure n.1.12 - Cartoon and surface representation of the ATP-bound open conformation of *Escherichia coli* DnaK (PDB code 4B9Q), in two orientations as indicated, and of the ADP-bound closed conformation of *E. coli* DnaK (PDB code 2KHO; lower panel); NBD is in the same orientation as upper right panel. Subdomains of the NBD are shown in dark red (IA), red (IIA), orange (IB), and yellow (IIB); the linker is in magenta, SBDβ is dark blue and SBDα is cyan, and the substrate is green. The loops of the SBDβ are designated as L1,2 to L7,8 (Bertelsen, E.B. et al. 2009)

## 2 AIM OF THE PROJECT

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The aim of this study was to increase the understanding of the basic cellular processes responsible for enhanced stress tolerance that may enable organisms to tolerate climate change. In particular, for reef-building corals dramatic declines in abundance are expected to worsen as anthropogenic climate change intensifies. Recent evidence has called into question whether corals have the capacity to acclimatize or adapt to global climate change and some groups of corals may possess inherent physiological tolerance to environmental stress. Such a physiological tolerance would enable specific corals to cope with future climate change. Different corals differ substantially in physiological resilience to environmental stress, but the molecular mechanisms behind enhanced coral resilience remain unclear.

At the molecular level, enhanced thermotolerance in other marine organisms, including intertidal limpets, mussels, and sea cucumbers, has been related to higher basal expression of heat shock proteins, mainly HSP70, together with the ability to develop an inducible HSP response, activation of some enzymes, cytoskeletal reorganization, cell signaling and transcriptional modulation. Recently, a wide transcriptomic approach was carried out in *Acropora hyacinthus* addressing the genomic basis for coral resilience.

The present work focused on three specific gene products (namely ATP synthase; NADH dehydrogenase and Heat Shock Protein 70kDa ) and mRNA expression patterns underlying differences in thermal resilience were investigated in specimen of the common reef-building coral *Pocillopora verrucosa* collected at different locations in Bangka Island waters (North Sulawesi; Indonesia). Moreover, to identify potential mechanisms behind physiological resilience a thermal stress experiment using 174 colonies of *P. verrucosa* was performed under controlled conditions.

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# 3 MATERIALS AND METHODS

## Summary

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- **Samples Collection**  
Each site was been characterized in order to understand the percentage cover of the chosen species and the principal categories of organism.
- **Animal holding conditions and experimental setup**  
Experimental design, methods and equipment to perform the experiment.
- **mRNA Extraction, and cDNA preparation**  
Techniques utilized to prepare the mNA for the Gene Expression Analysis.
- **Real Time PCR assays**  
Instrument and methods
- **Statistical Analysis**

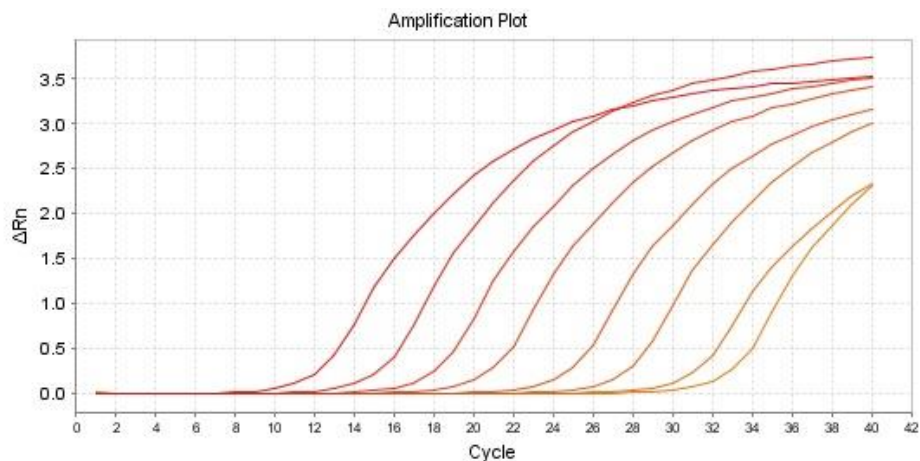


Figure n.3.1 - Amplification plot of the RT-qPCR “Coralli 33\_22 Gen 2014”

### 3.1 Samples Collection

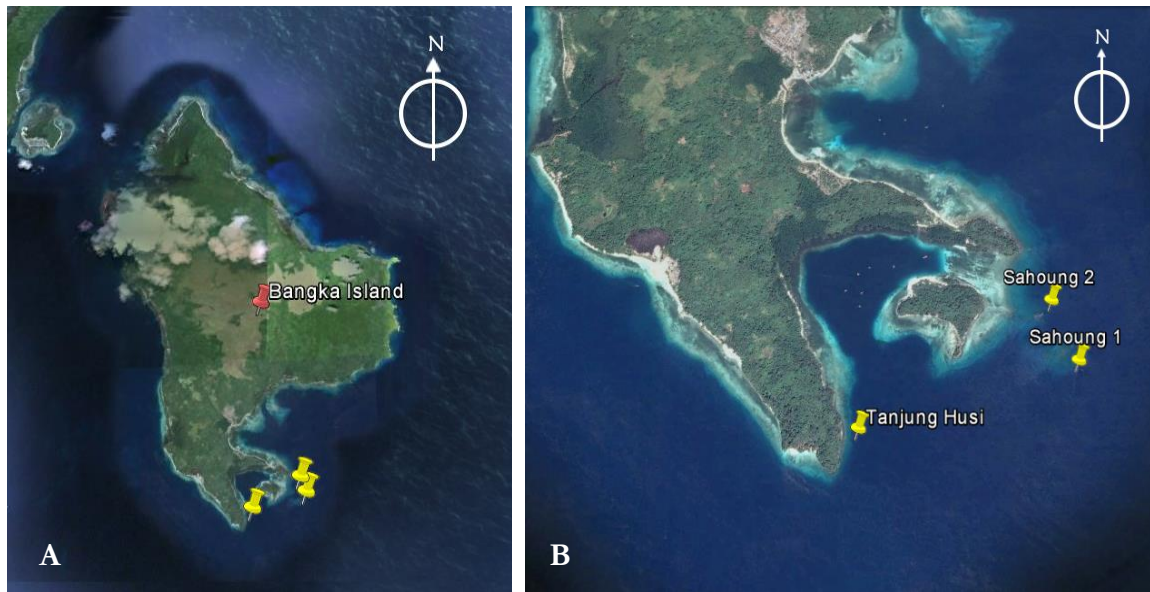


Figure n. 3.2 a and b – Geographic location of the three sampling sites Sahoung 1 (SA1), Sahoung 2 (SA2), and Tanjung Husi (TA) located in the South-Est side of the island. Relative distance between SA1 and SA2: 210m; Relative distance between SA1 and TA: 1350m; Relative distance between SA2 and TA: 1370m. (Base Map: Google earth).

Experiments were performed at the CoralEye Reef Research Outpost (Bangka Island, North Sulawesi; Indonesia). Corals were collected at three different sampling sites, namely Sahoung 1 (SA1), Sahoung 2 (SA2), and Tanjung Husi (TA) located in the South-Est side of the island and sharing similar chemical-physical conditions (Table n. 3.1).

	Sahoung 1 (SA1)		Sahoung 2 (SA2)		Tanjung Husi (TA)	
GPS position	1°44'38.026" N 125° 9'49.845" E		1°44'39.956" N 125° 9'43.298" E		1°44'14.94" N 125° 6.162" E	
Orientation	N-E		N-E		N-E	
	Low depth (L)	High depth (H)	Low depth (L)	High depth (H)	Low depth (L)	High depth (H)
Depth (m)	3	12	3	12	3	12
Temperature (°C)	27	27	27	27	28	27
pH	8.81	8.57	8.68	8.85	8.77	8.72
Salinity (psu)	34	35	35	34	35	35

Table n. 3.1 – Water parameters of the sampling sites

A preliminary characterization of the sampling sites was also performed to assess whether a suitable percentage of cover for the selected coral species occurred. For each site, 5 x 10 m Line Intercept Transects (LIT) (Bianchi et al. 2003) (Figure n.3.3 and n.3.4) were made at a 3-m and 12-m LLT depth. The 3 m depth was been chosen for the proximity to the surface during the Lowest Low Tide (LLT), when the organisms could be exposed out of

the water. The 12 m depth was been chosen because the temperature becomes stable below 10 m.

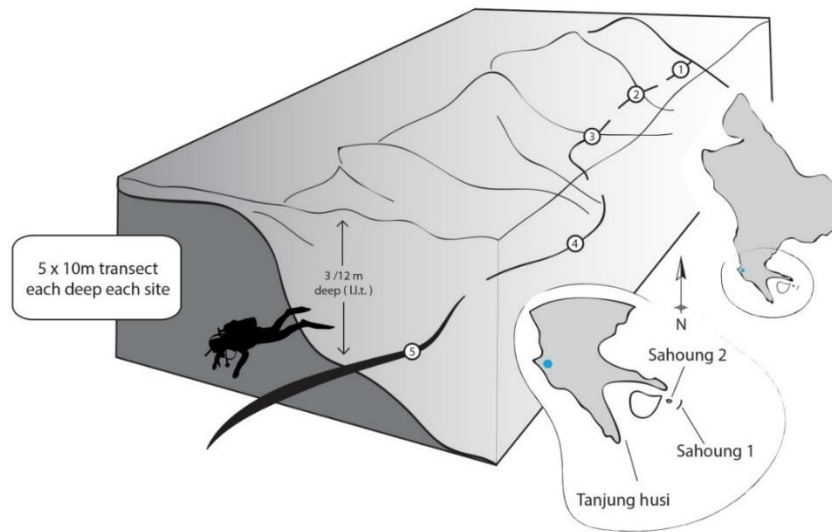


Figure n. 3.3 – Scheme of the characterization method. 5 Line Intercept Transect (LIT) of 10m each one were made. In order to ensure the data independence the LIT were separated each other by 10m without investigation. (Davide Poli 2014).

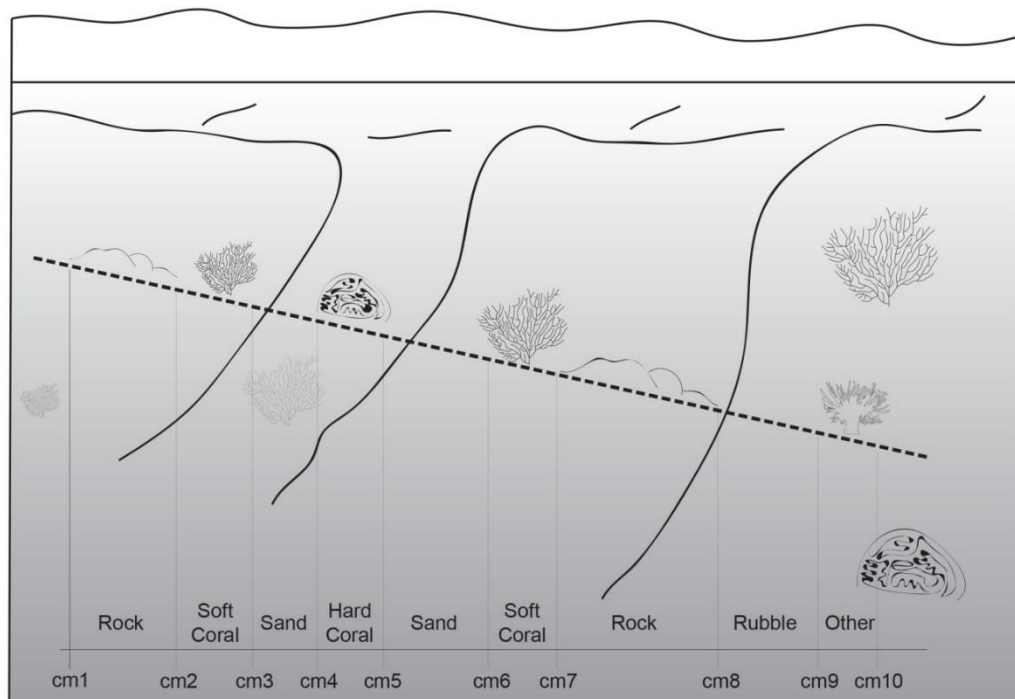


Figure n. 3.4 – Line Intercept Transect. It is recorded the intercept point, at the centimeter details, where the category of organisms or substratum changes below the metric line. (Davide Poli 2014).

Seven categories of organism were visually classified: rocky corals (R), sand corals (G), soft corals (Sc), hard corals (HD), *Acroporidae* (A), *Pocilloporidae* (P), Others (O). The two genus *Acroporidae* and *Pocilloporidae* were chosen because of their relevant role as

reef constructors and wide distribution in the Indo-Pacific area (Wallace C.C. and Wolstenholme J. 1998; Hoeksema B.W. et al. 2014).

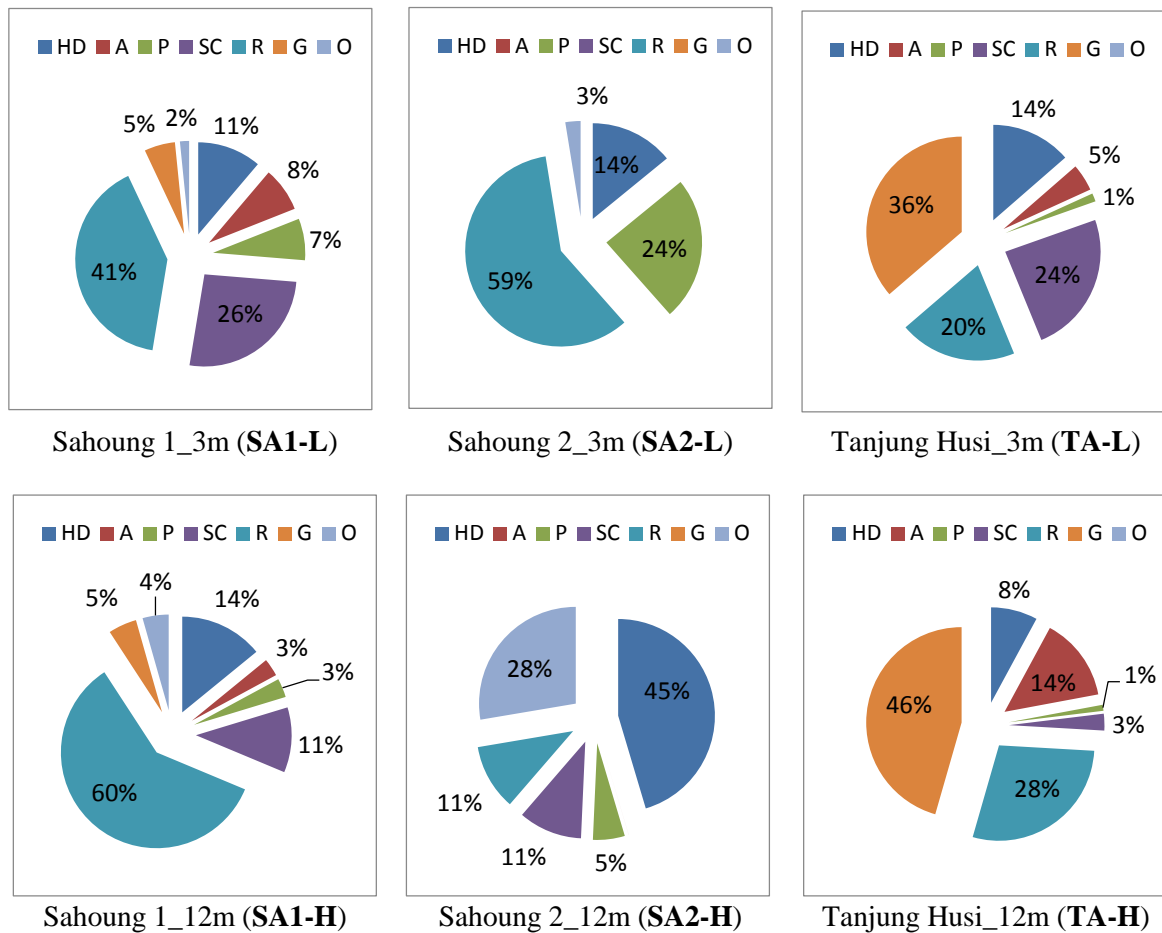


Figure n. 3.5 – Percentage cover of the selected categories of organisms [rocky corals (R), sand corals (G), soft corals (Sc), hard corals (HD), Acroporidae (A), Pocilloporidae (P), Others (O)] at the selected sampling sites (Sahaoung 1, Sahaoung 2, and Tanjung Husi) at low (3 m) and high (12 m) depths. To evaluate the Percentage cover 5 Line Intercept Transect (LIT) of 10m each one were made. In order to ensure the data independence the LIT were separated each other by 10m without investigation.

Data characterization reported in Figure n. showed similar compositions between SA1 and SA2, while TA showed a different distribution despite displaying temperature, salinity, pH, orientation (Table n).

### 3.2 Animal holding conditions and experimental setup

Small branches (~ 3cm<sup>2</sup>) of *P. verrucosa* were collected from 174 different coral colonies from the three different sites at the established depths (29 for each sampling site and depth). Five random branches for each site and depth were immediately immersed in a suitable volume of the RNAlater® stabilization reagent (Sigma-Aldrich, Milan, Italy) and stored at -20°C until analysed. These samples were further employed to assess

transcriptional levels of corals under field conditions. The remaining 24 branches per site per depth were randomly divided in groups of three branches, placed in eight experimental aquaria (Figure n.3.6) filled with 20L of natural sea water, and maintained with a movement pump (75 l/h) and continuous oxygenation. Corals spent a 14-days period of acclimatization to the laboratory conditions; in particular, a constant water temperature of  $28\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$  close to the mean value at the collection sites was employed along with a natural photoperiod (light-dark cycle, 10L:14D). The aquaria were filled through a system of two 150-L tanks which were constantly filled with seawater taken on-site at ~100 m from the shore (Figure n.3.7a and b). To prevent the stagnation, the water was moved by movement pump. It was not necessary to use a filtering system because the water was gradually but constantly flowed through the aquaria with a complete water change every 6 hours, so that the elimination of metabolites was constant while maintaining unchanged the water parameters, which were monitoring every 6 hours through a multi-parametric instrument (ADWA AD12) and densimeter (Milwaukee MR100 ATC).

After the acclimatization period, coral samples were collected from each treatment condition to account for animal physiological status at the onset of the experimental exposure to increased water temperature, thus representing the “zero time” ( $T_0$ ) of the time-course evaluation. At the onset of the experiments, aquaria were randomly divided in two groups of controls (temperature  $28\text{ }^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ) and thermally-challenged corals, in which water temperature was elevated at  $31\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$  according to Barshis et al. (2013) and Kenkel et al. (2013). The experimental treatment were brought to  $31\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$  over 12 h during the day, after which the temperature was reduced to ambient condition of  $28\text{ }^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$  over 12 h during the darkness according to Mayfield et al. 2013.

Samplings were performed after 3 days and 7 days of exposure to the increased temperatures both in controls and thermally-challenged corals, and samples were immediately preserved in the RNAlater® solution (Sigma-Aldrich) and stored at  $-20\text{ }^{\circ}\text{C}$  until subsequent analysis. Four replicates were employed for each experimental condition ( $N = 4$ ).

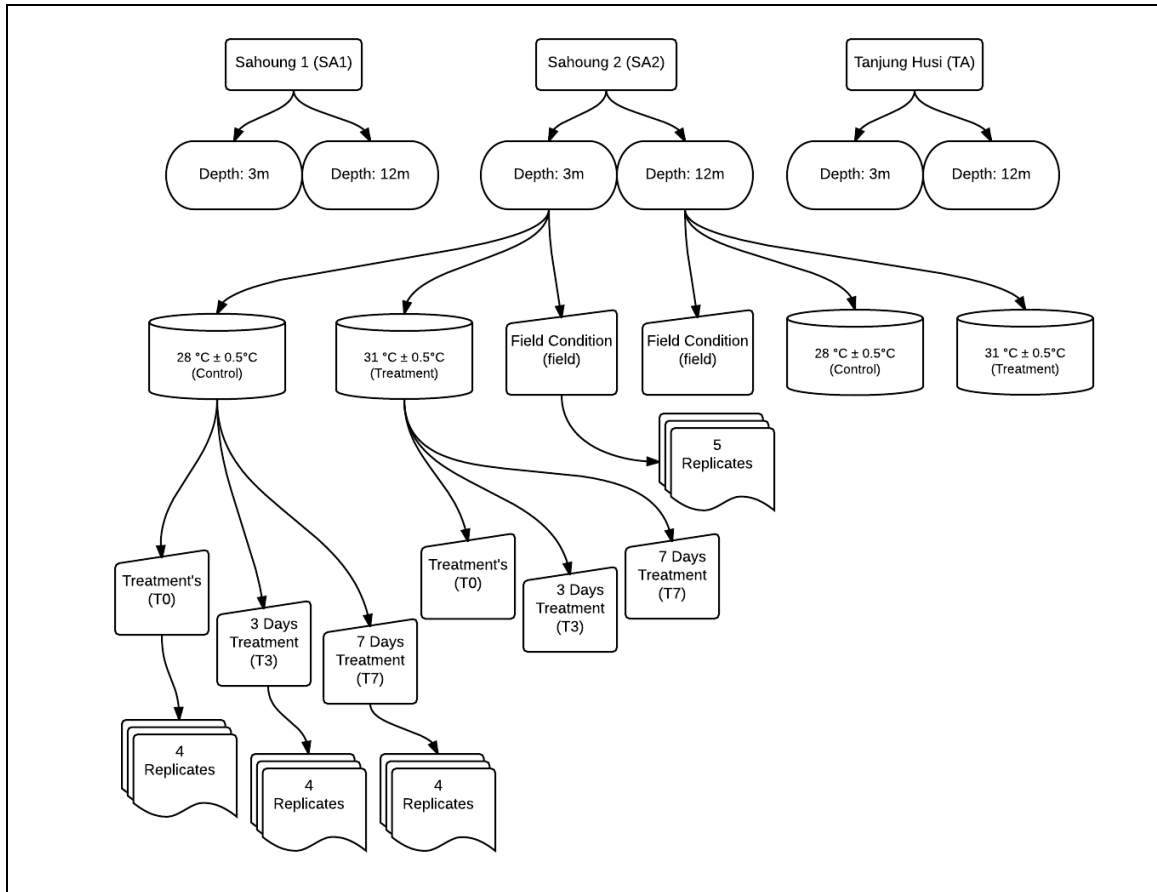


Figure n.3.6 - Experimental design flow chart. All the procedures were developed for each site (SA1, SA2, TA). Small branches of *P. verrucosa* were collected from 28 different coral colonies from each of the three different sites (SA1, SA2, TA) at each established depth (3 m and 12 m). Five randomly selected branches for each site and depth were immediately immersed in RNALater® to further assess transcriptional levels showed by corals under field conditions. The remaining 24 branches per site per depth were randomly divided in groups of three branches and placed in eight experimental aquaria (4 each experimental condition).

T0 are coral samples collected at the onset of the experimental exposure to increased water temperature and representing the “zero time”. T3 and T7 are the coral samples collected after respectively 3 and 7 days of exposure to the experimental conditions (31 °C ± 0.5 °C over 12 h during the day, 28 °C ± 0.5 °C over 12 h during the darkness).

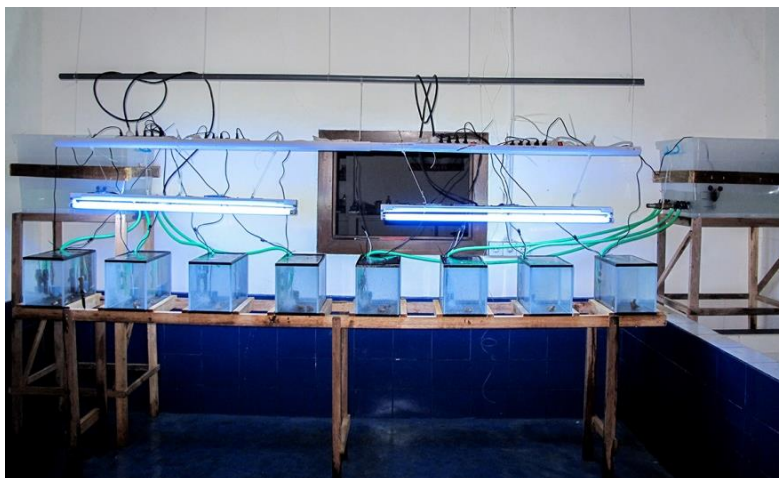


Figure n. 3.7 a – Scheme of the experimental equipment (Davide Poli 2014)

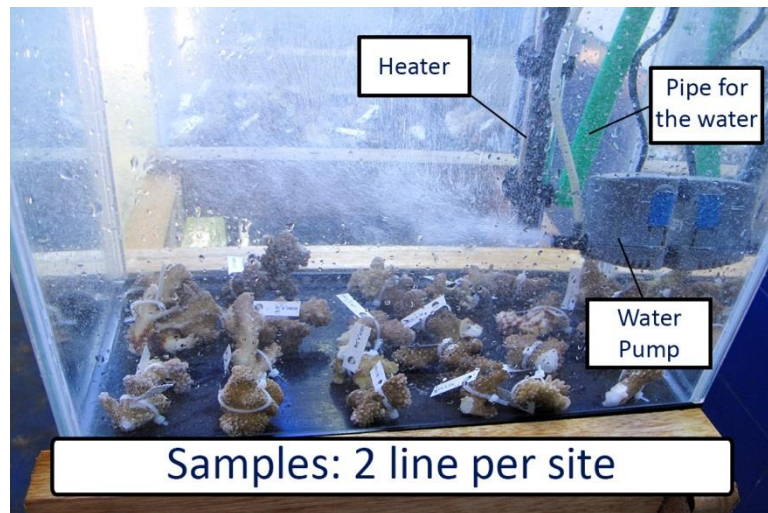


Figure n.3.8 b – Scheme of the setup of the aquariums ( Davide Poli 2014)

### 3.3 RNA Extraction and cDNA preparation

A small piece of coral branch (about 1 cm<sup>3</sup>) was mechanically homogenized in a suitable volume of the Tri-Reagent® (Sigma Aldrich), and total RNA was extracted according to the manufacturer's protocol (Sigma Aldrich). The total RNA was resuspended in a suitable volume of DEPC-treated molecular-grade milliQ water (Life Technologies, Milan, Italy). RNA concentration and quality were verified using the Qubit RNA assay (Life Technologies, Milan, Italy) and electrophoresis using a 1.2% agarose gel under denaturing conditions. First strand cDNA for each sample was synthesized from 600 ng total RNA using the iScript supermix following manufacturer's protocol (Biorad Laboratories, Milan, Italy). cDNA samples were further stored at – 20°C until analysed.

### 3.4 Real time PCR assays

Transcriptional analyses were conducted using Real Time quantitative Polymerase Chain Reaction (qPCR) assays. Expression levels of three target transcripts were evaluated in *P. verrucosa*; these transcripts encode an ATP synthase (*ATPs*), a NADH dehydrogenase (*NDH*) and a 70kDa heat-shock protein (*HSP70*). Target specific primers were designed using Primer Express (Life Technologies, Milan, Italy) using nucleotide retrieved from the GeneBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) for *P. verrucosa* (Table n.3.2).

<b>Table n.3.2</b>				
Transcript	Abbreviation	GeneBank Ac. Numb	T <sub>m</sub> (°C)	qPCR Efficiency
ATP synthase	ATPs	JX985612	60°C	122.60%
NADH dehydrogenase	NDH	Primer pair was deduced* from a partial sequence of <i>P. damicornis</i> (GeneBank Accession DQ351262)	60°C	120.06%
Heat Shok Proteine 70kDa	HSP70	Primer pair was deduced* from a partial sequence of <i>P. damicornis</i> (GeneBank Accession JX624896.1)	60°C	118.26%
*sequences actually under submission process to the GeneBank database				

The qPCR technique is based on real-time monitoring of amplicon formation by a reporter molecule (e.g., SYBR green dye). Fluorescence is measured after each temperature cycle and is proportional to the amount of synthesized amplicon. The exponential growth of the amplicon concentration in the reaction mixture is described as an exponential function of the template starting concentration (Bustin et al. 2004). Two parameters are essential for quantification: the threshold cycle,  $C_T$ , and the qPCR efficiency (E). The  $C_T$  is the number of cycles necessary to reach a certain threshold fluorescence. In one experimental setup, the threshold is the same for all samples. Since fluorescence is a relative measure of the DNA content, samples containing higher amounts of target DNA will have lower  $C_T$ , while samples holding lower amounts of target DNA will have higher  $C_T$  values. E is a measure of amplification quality and depends on factors such as the primer GC content, primer mismatch, and the presence of PCR inhibitors. If E equals 2, the number of amplicons doubles per cycle, i.e., the efficiency is 100%.

At a first stage, we attempted to set-up a relative quantification protocol to infer changes in mRNA expression of the selected transcripts. Relative quantification using the  $\Delta\Delta C_T$  method is the most common method used in mRNA expression analyses (VanGuilder, et al. 2008). The method determines the expression ratio (or fold change) of a target transcript in a sample compared to a control, normalizing with the expression ratio of an internal



reference transcript, to control for sample-to-sample variations enabling comparisons of mRNA concentrations across different experimental conditions. Hypothetically, the ideal reference gene product is expressed at stable levels irrespective of tissue type, species, treatment, metabolism or sampling conditions, and computational approaches have to be used to infer suitability of a transcript as a putative reference transcript. According to the standardized guidelines (MIQE guidelines; Bustin et al., 2009), this is a mandatory prerequisite for qPCR data publication in mammalian model species. Although in studies with non-model organisms, the lack of large set of genomic data has heavily limited these approaches, the increased awareness of the importance of systematic validation of reference gene selection is raised also considering the potential confounding factors given by environmental pressures on animal transcriptional profiles evaluated under field conditions (Maes et al. 2013).

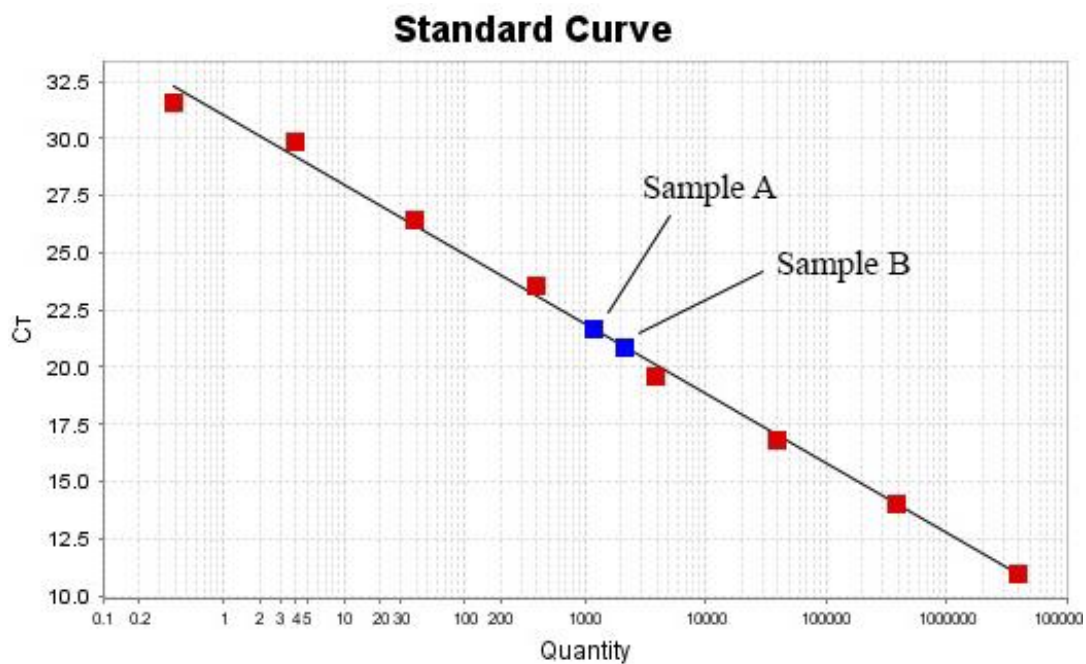
We identified three candidate reference transcripts to be used in qPCR experiments with *P. verrucosa* samples. These transcripts encoded the ribosomal proteins L22, L40 and P0. Primers were designed basing on homologous sequences of *P. damicornis*. Representative PCR products for each gene product were cloned and sequenced to verify primer specificity in *P. verrucosa* (sequences are actually under submission process to the GeneBank database) Unfortunately, stability analysis performed using four different computational approaches (Franzellitti et al. submitted) showed that none of those selected transcripts were suitable as reference transcripts for qPCR data normalization in *P. verrucosa* under the experimental conditions employed in this study. As normalization is a critical step representing a relevant methodological bias in our transcriptional investigations with *P. verrucosa*, we decided avoiding the semi-quantitative approach and going through the application of absolute quantification protocols for the selected target gene products.

Absolute quantification by the standard curve method employs a dilution series of known template concentrations ( $N_0$ , expressed as copy numbers) in the qPCR assay. Linear regression of  $\log(N_0)$  versus  $C_T$  gives the standard curve, and this is then used to calculate template concentrations,  $N_0$ , of the samples. This method requires that  $E$  of the sample is the same as  $E$  of the standard.

qPCR standards for each target transcript were prepared by serial dilution of a purified and quantified PCR product to obtain a standard curve of  $C_t$  values vs the logarithmic DNA amount (Figure n3.9).  $R^2$  values for all standard curves were always  $> 0.988$ , and PCR efficiencies of the standards were not significantly different from those of the samples.

Absolute mRNA abundance was calculated from the standard curves and expressed as copy number/ng RNA (mean  $\pm$  SEM).

Real-time PCR reactions were performed in duplicate, in a final volume of 10  $\mu$ L containing 5  $\mu$ L Fast Sybr Green reaction mix with ROX (Life Technologies, Milan, Italy), 2  $\mu$ L diluted cDNA, and 0.2  $\mu$ M specific primers (Table n). A control lacking the DNA template (no template) and a minus-reverse transcriptase (no RT) control were included in the real-time PCR analysis to determine the specificity of target cDNA amplification. Amplification was detected with a StepOne real time PCR system apparatus (Life Technologies, Milan, Italy) using a standard “fast mode” thermal protocol (Figure n 3.10). For each target mRNA, melting curves, gel pictures and sequences were further analysed to verify the specificity of the amplified products and the absence of artifacts. The amplification efficiency of each primer pair was calculated using a dilution series of samples cDNAs or of the qPCR standards (Table n).



Target: <i>P. verrucosa</i> HSP70	Slope: -3.055	Y-Intercept: 31.036	R <sup>2</sup> : 0.996	Eff %: 112.503
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Figure n.3.9 Example of a standard curve obtained for the *HSP70* gene product of *P. verrucosa*. Blue squares represent the samples analyzed related to the standard curve; red squares represent the standards, with 10-fold dilutions of a known concentration of template ( $10^6$ - $10^{-1}$ ), on which the standard curve is constructed

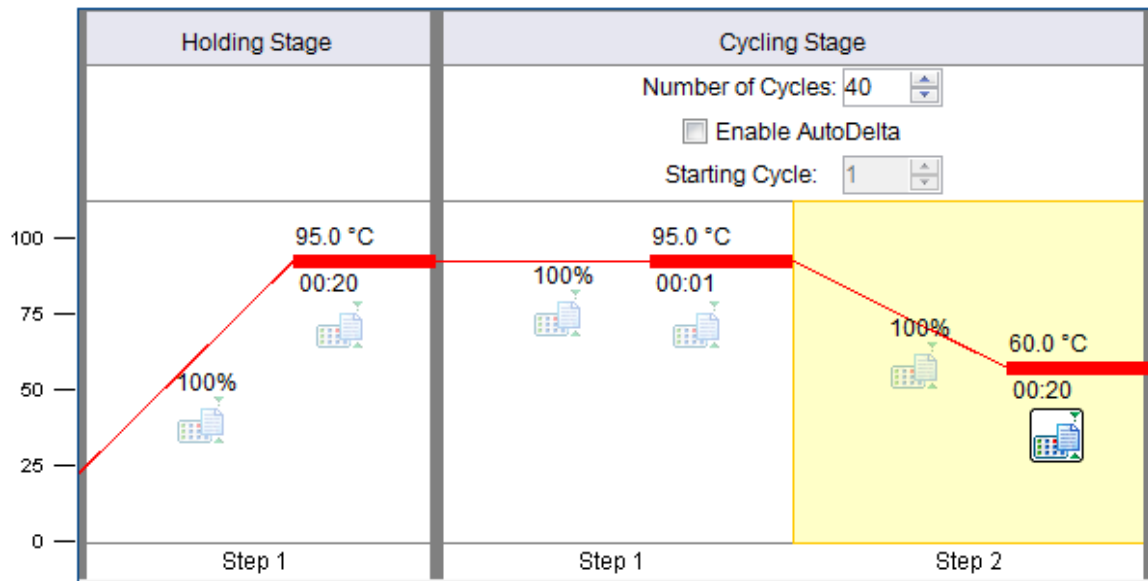


Figure n.3.10 - Standard “fast mode” qPCR thermal protocol

### 3.5 Statistical analyses

Data were analyzed using the SigmaStat statistical package (SPSS Science, ver.3.1). Significant differences between samples were determined using 1-way ANOVA followed by the multiple comparison Bonferroni’s test. Statistical differences were accepted when  $p < 0.05$ .

Factor Analysis using the Principal Component Analysis extraction procedure was performed using the STATISTICA software package (StatSoft) to determine whether coral sampled in different sites and/or at different depths can be discriminated by means of overall variations of mRNA transcriptional profiles.

Clustering of qPCR data was performed to create heatmaps using the Gene Cluster software ver2.0 (Eisen, M. B. et al 1998) and the TreeView software for cluster visualizations. Both software packages are specifically written for the analysis of mRNA expression patterns obtained from microarrays as well as qPCR assays. Similarity was measured by standard correlation.



## 4 RESULTS

### Summary

- Transcriptional profiles under field conditions
- Effects of the acclimatization to the laboratory conditions
- Transcriptional-responses following *P. verrucosa* exposure to seawater temperature increase under laboratory conditions
- Cluster analysis

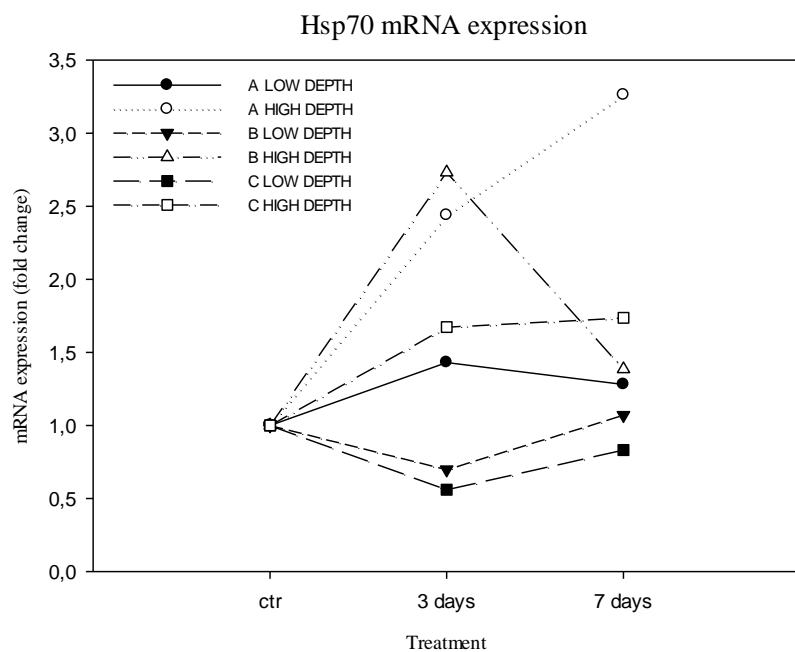


Figure n. 4.1 - example of mRNA expression



#### 4.1 Transcriptional profiles under field conditions

Using absolute quantitative real time PCR, mRNA levels for three selected gene products encoding an ATP synthase (ATPs), a NADH dehydrogeanse (NDH) and a 70kDa heat-shock protein (HSP70), were evaluated in *P. verrucosa* samples immediately after their collection from the selected sampling sites from low depths (3 m) and high depths (12 m). Results reported in Figures 4.2,4.3,4.4 showed that levels of the three transcripts were not significantly different between low- and high-depths collected samples at sites A and B, although mean *HSP70* mRNA levels were always lower in high-depths that in low-depths samples (Figure 4.4). At site C, transcription levels of three gene products were significantly lower in high-depths collected corals with respect to low-depths samples ( $p < 0.05$ ), with a mean 0.47 (*ATPs*), 0.45 (*NDH*) and 0.11 (*HSP70*) fold difference.

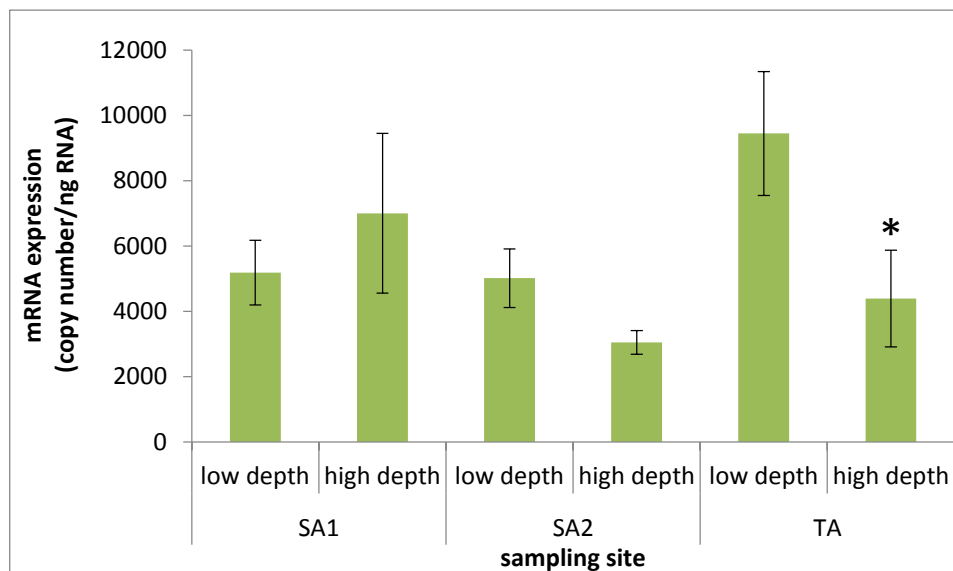


Figure 4.2 – Expression of a *ATP synthase (ATPs)* gene product in *P. verrucosa* immediately after coral collection at the selected sampling sites (Sahaoung 1, Sahaoung 2, and Tanjung Husi) at low (3 m) and high (12 m) depths. mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  sems ( $N = 4$ ) of the copy numbers of each gene product normalized over the nanograms of total RNA employed in a single PCR reaction. SA1: Sahaoung 1; SA2: Sahaoung 2; TA: Tanjung Husi. \* $p < 0.05$  low depth vs high depth within each sampling site (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).

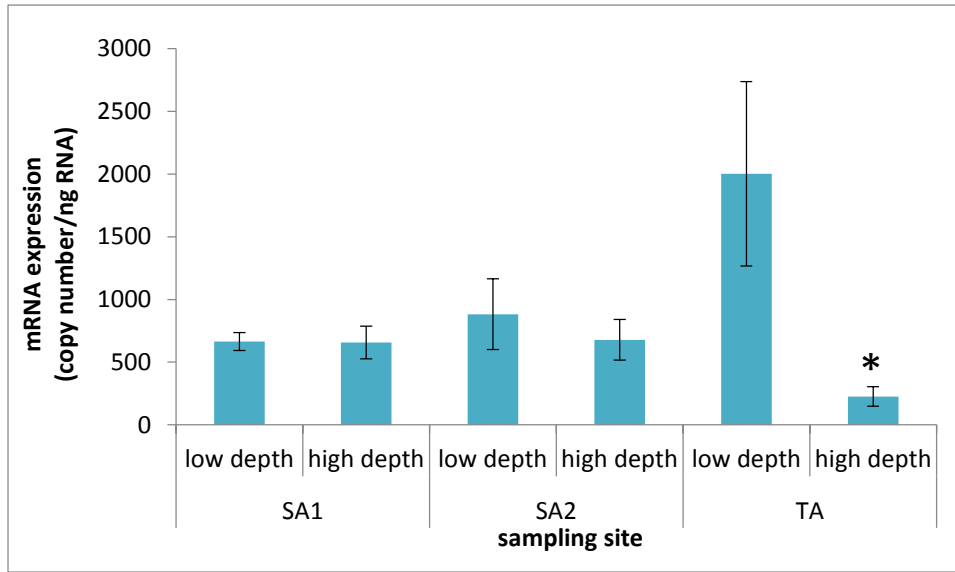


Figure 4.3 – Expression of a *NADH dehydrogenase (NDH)* gene product in *P. verrucosa* immediately after coral collection at the selected sampling sites (Sahaoung 1, Sahaoung 2, and Tanjung Husi) at low (3 m) and high (12 m) depths. mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the copy numbers of each gene product normalized over the nanograms of total RNA employed in a single PCR reaction. SA1: Sahaoung 1; SA2: Sahaoung 2; TA: Tanjung Husi. \* $p < 0.05$  low depth vs high depth within each sampling site (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).

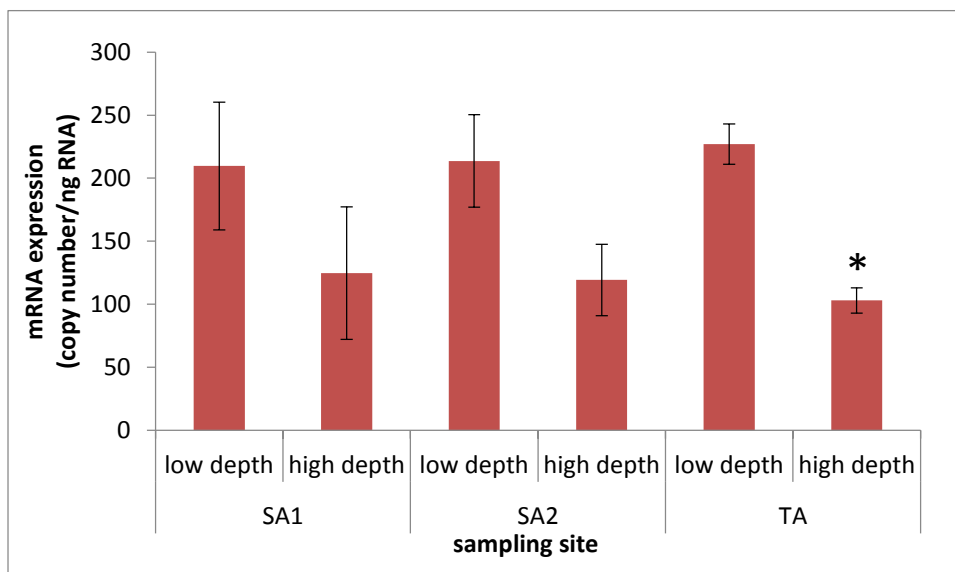
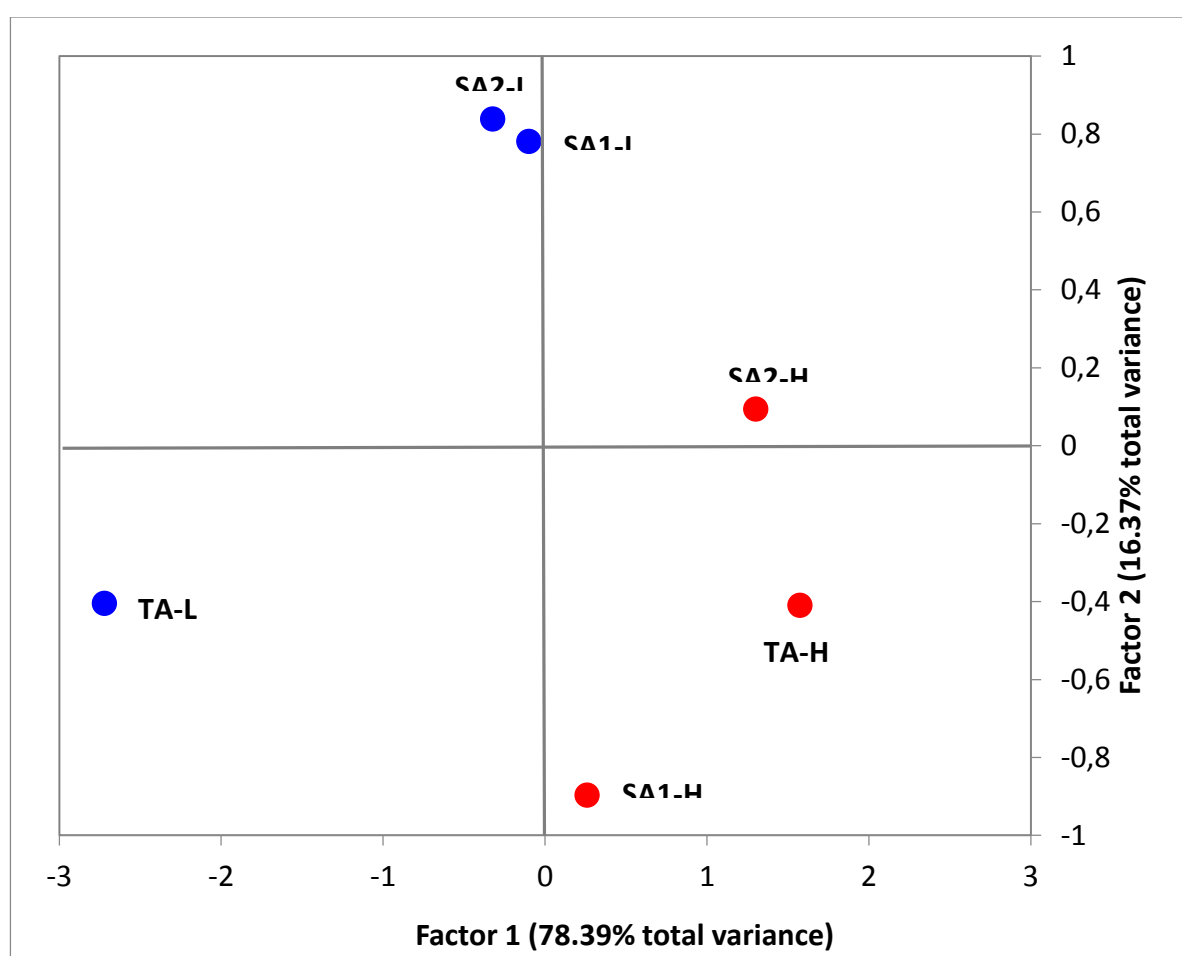


Figure 4.4 – Expression of a gene product encoding a 70-kDa *heat shock protein (HSP70)* in *P. verrucosa* immediately after coral collection at the selected sampling sites (Sahaoung 1, Sahaoung 2, and Tanjung Husi) at low (3 m) and high (12 m) depths. mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the copy numbers of each gene product normalized over the nanograms of total RNA employed in a single PCR reaction. SA1: Sahaoung 1; SA2: Sahaoung 2; TA: Tanjung Husi. \* $p < 0.05$  low depth vs high depth within each sampling site (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).



A factor analysis using the principal component extraction procedure was further applied to the mRNA expression profiles measured in *P. verrucosa* under field conditions to determine whether coral sampled in different sites and/or at different depths can be discriminated by means of overall variations of mRNA expressions for the selected gene products (Figure 4.5). Two factors (factor 1 and factor 2) were observed to explain 94.76% of total variance (78.39% and 16.37%, respectively). Samples from SA1 and SA2 collected at low depth (L), formed one group with high similarity, and showed evident separation from TA-L. For all sampling site, high-depth (H) samples showed separation from the low depth conditions, displaying also a less pronounced site-to-site separation.



**Figure 4.5** – Factor analysis bi-plot of ATPs, NDH and HSP70 mRNA expressions in *P. verrucosa* from the different sampling sites and depths (blue circles: low depth; red circles: high depth). Factor analysis was carried out using the principal component analysis extraction procedure. The Varimax normalized rotation method was used for the graphical representation. SA1-L: samples from Sahaoung 1 collected at low depth (3 m); SA1-H: samples from Sahaoung 1 collected at high depth (12 m); SA2-L: samples from Sahaoung 2 collected at low depth (3 m); SA2-H: samples from Sahaoung 2 collected at high depth (12 m); TA-L: samples from Tanjung Husi collected at low depth (3 m); TA-H: samples from Tanjung Husi collected at high depth (12 m).

#### 4.2 Effects of acclimatization to the laboratory conditions

Expression profiles of the selected transcripts were evaluated after a 14-days acclimatization period of corals to the laboratory conditions (temperature 28°C; salinity 1026 psu, light/dark cycle 10L:14D); these samples also accounted for the mRNA expression levels at the onset of the experimental exposure to increased water temperature, thus representing the “zero time” (T0) condition in the time-course analysis of transcriptional responses to the increased seawater temperature. The comparisons between field samples and T0 samples are reported in Figure n. 4.6, 4.7 and 4.8. The *ATPs* and *NDH* gene products showed no significant variations in field samples vs T0 samples at all the sampling sites and depths (Figures 4.6, 4.7), despite lower values for both transcripts can be observed in T0 samples from corals collected at the low depth in the TA site. Compared with their respective field samples, T0 samples from SA1 and TA sites collected at the low depth, and in T0 samples from the SA2 site collected at the high depth showed significantly lower *HSP70* expression levels (Figure 4.8).

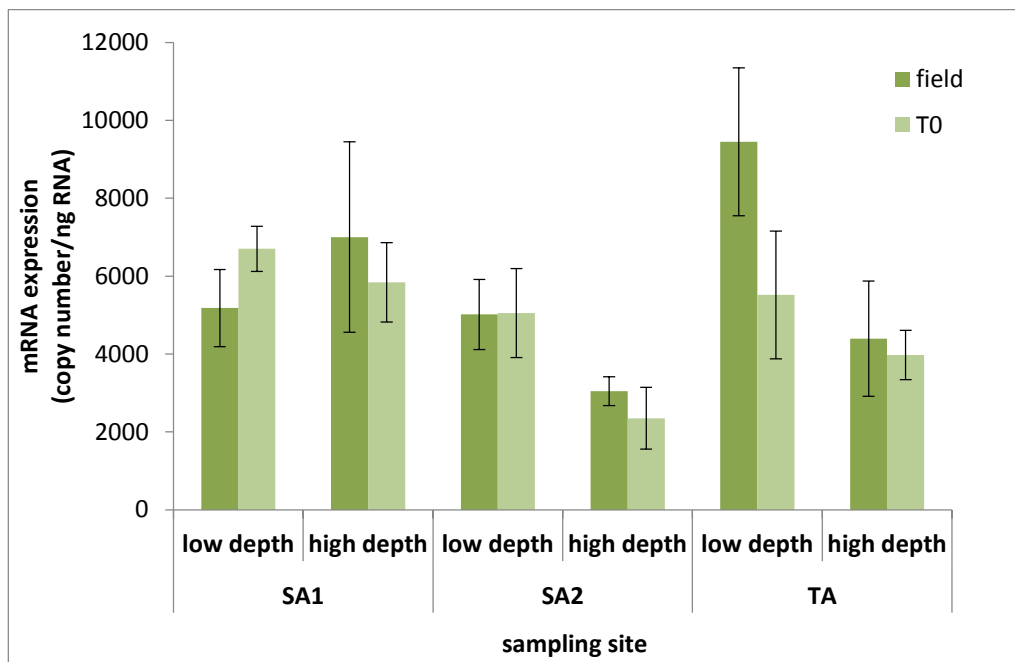


Figure n.4.6 – Comparison of *ATPs* mRNA levels measured in *P. verrucosa* immediately after coral collection at the selected sampling at low (3 m) or high (13 m) depths (field) and following a 14-days acclimatization to laboratory conditions (T0). mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the copy numbers of each gene product normalized over the nanograms of total RNA employed in a single PCR reaction. SA1: Sahaoung 1; SA2: Sahaoung 2; TA: Tanjung Husi.

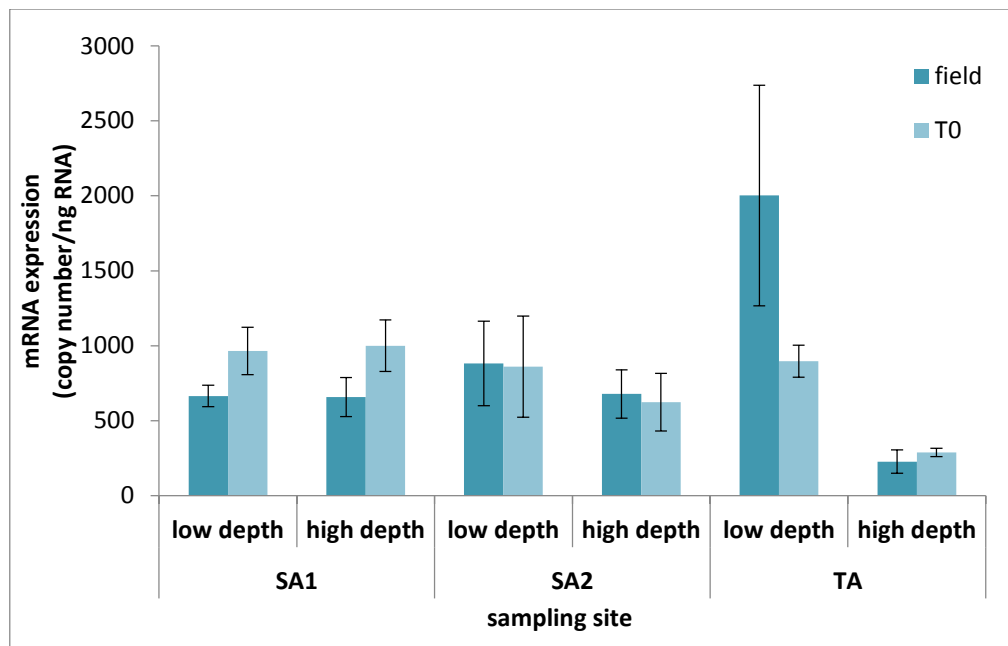


Figure n. 4.7 – Comparison of *NDH* mRNA levels measured in *P. verrucosa* immediately after coral collection at the selected sampling at low (3 m) or high (13 m) depths (field) and following a 14-days acclimatization to laboratory conditions (T0). mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the copy numbers of each gene product normalized over the nanograms of total RNA employed in a single PCR reaction. SA1: Sahaoung 1; SA2: Sahaoung 2; TA: Tanjung Husi.

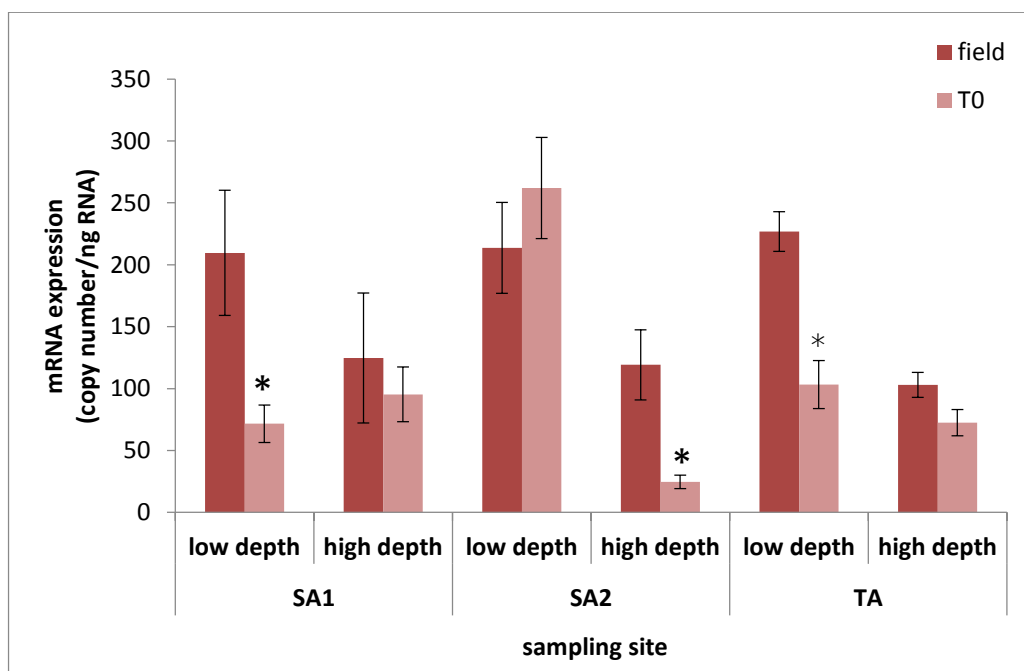


Figure n. 4.8 – Comparison of *HSP70* mRNA levels measured in *P. verrucosa* immediately after coral collection at the selected sampling at low (3 m) or high (13 m) depths (field) and following a 14-days acclimatization to laboratory conditions (T0). mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the copy numbers of each gene product normalized over the nanograms of total RNA employed in a single PCR reaction. SA1: Sahaoung 1; SA2: Sahaoung 2; TA: Tanjung Husi. \*  $p < 0.05$  T0 vs field (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).



### 4.3 Transcriptional-responses following *P. verrucosa* exposure to seawater temperature increase under laboratory conditions

The effects of elevated seawater temperature were evaluated in *P. verrucosa* by analysing changes of mRNA expression levels following a 3-days and a 7-days coral exposure to 31,5 °C. Control samples (ctr) representing corals maintained under the same laboratory conditions of thermally challenged corals (T) but at a water temperature of 28°C, which was the mean temperature value measured in the sampling sites at the time of coral collection.

Control samples showed significant variations when compared with expression levels measured in T0 samples. In general, expression levels of the three gene products resulted decreased with respect to T0 in control corals maintained under the described laboratory conditions for 3 and 7 days, with the exception of *ATPs* and *NDH* mRNA levels measured at the 7-days time point in control corals collected at the low depth in site SA1, and *ATPs* levels measured at the 3-days and 7-days time points in control corals collected at the low depth in site SA2 (Figure 4.9, 4.10, 4.11)

In general, expression of all the three analysed gene products was significantly modified by temperature increase if compared with their respective controls at each time point. After a 3-days exposure to increased temperature, corals from site SA1 showed significant up-regulation of *ATPs* in high-depth collected corals (Figure 4.9a). Moreover, also *HSP70* was significantly up-regulated both in low- and high-depth collected corals after a 7-days treatment (Figure 4.9c). Low-depth collected corals from site SA2 showed significant down-regulation of *ATPs* and *NDH* after 3-days of exposure to increased temperature (Figure 4.10a,b), while all the three analysed gene products resulted significantly down-regulated after a 7-days treatment (Figure 4.10). High-depth collected corals from the same sampling site showed significant up-regulation of *NDH* at 3 days of coral exposure to the temperature increase, while at the 7-days time point all the three analysed transcripts were significantly up-regulated with an average 3 (*ATPs*, *HSP70*) and 2 (*NDH*) fold change variation (Figure 4.10). Low-depth collected corals from site TA showed significant up-regulation of *NDH* and *HSP70* gene products after a 3-days exposure to increased temperature, whereas at the 7-days time point *NDH* resulted significantly up-regulated, while *ATPs* and *HSP70* unchanged (Figure 4.11). High-depth collected corals from the same sampling site showed significant down-regulation of the *ATPs* gene product after a

3-days exposure to increased temperature, whereas at the 7-days time point the three transcripts were significantly up-regulated (Figure 4.11).

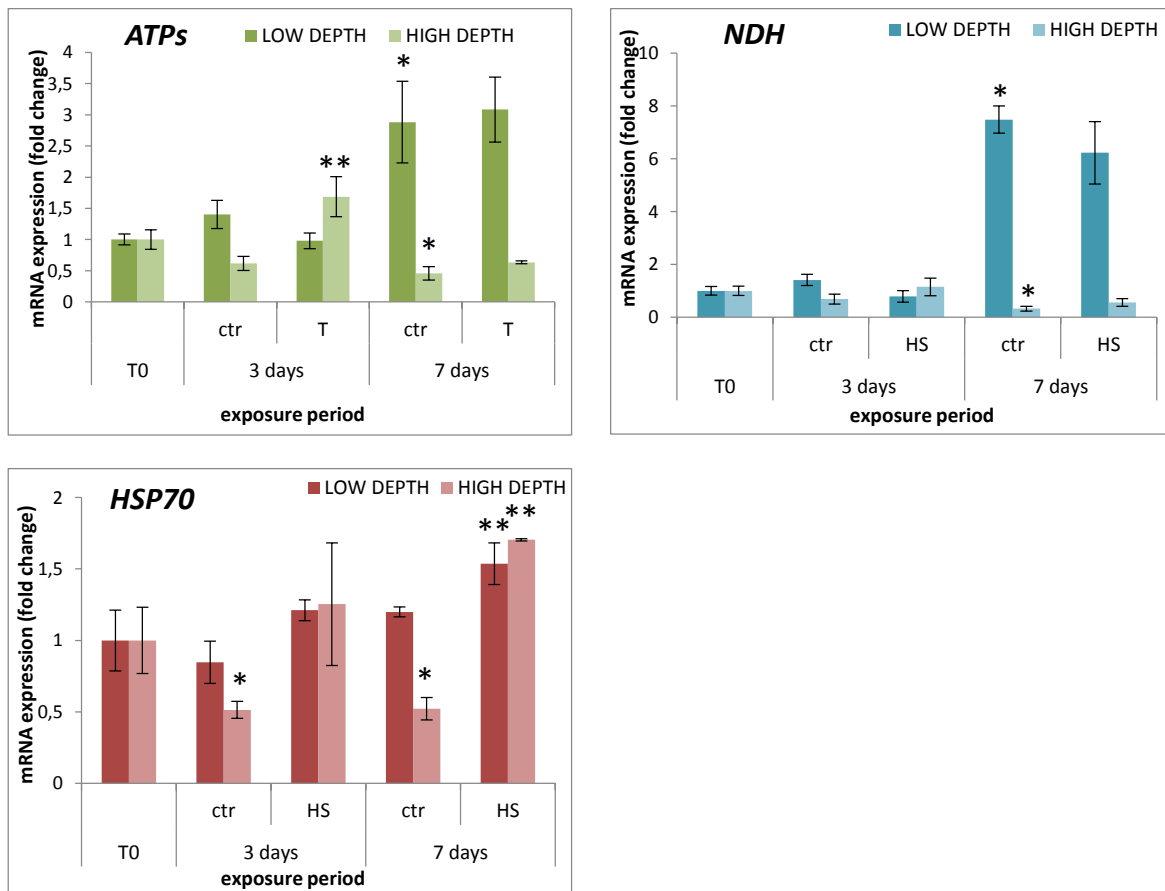


Figure 4.9 – Effects of a 7-days exposure to increased seawater temperature on mRNA levels of *ATPs*, *NDH*, and *HSP70* gene products in *P. verrucosa* corals collected at site Sahaoung 1 (SA1) at low (3 m) and high (12 m) depths. mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the relative variation (fold change) with respect to “time zero” (T0) samples. Ctr: control corals kept under normal temperature conditions (28°C); T: corals exposed to an increased seawater temperature (31,5°C). \* $p < 0.05$  ctr vs T0 (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test); \*\* $p < 0.05$  T vs ctr at each time point (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).

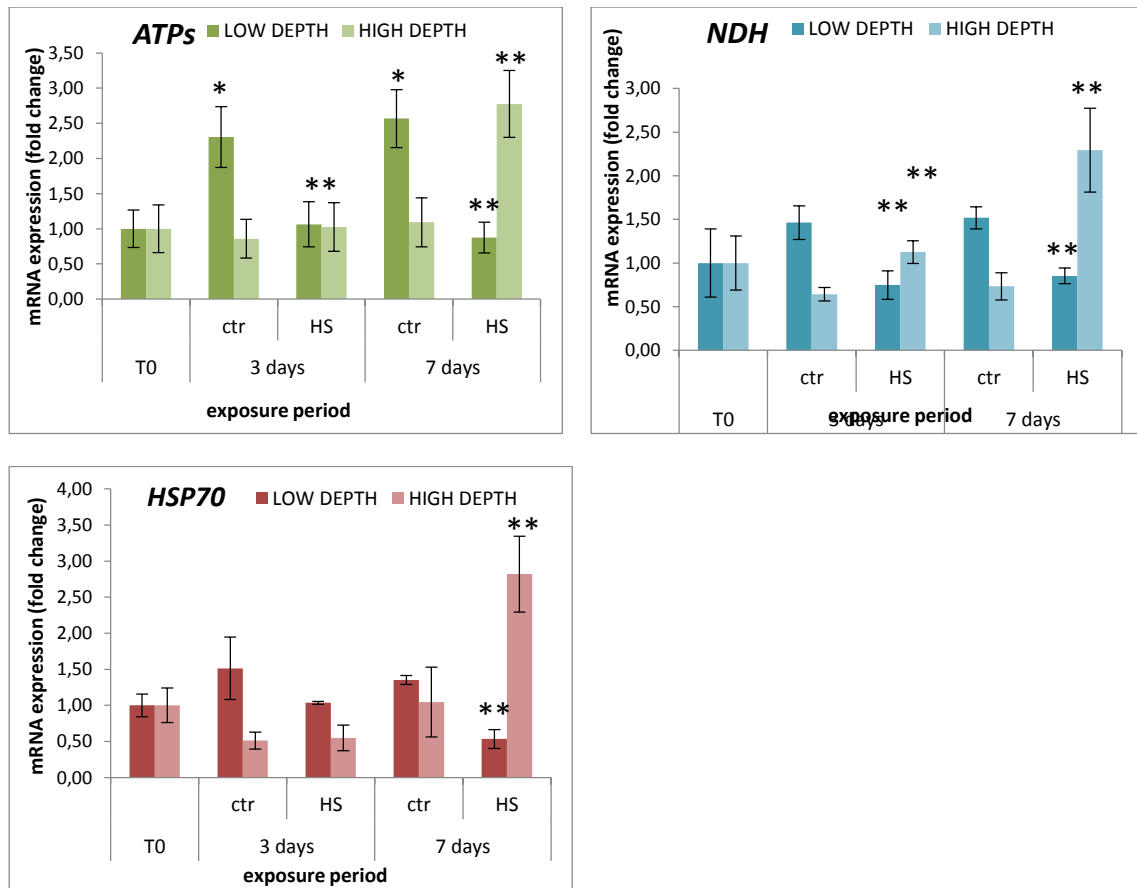


Figure 4.10 – Effects of a 7-days exposure to increased seawater temperature on mRNA levels of *ATPs*, *NDH*, and *HSP70* gene products in *P. verrucosa* corals collected at site Sahaoung 2 (SA2) at low (3 m) and high (12 m) depths. mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the relative variation (fold change) with respect to “time zero” (T0) samples. Ctr: control corals kept under normal temperature conditions (28°C); T: corals exposed to an increased seawater temperature (31,5°C). \* $p < 0.05$  ctr vs T0 (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test); \*\* $p < 0.05$  T vs ctr at each time point (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).

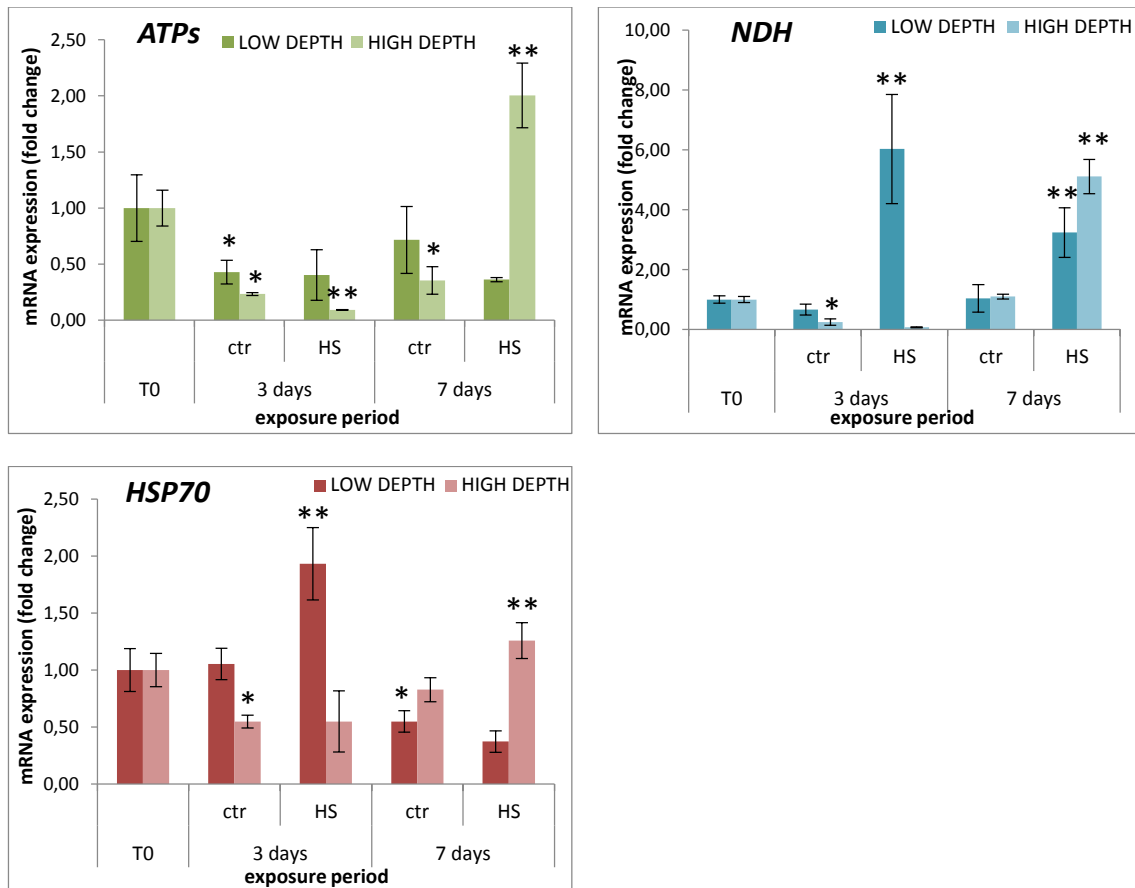
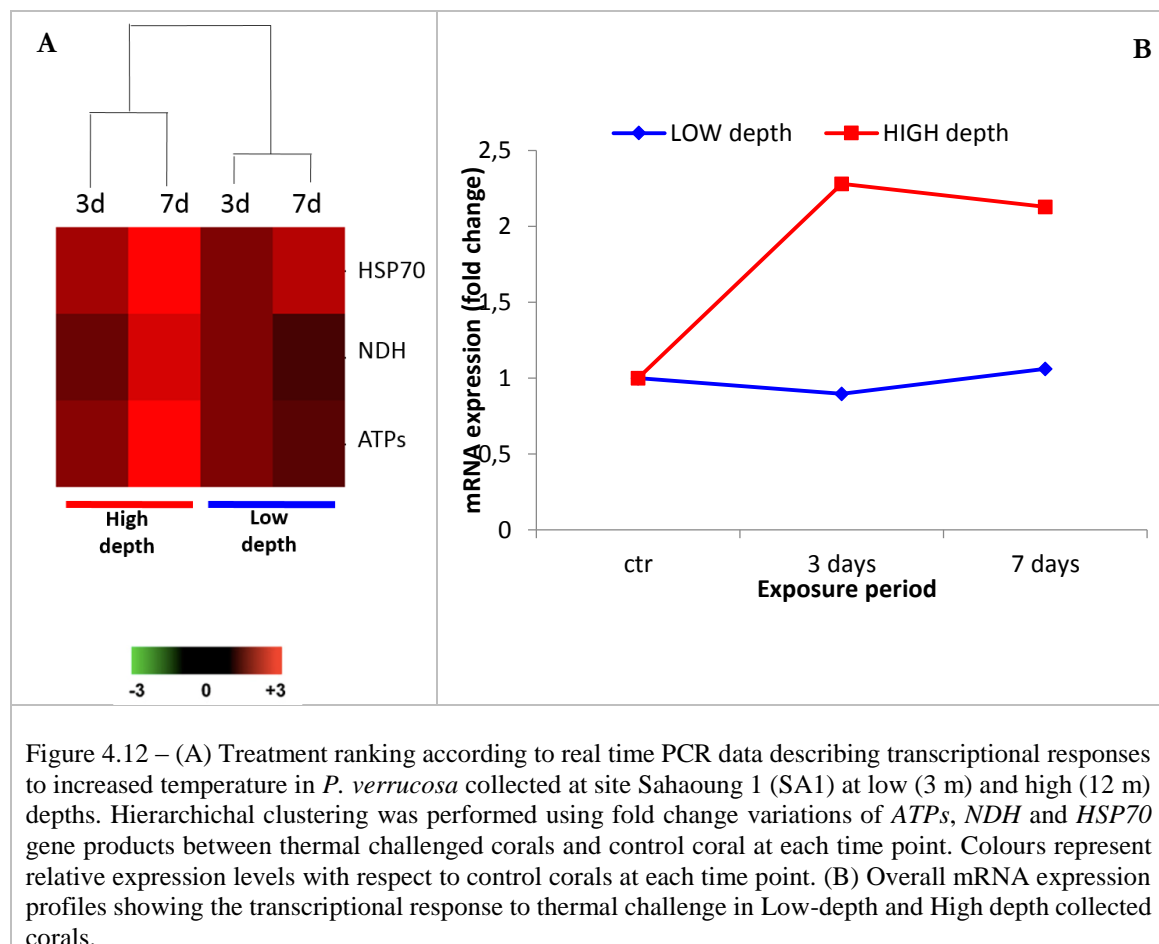


Figure 4.11 – Effects of a 7-days exposure to increased seawater temperature on mRNA levels of *ATPs*, *NDH*, and *HSP70* gene products in *P. verrucosa* corals collected at site Tanjung Husi (TA) at low (3 m) and high (12 m) depths. mRNA levels were evaluated using absolute quantitative real time PCR. Data are expressed as means  $\pm$  SEMs (N = 4) of the relative variation (fold change) with respect to “time zero” (T0) samples. Ctr: control corals kept under normal temperature conditions (28°C); T: corals exposed to an increased seawater temperature (31,5°C). \* $p < 0.05$  ctr vs T0 (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test); \*\* $p < 0.05$  T vs ctr at each time point (1-way ANOVA followed by the Bonferroni *post hoc* multi comparison test).



#### 4.4 Clustering Analysis

A cluster analysis was performed on fold change variations of *ATPs*, *NDH* and *HSP70* gene products between thermal challenged and control corals at each time point within each sampling site and depth. Clustering of data for sites SA1 and SA2 showed substantial time-course variations of the analysed transcriptional responses, with samples from low-depth and high-depth collected corals forming distinct clusters (Figure 4.12A, 12A) and showing unchanged (SA1) or down-regulated (SA2) overall expression profiles at low-depth and remarkable up-regulation at high depth (Figures 4.12B, 4.13B).



As to the site TA, no distinct clusters according to the depth at which corals were collected can be observed, probably due to the complex transcriptional profile of low-depth collected corals (Fig. 4.14,B); furthermore, high-depth collected corals showed the same up-regulation trend observed at the formerly described sampling sites (Fig. 4.14B).

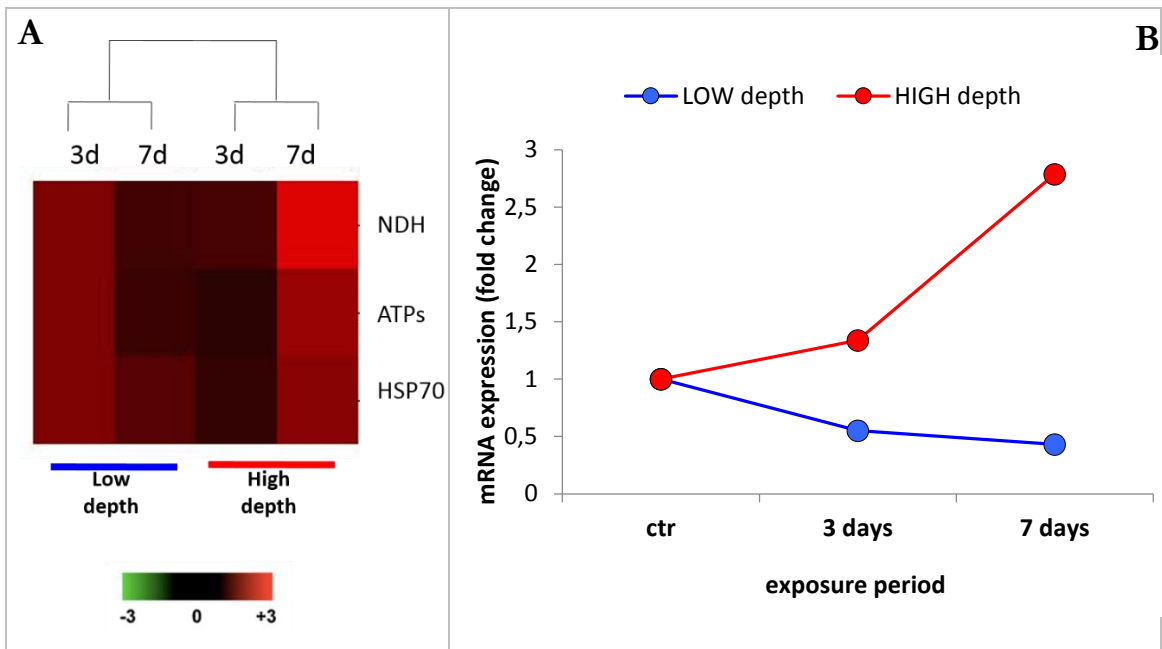


Figure 4.13 – (A) Treatment ranking according to real time PCR data describing transcriptional responses to increased temperature in *P. verrucosa* collected at site Sahaoung 2 (SA2) at low (3 m) and high (12 m) depths. Hierarchical clustering was performed using fold change variations of *ATPs*, *NDH* and *HSP70* gene products between thermal challenged corals and control coral at each time point. Colours represent relative expression levels with respect to control corals at each time point. (B) Overall mRNA expression profiles showing the transcriptional response to thermal challenge in Low-depth and High depth collected corals.

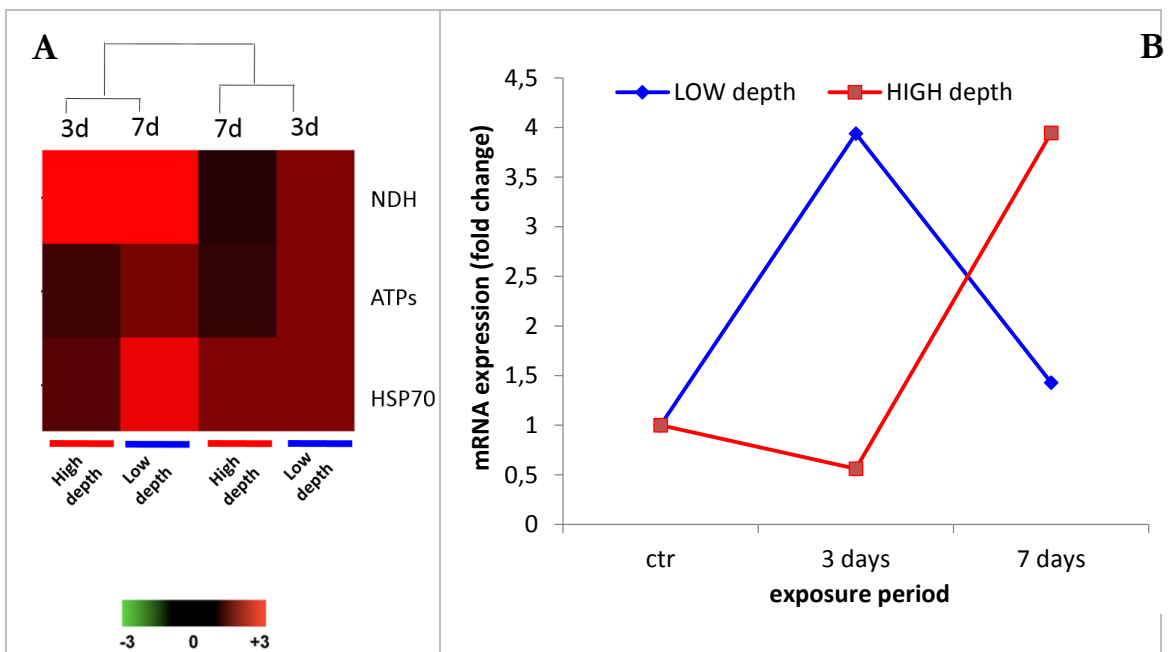


Figure 4.14 – (A) Treatment ranking according to real time PCR data describing transcriptional responses to increased temperature in *P. verrucosa* collected at site Tanjung husi (TA) at low (3 m) and high (12 m) depths. Hierarchical clustering was performed using fold change variations of *ATPs*, *NDH* and *HSP70* gene products between thermal challenged corals and control coral at each time point. Colours represent relative expression levels with respect to control corals at each time point. (B) Overall mRNA expression profiles showing the transcriptional response to thermal challenge in Low-depth and High depth collected corals.

## 5 DISCUSSION and CONCLUSIONS

### Summary

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- General view
- Evaluation of the transcript immediately after their collection
- Evaluation of changes in mRNA expression in response to increasing temperatures
- Conclusion

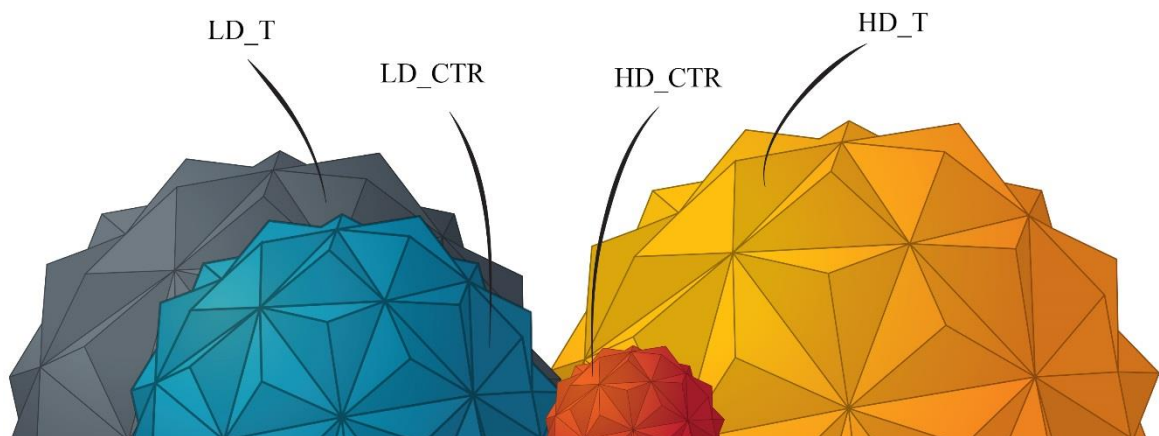


Figure n.5.1 – Artistic representation of the difference between constitutive expression and induced expression of Hsp70 for the samples of the Site A. The LD\_CTR is higher than its corresponding HD\_CTR. However, the variation between the CTR and T shows a higher variation rate for the HD specimens. Image credit: Davide Poli 2015



Global climate change is impacting on coral reefs worldwide, with approximately 19% of reefs being permanently degraded, 15% showing symptoms of imminent collapse, and 20% at risk of becoming critically affected in the next few decades (Costanza R, et al 1997). This alarming level of reef degradation is mainly due to an increase in frequency and intensity of natural and anthropogenic disturbances (Wilkinson C 2004). Among these, ocean pH decrease, also termed as “ocean acidification” (Fitt WK, 2001, Glynn PW 1984), sea level rise and alteration of seawater temperature were shown to represent critical factors affecting physiological status of reef-building corals (Vidal-Dupiol et al. 2013). The point at which physiological tolerance is exceeded and stress occurs (e.g. bleaching) varies among coral populations and species (Hoegh-Guldberg O, et al 2007, Smith DJ, et al. 2005), Weis VM, 2008).

During mass coral bleaching events, survival of scattered coral colonies suggests that some groups of corals may possess inherent physiological tolerance to environmental stress (Marshall PA, Baird AH 2000; West JM, Salm RV 2003). In addition, some high-temperature environments naturally retain healthy growing corals (Coles S 1997; Oliver TA, Palumbi SR 2011) showing enhanced bleaching tolerances (Jokiel PL, Coles SL 1990; Oliver TA, Palumbi SR 2011).

As a first step, transcript levels were evaluated in corals immediately after their collection to account for differences in mRNA expression under field conditions between corals collected at low- and high-depth and between sites SA1-SA2 and TA.

The present study showed remarkable differences in overall transcript levels between sampling sites when low-depth collected corals are considered. In particular, factor analysis reported in Figure 4.12 and 4.13 showed that sites SA1 and SA2 are similar between each other, while site TA showed evident separation from the former sampling sites. Indeed, observations from the characterization analysis (Table 3.1; Figure 3.2) show differences in the assemblages and higher relative distance between TA and the other two sites SA1 and SA2. Furthermore, during the collecting dives was observed a constant current related with tides changes not detected for the site SA1 and SA2.

Therefore, adaptation to peculiar local environmental conditions may be at the basis of the observed transcriptional differences showed by corals belonging to the different sampling sites. Moreover, corals collected at the different depths from the same sampling site displayed altered transcript levels, and factor analysis highlights that high-depth collected corals from the different sampling sites showed a less pronounced site-to-site

separation (Figure n. 4.12, 4.13, 4.14). This last finding may be explained considering that at high depths environmental conditions may be more homogenous, even between more distant sampling sites (i.e. between SA1-SA2 vs TA). Low-depth adapted corals are likely to face more relevant fluctuations in chemical-physical parameters. To minimize the effects of these environmental stressors, they may have stress-inducible gene products physiologically expressed at relatively high levels compared with organisms inhabiting less stressful environments, so that these may be promptly activated as the animal experiences adverse environmental changes. As previously observed this general broad-spectrum protective mechanism is commonly observed in many organisms living in rapidly changing environmental conditions where protection from natural and anthropogenic-induced stressors is essential for survival (Bedulina et al., 2010; Dong et al., 2008, 2010; Gehring, 1995). A support to this hypothesis is provided by the relative expression levels of the *HSP70* gene product observed in low- vs high- depth collected corals. Indeed, the *HSP70* gene product was always expressed at relatively higher levels in low-depth collected corals, with a similar mean value of about 200 copy number/ng RNA shared by the different sampling sites, while high-depth collected corals showed a mean transcript level of about 100 copy number/ng RNA, independently from the sampling site investigated (Figure n. 4.4). That said, significantly different *HSP70* mRNA levels were observed between low- vs high-depth collected corals only at site TA. These could be related to peculiar features of the site, however salinity, pH and temperature were similar to sites SA1 and SA2. However the different exposure to the open sea could be considered as variable affecting the site TA, also related to different assemblages found during the sites characterization (Figure n. 3.5). The lack of statistical significance in SA1 and SA2 sampling sites could be explained considering the high level of variation that is occurring in natural environments, as demonstrated by recent investigations (Granados-Cifuentes et al. 2013; Bay L.K., et al. 2009).

A second experiment was addressed to evaluate changes in mRNA expression in response to increasing temperatures. Thermal history was showed to affect coral responses to further stressors, as reported by several investigations demonstrating the effects of thermal preconditioning on later bleaching susceptibility during natural heat stress events (Brown B.E., et al. 2002), (Carilli J., 2010), (Maynard J.A. et al. 2008), (Thompson D.M., and van Woesik R., 2009), (Williams G.J., et al. 2010). Moreover, transcriptomic signatures underlying tolerance associated with coral thermal history has also been assessed

(Bellantuono et al., 2012). Therefore, the different transcriptional responses to increased seawater temperature between low-depth and high-depth collected corals observed in this study under laboratory controlled conditions (Figure. 4.12, 4.13, 4.14) may be explained considering the different transcriptional profiles assessed under field conditions. In general, cluster analysis and overall mRNA expression profiles clearly showed that corals sampled at the low-depth showed unaffected or down-regulated mRNA expression profiles, with the exception of corals collected at site TA, which displayed a more complex transcriptional profile likely to reflect peculiar micro-habitat conditions. Furthermore, high-depth collected corals from all the sampling sites reacted to thermal challenge by over-expressing the selected gene products. These findings agree with the possibility that corals adapted to the high depth faced more stable environmental conditions and are more sensitive to the effects of increased temperature, while low-depth adapted corals being continuously exposed to fluctuating environmental conditions, are more resilient to the stress stimulus. Increased *HSP70* mRNA expression represents a canonical response to temperature challenges, in which stress-inducible *HSP70* isoforms are rapidly induced assisting to repair and to protect cellular proteins from stressor-induced damages and to minimize protein aggregation (reviewed in Fabbri et al., 2008 *Invertebrate Survival Journal*). Increases of *ATPs* and *NDH* mRNA levels were also expected considering the involvement of these transcripts in metabolic processes. Indeed, plasticity in metabolic rates is required to balance energy uptake with energy expenditure and the metabolic transcriptional apparatus is likely to reflect this balance (Hochachka 1990; Storey & Storey 2004). In this case, both *ATPs* and *NDH* might be over-expressed as a response to the increased metabolic needs in order to counteract or adapt to the changed physiological status.

## 5.1 Conclusion

Overall, our results suggest that *P. verrucosa* could acclimated to increasing temperatures up to 31.5°C (1.5°C higher than the highest seawater temperatures registered during the summer) when naturally exposed to thermally variable habitats. This was in line with previous observations reported for other coral species (e.g. Jones R.J. et al., 2000, Mayfield A.M. et al. 2013). However, a temperature increase greater than 2°C with respect to the highest seawater temperatures registered during the summer causes significant mortality in *P. verrucosa*, as observed in our preliminary experiments performed to set up the experimental conditions (data not shown). Interestingly, similar results were obtained

for other coral species exposed to the field (e.g., Jokiel P.L. and Coles S.L., 1990) or to the laboratory (e.g., Vidal-Dupiol J. et al., 2009). Our results also highlight that mRNA expression is modulated by natural seasonal temperature changes as small as 1.5 °C. Mild stress exposures are known to induce subsequent increases in stress tolerance in a broad suite of organisms [e.g., humans (Hahn G.M., et al. 1989), plants (Durrant W.E., et al. 2004), bacteria (Hecker M, et al 2007), fungi (Berry D.B., Gasch A.P. 2008), corals (Bellantuono A.J., 2011), fish (Todgham A.E., 2005)]. The higher expression values found in low-depth specimens from the sites SA1 and SA2, suggest that “frontloaded genes” (according to Barshis D.J. et al. 2012) could enable corals to maintain physiological resilience during frequently encountered environmental stress, an idea that has strong parallels in model systems such as yeast (Berry, D.B., Gasch, A.P. 2008).







## 6 REFERENCES

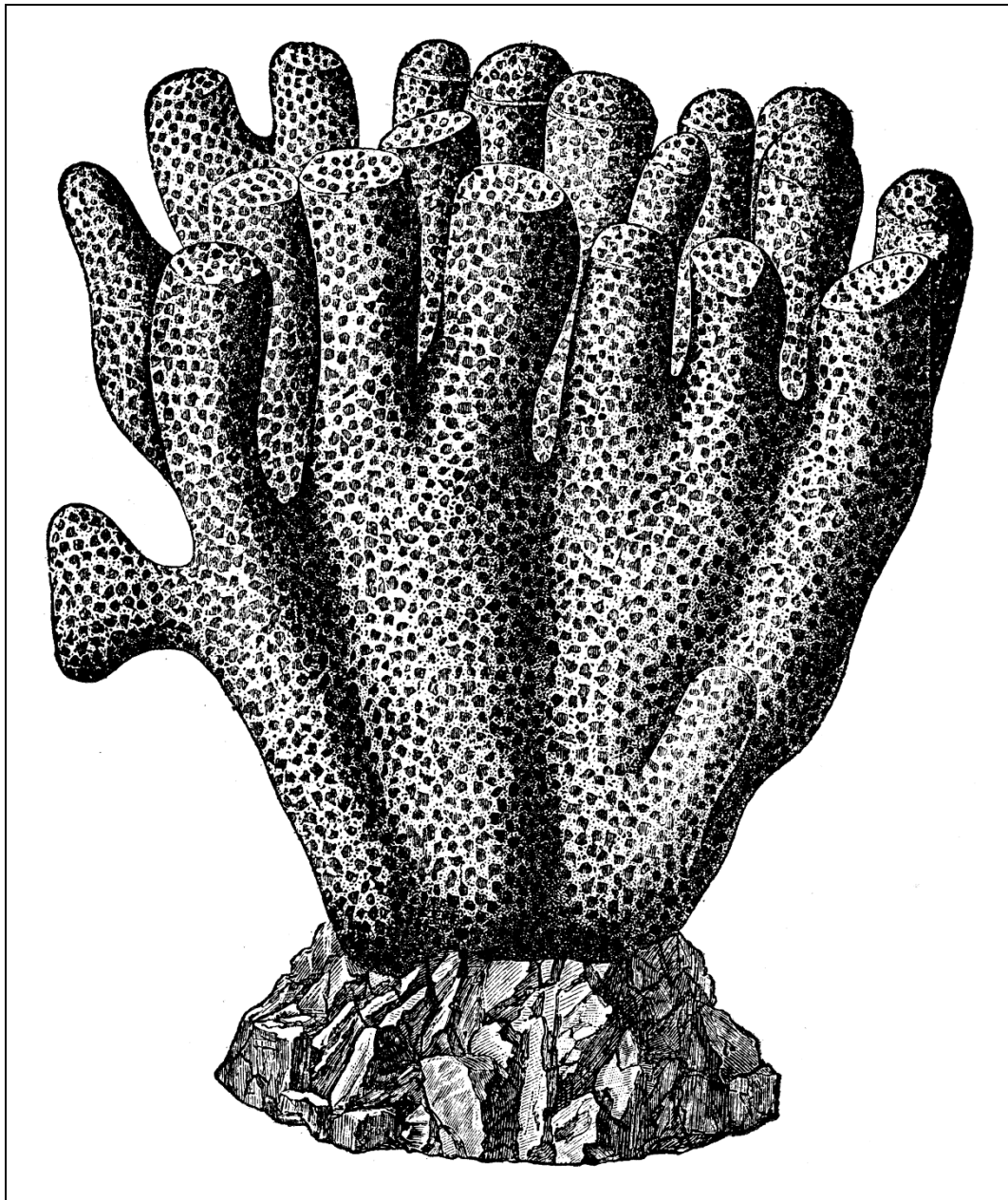


Figure n. – Diagram of *Pocillopora verrucosa* (Hoeksema, B. 2014)



- Allen G.R. (2007) - Conservation hotspots of biodiversity and endemism for Indo-Pacific coral reef fishes. *Aquat Conserv Mar Freshwater Ecosyst* 18:541–556
- Baker A.C. (2003) - Flexibility and Specificity in Coral-Algal Symbiosis: Diversity, Ecology, and Biogeography of Symbiodinium. *Annu. Rev. Ecol. Evol. Syst.* 2003. 34:661-89
- Barange M., Field J.G., Harris R.P., Hofmann E.E., Perry R.I, Werner F. (2010) - *Marine Ecosystems and Global Change*. Oxford University Press Inc., New York, USA.
- Barber P.H. (2009) - The challenge of understanding the Coral Triangle biodiversity hotspot. *J. Biogeogr.* 36: 1845–1846
- Barshis D.J. et al. (2013) - Genomic basis for coral resilience to climate change. *PNAS*, 110(4): 1387–1392.
- Bartholomew J.G., Clark W.E., Grimshaw P.H. (1911) - *Atlas of zoogeography*. Bartholomew, Edinburgh
- Bay, L. K., Guerecheau, A., Andreakis, N., Ulstrup, K. E., & Matz, M. V. (2013) - Gene expression signatures of energetic acclimatisation in the reef building coral *Acropora millepora*. *PloS one*, 8(5), e61736.
- Bay, L. K., Ulstrup, K. E., NIELSEN, H. B., Jarmer, H., Goffard, N., Willis, B. L., Miller, D.J., & Van Oppen, M. J. (2009) - Microarray analysis reveals transcriptional plasticity in the reef building coral *Acropora millepora*. *Molecular ecology*, 18(14), 3062-3075.
- Bay, L. K., Ulstrup, K. E., NIELSEN, H. B., Jarmer, H., Goffard, N., Willis, B. L., Miller, D.J., & Van Oppen, M. J. (2009) - Microarray analysis reveals transcriptional plasticity in the reef building coral *Acropora millepora*. *Molecular ecology*, 18(14), 3062-3075.
- Beaugrand G., Reid P.C., Ibanez F., Lindley J.A., Edwards M. (2002) - Reorganization of North Atlantic marine copepod biodiversity and climate. *Science* 296:1692–94
- Bedulina, D. S., Zimmer, M., & Timofeyev, M. A. (2010) - Sub-littoral and supra-littoral amphipods respond differently to acute thermal stress. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 155(4), 413-418.
- Bedulina, D. S., Zimmer, M., & Timofeyev, M. A. (2010) - Sub-littoral and supra-littoral amphipods respond differently to acute thermal stress. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 155(4), 413-418.
- Bellantuono, A.J., Granados-Cifuentes, C., Miller, D.J., Hoegh-Guldberg, O., Rodriguez-Lanetty, M., (2012) - Coral Thermal Tolerance: Tuning Gene Expression to Resist Thermal Stress. *PLoS ONE* 7(11): e50685. doi:10.1371/journal.pone.0050685
- Bellantuono, A.J., Hoegh-Guldberg, O., Rodriguez-Lanetty, M., (2011) - Resistance to thermal stress in corals without changes in symbiont composition. *Proc Biol Sci* 279(1731):1100–1107.
- Bellantuono, A.J., Hoegh-Guldberg, O., Rodriguez-Lanetty, M., (2011) - Resistance to thermal stress in corals without changes in symbiont composition. *Proc Biol Sci* 279(1731):1100–1107.

- Berry, D.B., Gasch, A.P. (2008) - Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Mol Biol Cell* 19(11):4580–4587.
- Berry, D.B., Gasch, A.P. (2008) - Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Mol Biol Cell* 19(11):4580–4587.
- Berry, D.B., Gasch, A.P., (2008) - Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Mol Biol Cell* 19(11):4580–4587.
- Berry, D.B., Gasch, A.P., (2008) - Stress-activated genomic expression changes serve a preparative role for impending stress in yeast. *Mol Biol Cell* 19(11):4580–4587.
- Bertelsen E.B. et al. (2009) - Solution conformation of wild-type E. coli Hsp70 (DnaK) chaperone complexed with ADP and substrate. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8471–8476
- Bianchi CN, Pronzato R, Cattaneo-Vietti R, Benedetti Cecchi L, Morri C, Pansini M, Chemello R, Milazzo M, Frascchetti S, Terlizzi A, Peirano A, Salvati E, Benzoni F, Calcinaï B, Cerrano C, Bavestrello G 2003. *Biol. Mar. Medit.*, 10: 199-232
- Bijlsma, L., Ehler, C. N., Klein, R. J. T., Kulshrestha, S. M., McLean, R. F., Mimura, N., Nicholls, R. J., Nurse, L. A., Perez Nieto, H., Stakhiv, E. Z., Turner, R. K., and Warrick, R. A. (1995) - Coastal zones and small islands. In *Climate Change 1995 -Impacts, adaptations and mitigations of climate change: scientific-technical analyses: the second assessment report of the Inter-Governmental Panel on Climate Change*. (Eds R. T. Watson, M. C. Zinyowera and R. H. Moss.) pp. 6ñ12. (Cambridge University Press: New York.)
- Bindoff N.L., Willebrand J., Artale V., et al. (2007) - Observations: oceanic climate change and sea level. In: Solomon, S., Qin, D., Manning, M., et al., eds. *Climate change 2007: the physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge University Press, Cambridge and New York.
- Boutet I, Tanguy A, Rousseau S, Auffret M, Moraga D (2003) - Molecular identification and expression of heat shock cognate 70 (hsc70) and heats shock protein 70 (hsp70) genes in the Pacific oyster *Crassostrea gigas*. *Cell Stress Chaperones*, 8, 76–85.
- Boyer P.D. (1997) – The ATP Syntase – A splendid Molecular machine. *Annu. Rev. Biochem.* 1997. 66:717–49
- Brown B. E. (1997) - Coral bleaching: causes and consequences. *Coral Reefs* 16:s129–138.
- Brown BE, Cossins AR (2011) The Potential for Temperature Acclimatisation of Reef Corals in the Face of Climate Change In: Dubinsky Z, Stambler N, editors. *Coral Reefs: An Ecosystem in Transition*. Heidelberg: Springer pp. 421–434.
- Brown, B.E., (1997) - Coral bleaching: causes and consequences. *Coral Reefs* 16, S129–S138.
- Brown, B.E., Dunne, R.P., Goodson, M.S., Douglas, A.E. (2002) - Experience shapes the susceptibility of a reef coral to bleaching. *Coral Reefs* 21: 119–126.

- Bruno J.F., Selig E.R. (2007) - Regional decline of coral cover in the Indo-Pacific: timing, extent, and subregional comparisons. *Plos One* 8:e711:1–8
- Burke L.E., Selig M., Spalding M. (2002) - Reefs at risk in Southeast Asia. World Resources Institute, Washington, DC
- Bustin, S. A., & Nolan, T. (2004) - Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *Journal of biomolecular techniques: JBT*, 15(3), 155.
- Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., ... & Wittwer, C. T. (2009) - The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical chemistry*, 55(4), 611-622.
- Cairns S.D. (2007) - Deep-water corals: an overview with special reference to diversity and distribution of deep-water scleractinian corals. *Bull Mar Sci* 81: 311-322
- Capaldi R.A. and Aggeler R. (2002) - Mechanism of the F1F0-type ATP synthase, a biological rotary motor. *TRENDS in Biochemical Sciences* Vol.27 No.3 March 2002
- Caplan A.J., Cyr D.M. and Douglas M.G. (1993) - Eukaryotic homologues of *Escherichia coli* dnaJ: a diverse protein family that functions with hsp70 stress proteins. *Mol. Biol. Cell* 4: 555–563.
- Carilli, J., Donner, S.D., Hartmann, A.C., (2012) - Historical Temperature Variability Affects Coral Response to Heat Stress. *PLoS ONE* 7: e34418.
- Chen C.A., Wang A.T., Fang L.S., Yang Y.W. (2005) - Fluctuating algal symbiont communities in *Acropora palifera* (Scleractinia: Acroporidae) from Taiwan. *Marine Ecology-Progress Series*, 295,113–121.
- Cheviron Z.A., Whitehead A., Brumfield R.T. (2008) - Transcriptomic variation and plasticity in rufous-collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient. *Molecular Ecology*, 17, 4556–4569.
- Cheviron Z.A., Whitehead A., Brumfield R.T. (2008) - Transcriptomic variation and plasticity in rufous-collared sparrows (*Zonotrichia capensis*) along an altitudinal gradient. *Molecular Ecology*, 17, 4556–4569.
- Coles S. (1997) - Reef corals occurring in a highly fluctuating temperature environment at Fahal Island, Gulf of Oman (Indian Ocean). *Coral Reefs* 16(4):269–272.
- Coles S. L., and Jokiel P.L. (1978) - Synergistic effects of temperature, salinity and light on the hermatypic coral *Montipora verrucosa*. *Marine Biology* 49, 187-95.
- Coles S. L., and Jokiel P.L. (1977) - Effects of temperature on photosynthesis and respiration in hermatypic corals. *Marine Biology* 43, 209-16.
- Coles S.L., Brown B.E. (2003) - Coral bleaching - capacity for acclimatization and adaptation. *Advances in Marine Biology*, 46: 183–223.
- Costanza, R., d'Arge, R., deGroot, R., Farber, S., Grasso, M., et al. (1997) - The value of the world's ecosystem services and natural capital. *Nature* 387: 253–260.
- Craig, P., Birkeland, C., Belliveau, S., (2001) - High temperatures tolerated by a diverse assemblage of shallow-water corals in American Samoa. *Coral Reefs* 20(2):185–189.

- Craig, P., Birkeland, C., Belliveau, S., (2001) - High temperatures tolerated by a diverse assemblage of shallow-water corals in American Samoa. *Coral Reefs* 20(2):185–189.
- Dana J.D. (1853) - On an isothermal oceanic chart illustrating the geographical distribution of marine animals. *Am J Sci* 16:314-327
- Darwin C. (1859) - The origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. John Murray, London
- DeSalvo M.K, et al. (2008) - Differential gene expression during thermal stress and bleaching in the Caribbean coral *Montastraea faveolata*. *Mol Ecol* 17(17):3952–3971.
- deYoung B., Harris R., Alheit J., Beaugrand G., Mantua N., Shannon L. (2004) - Detecting regime shifts in the ocean: data considerations. *Prog. Oceanogr.* 60:143–64
- Djafarzadeh R., Kerscher S., Zwicker K., Radermacher M., Lindahl M., Schagger H., Brandt U. (2000) - Biophysical and structural characterization of proton-translocating NADH-dehydrogenase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*. *Biochimica et Biophysica Acta* 1459 (2000) 230^238
- Domingues C.M., Church J.A., White N.J., et al. (2008) - Improved estimates of upper-ocean warming and multidecadal sea-level rise. *Nature*, 453, 1090–3.
- Dong, Y. W., Ji, T. T., Meng, X. L., Dong, S. L., & Sun, W. M. (2010) - Difference in thermotolerance between green and red color variants of the Japanese sea cucumber, *Apostichopus japonicus* Selenka: Hsp70 and heat-hardening effect. *The Biological Bulletin*, 218(1), 87-94.
- Dong, Y., Miller, L. P., Sanders, J. G., & Somero, G. N. (2008) - Heat-shock protein 70 (Hsp70) expression in four limpets of the genus *Lottia*: interspecific variation in constitutive and inducible synthesis correlates with in situ exposure to heat stress. *The Biological Bulletin*, 215(2), 173-181.
- Drinkwater K.F., Beaugrand G., Kaeriyama M., et al. (2009) - On the processes linking climate to ecosystem changes. *Journal of Marine Systems*, 79, 374–88.
- Drinkwater K.F., Belgrano A., Borja A., et al. (2003) - The response of marine ecosystems to climate variability associated with the North Atlantic Oscillation. In: Hurrell J., Kushnir Y., Ottersen G., et al. eds. *The north Atlantic oscillation, climatic significance and environmental impact*. Geophysical Monograph Series 134. AGU Press, Washington, DC, pp. 211–34.
- Dubinsky Z. and Jokiel P.L. (1994) - Ratio of Energy and Nutrient Fluxes Regulates Symbiosis between Zooxanthellae and Corals. *Pacific Science* (1994). vol. 48, no. 3: 313-324
- Durrant, W.E., Dong, X. (2004) - Systemic acquired resistance. *Annu Rev Phytopathol* 42: 185–209.
- Eisen M. B., Spellman P. T., Brown P. O., and Botstein D. (1998) - Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95, 14863–14868
- Ekman S. (1935) - *Tiergeographie des Meeres*. Akademische Verlagsgesellschaft, Leipzig



- Fabbri, E., Valbonesi, P., & Franzellitti, S. (2008) - HSP expression in bivalves. *ISJ*, 5, 135-161.
- Falkowski P.G., Dubinsky Z., Muscatine L., and Porter J., (1984) - Light and the bioenergetics of a symbiotic coral. *BioScience* 34: 705-709.
- Fearnley I. M. & Walker J. E. (1992) - Conservation of sequences of subunits of mitochondrial complex I and their relationships with other proteins. *Biochim. Biophys. Acta*, 1140, 105±34.
- Fitt, W.K., Brown, B.E., Warner, M.E., Dunne, R.P. (2001) - Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. *Coral Reefs* 20: 51–65.
- Forbes E. (1856) - Map of the distribution of marine life. The physical atlas of natural phenomena, plate 31. Johnston W, Johnston A.K, Edinburgh
- Franzellitti S, Kiwan A, Fabbri E. Selection of the best performing reference gene products for investigating transcriptional regulation during life stage transformations in the European eel (*Anguilla anguilla*). *Marine Environmental Research* submitted for publication.
- Franzellitti, S., & Fabbri, E. (2005) - Differential HSP70 gene expression in the Mediterranean mussel exposed to various stressors. *Biochemical and biophysical research communications*, 336(4), 1157-1163.
- Gässler C.S. et al. (2001) Bag-1M accelerates nucleotide release for human Hsc70 and Hsp70 and can act concentration-dependent as positive and negative cofactor. *J. Biol. Chem.* 276, 32538–32544
- Gates R. D., Baghdasarian G., and Muscatine L. (1992) - Temperature stress causes host cell detachment in symbiotic cnidarians: implications for coral bleaching. *Biological Bulletin* 182:324–332.
- Gehring, W. J., & Wehner, R. (1995). Heat shock protein synthesis and thermotolerance in *Cataglyphis*, an ant from the Sahara desert. *Proceedings of the National Academy of Sciences*, 92(7), 2994-2998.
- George W. (1981) - Wallace and his line. Wallace's Line and plate tectonics. Clarendon Press, Oxford
- Glynn P. W., and D'Croz, L. (1990) - Experimental evidence for high temperature stress as the cause of El Niño coincident coral mortality. *Coral Reefs* 8, 181.91.
- Glynn P.W. (2001) - Eastern Pacific coral reefs: New revelations in the Twentieth Century. *Atoll Research Bulletin* 494: 119-158.
- Glynn P.W., Cortes J., Guzman H.M., and Richmond R.H. (1988) - El nino (1982-1983) associated coral mortality and relationship to sea surface temperature deviations in the tropical eastern Pacific. 6th International Coral Reef Symposium. Townsville Australia.
- Glynn, P.W., (1984) - Widespread coral mortality and the 1982–83 El Niño warming event. *Environ Conserv* 11: 133–146.
- Glynn, P.W., (1993) - Coral reef bleaching: ecological perspectives. *Coral Reefs* 12, 1–17.

- Granados-Cifuentes, C., Bellantuono, A. J., Ridgway, T., Hoegh-Guldberg, O., & Rodriguez-Lanetty, M. (2013) - High natural gene expression variation in the reef-building coral *Acropora millepora*: potential for acclimative and adaptive plasticity. *BMC genomics*, 14(1), 228.
- Grave de S. (2001) - Biogeography of Indo-Pacific Pontoniinae shrimps (Crustacea: Decapoda): a PAE analysis. *J Biogeogr* 28: 1239-1253
- Gray V. (2002) - *The Greenhouse Delusion: A Critique of "Climate Change" 2001*. Multi-Science Pub. UK, 95 pp.
- Guinotte J.M., Buddemeier R.W., Kleypas J.A. (2003) - Future coral reef habitat marginality: temporal and spatial effects of climate change in the Pacific basin. *Coral Reefs* 22:551–558
- Hahn, G.M., Ning, S.C., Elizaga, M., Kapp, D.S., Anderson, R.L. (1989) - A comparison of thermal responses of human and rodent cells. *Int J Radiat Biol* 56(5):817–825.
- Hansen J. Et al. (2005) - Earth's Energy Imbalance: Confirmation and Implications. *Science* 308(5727): 1431-1435
- Hansen, J.M., Sato, R. Ruedy, L. Nazarenko, A. Lacis, and 40 others (2005) - Efficacy of climate forcings. *Journal of Geophysical Research-Atmospheres* 110:18104
- Harrison D.E. and Carson M. (2007) - Is the world ocean warming? Upper-ocean temperature trends: 1950–2000. *Journal of Physical Oceanography*, 37, 174–87.
- Hartl F.U., Bracher A. and Hayer-Hartl M.(2011) - Molecular chaperones in protein folding and proteostasis. *Nature* 475: 324-332
- Hartmann, D.L., A.M.G. Klein Tank, M. Rusticucci, L.V. Alexander, S. Brönnimann, Y. Charabi, F.J. Dentener, E.J. Dlugokencky, D.R. Easterling, A. Kaplan, B.J. Soden, P.W. Thorne, M. Wild and P.M. Zhai, (2013) - Observations: Atmosphere and Surface. In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G.K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press,
- Hays G.C., Richardson A.J., Robinson C. (2005) - Climate change and marine plankton. *Trends Ecol. Evol.* 20:337–44
- Hecker, M., Pané-Farré, J., Völker, U. (2007) - SigB-dependent general stress response in *Bacillus subtilis* and related gram-positive bacteria. *Annu Rev Microbiol* 61:215–236.
- Hochachka P., (1990) - Design of energy metabolism. In: *Environmental and Metabolic Animal Physiology* (ed. Prosser L), pp. 325–352. Wiley-Liss, New York.
- Hoegh-Guldberg O. (1999) - Climate change, coral bleaching and the future of the world's coral reefs. *Mar. Freshwater Res.*, 1999, 50, 839-66
- Hoegh-Guldberg O., Hoegh-Guldberg H., Veron JEN. et al (2009) - The Coral Triangle and climate change: ecosystems, people and societies at risk. World Wildlife Fund
- Hoegh-Guldberg O., Mumby P.J., Hooten A.J. et al. (2007) - Coral reefs under rapid climate change and ocean acidification. *Science*, 318: 1737–1742.

- Hoegh-Guldberg, O., Mumby, P.J., Hooten, A.J., Steneck, R.S., Greenfield, P., et al. (2007) Coral reefs under rapid climate change and ocean acidification. *Science* 318: 1737–1742.
- Hoeksema B.W. (2007) - Delineation of the Indo-Malayan centre of maximum marine biodiversity: the Coral Triangle. *Biogeography, time, and place: distributions, barriers, and islands*. Springer, Netherlands, 117-178
- Hoeksema B.W., Rogers A. & Quibilan M.C. (2014). *Pocillopora verrucosa*. The IUCN Red List of Threatened Species. Version 2014.3. <[www.iucnredlist.org](http://www.iucnredlist.org)>. Downloaded on 18 December 2014.
- Houghton J. (2005) - Global warming. *Rep. Prog. Phys.* 68 (2005) 1343–1403
- Houlbreque F, Ferrier-Pages C (2009) - Heterotrophy in Tropical Scleractinian Corals. *Biological Reviews* 84: 1–17.
- <http://eu.idtdna.com/Primerquest/Home/Index> - © Copyright 2014 Integrated DNA Technologies, Inc
- Hughes T.P, Baird A.H., Bellwood D.R. et al. (2003) - Climate change, human impacts, and the resilience of coral reefs. *Science*, 301: 929–933.
- IPCC (2007) - *Climate Change 2007: The Physical Science Basis*. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change (IPCC) [Solomon, S., D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor and H. L. Miller (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 996 pp. Cambridge, United Kingdom and New York, NY, USA.
- IPCC (HYPERLINK "<http://www.ipcc-wg2.org/index.html>") Intergovernmental Panel on Climate Change) - (2007a) *Climate change 2007: impacts, adaptation, and vulnerability*. Contribution of Working Group II to the Third Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, New York
- IPCC (Intergovernmental Panel Climate Change) (2001) - *Climate Change 2001: The Science of Climate Change*, Contribution of Working Group I to the Intergovernmental Panel on Climate Change Third Assessment Report. UK: Cambridge Univ. Press
- IPCC (Intergovernmental Panel on Climate Change) (2007b) - *Summary for Policymakers*. *Climate change 2007: the physical science basis*. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate change. Cambridge University Press, Cambridge, UK, New York
- Ishii M., and Kimoto M. (2009) - Reevaluation of historical ocean heat content variations with time-varying XBT and MBT depth bias corrections. *J. Oceanogr.*, 65, 287–299.
- Jokiel, P.L., Coles, S.L., (1990) - Response of Hawaiian and other Indo Pacific reef corals to elevated temperatures. *Coral Reefs* 8, 155e162
- Jones R. J., O. Hoegh-Guldberg A., Larkum W. and Scheiber U. (1998) - Temperature-induced bleaching of corals begins with impairment of the CO<sub>2</sub> fixation mechanism in zooxanthellae. *Plant, Cell and Environment* 21:1219–1230.

- Jones, R.J., Ward, S., Amri, A.Y., Hoegh-Guldberg, O., (2000) - Changes in quantum efficiency of Photosystem II of symbiotic dinoflagellates of corals after heat stress and of bleached corals after the 1998 Great Barrier Reef bleaching event. *Marine and Freshwater Research* 51, 63e71.
- Kenkel C. D., Meyer E. and Matz M. V. (2013) - Gene expression under chronic heat stress in populations of the mustard hill coral (*Porites astreoides*) from different thermal environments. *Molecular Ecology*, 10,1111-12390
- Khandekar M.L., Murty T.S. and Chittibabu P. (2005) - The Global Warming Debate: A Review of the State of Science. *Pure appl. geophys.* 162 (2005) 1557–1586
- Kiehl J.T, Trenberth K.E. (1997) - *Bull. Am. Met. Soc.* 78 197–208
- Lesser M. P. (1996) - Exposure of symbiotic dinoflagellates to elevated temperatures and ultraviolet radiation causes oxidative stress and inhibits photosynthesis. *Limnology and Oceanography* 41:271–283.
- Lesser M. P., Stochaj W. R., Tapley D. W., and Shick J. M. (1990) - Bleaching in coral reef anthozoans: effects of irradiance, ultraviolet radiation, and temperature on the activities of protective enzymes against active oxygen. *Coral Reefs* 8, 225.32.
- Levitus S., et al., (2012) - World ocean heat content and thermocline sea level change (0–2000m) 1955–2010. *Geophys. Res. Lett.*, 39, L10603.
- Lewis, S., Handy, R. D., Cordi, B., Billingham, Z., & Depledge, M. H. (1999) - Stress proteins (HSP's): methods of detection and their use as an environmental biomarker. *Ecotoxicology*, 8(5), 351-368.
- Liebler J. (2015) – RNA Polymerase. *The Art of Cell medical and scientific 3D animation.* [Http://www.artofthecell.com](http://www.artofthecell.com)
- Maes, G. E., Raeymaekers, J. A. M., Hellemaes, B., Geeraerts, C., Parmentier, K., De Temmerman, L., ... & Belpaire, C. (2013) - Gene transcription reflects poor health status of resident European eel chronically exposed to environmental pollutants. *Aquatic Toxicology*, 126, 242-255.
- Marshall P.A, Baird A.H. (2000) - Bleaching of corals on the Great Barrier Reef: Differential susceptibilities among taxa. *Coral Reefs* 19(2):155–163.
- Mayer M.P. et al. (2000) - Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat. Struct. Biol.* 7, 586–593
- Mayer M.P., (2010) - Gymnastics of Molecular Chaperones. *Molecular Cell* 39: 321-331
- Mayer M.P., and Bukau B. (2005) - Hsp70 chaperones: cellular functions and molecular mechanism. *Cell. Mol. Life Sci.* 62, 670–684.
- Mayer MP. (2013) - Hsp70 chaperone dynamics and molecular mechanism. *Trends in Biochemical Sciences* 38(10)
- Mayfield, A. B., Chen, M. N., Meng, P. J., Lin, H. J., Chen, C. S., & Liu, P. J. (2013) - The physiological response of the reef coral *Pocillopora damicornis* to elevated temperature:

results from coral reef mesocosm experiments in Southern Taiwan. *Marine environmental research*, 86, 1-11.

- Maynard, J.A., Anthony, K.R.N., Marshall, P.A., Masiri, I. (2008) - Major bleaching events can lead to increased thermal tolerance in corals. *Mar Biol* 155: 173–182.
- Meyer C.P., Geller J.B., Paulay G. (2005) - Fine scale endemism on coral reefs: archipelagic differentiation in turbinid gastropods. *Evolution* 59: 113-125
- Meyer E, Aglyamova G.V., Matz M.V (2011) - Profiling gene expression responses of coral larvae (*Acropora millepora*) to elevated temperature and settlement inducers using a novel RNA-Seq procedure. *Mol Ecol* 20(17):3599–3616.
- Mieog J.C., Van Oppen M.J.H., Berkelmans R., Stam W.T., Olsen J.L. (2009) - Quantification of algal endosymbionts (Symbiodinium) in coral tissue using real-time PCR. *Molecular Ecology Resources* (2009) 9, 74–82
- Mieog J.C., Van Oppen M.J.H., Cantin N.E., Stam W.T., Olsen J.L. (2007) - Real-time PCR reveals a high incidence of Symbiodinium clade D at low levels in four scleractinian corals across the Great Barrier Reef: implications for symbiont shuffling. *Coral Reefs*, 26, 449–457.
- Morris, J. P., Thatje, S., & Hauton, C. (2013) - The use of stress-70 proteins in physiology: a re-appraisal. *Molecular ecology*, 22(6), 1494-1502.
- Mudge F.B. (1997) - The development of greenhouse theory of global climate change from Victorian times. *Weather* 52 13–16
- Murray R.K., Rodwell V., Bender D., Botham K.M., Weil P.A., Kennelly P.J. (2009)- *Harper's Illustrated Biochemistry* (28th ed.) -McGraw-Hill Medical companies
- Muscatine, L. (1973) - Nutrition of corals. In *Biology and Geology of Coral Reefs*, Vol. 2. (Eds O. A. Jones and R. Endean.) pp. 77.115. (Academic Press: New York.)
- Muscatine, L. (1990) - The role of symbiotic algae in carbon and energy flux in reef corals. *Coral Reefs* 25, 1.29.
- Muscatine, L., and Porter, J. W. (1977). Reef corals: mutualistic symbioses adapted to nutrient-poor environments. *Bioscience* 27(7), 454.60.
- Nelson D.L. and Cox M.M. (2004) – *Lehninger Principles of Biochemistry* (4yh ed.). Zanichelli, Bologna, Italy
- O. Hoegh-Guldberg (1999) - *Mar. Freshw. Res.* 50, 839
- Odum, H.T., and Odum, E. P. (1955) - Trophic structure and productivity of windward coral reef community on Eniwetok Atoll. *Ecological Monographs* 25(3), 291.320.
- Oleksiak, M.F., Roach, J., Crawford, D.L. (2005) - Natural variation in cardiac metabolism and gene expression in *Fundulus heteroclitus*. *Nature Genetics*, 37, 67–72.
- Oliver T.A., Palumbi SR (2011) - Do fluctuating temperature environments elevate coral thermal tolerance? *Coral Reefs* 30(2):429–440.
- Oliver T.A., Palumbi, S.R. (2011) - Do fluctuating temperature environments elevate coral thermal tolerance? *Coral Reefs* 30(2):429–440.

- Palmer M., Haines K., Tett S., and Ansell T., (2007) - Isolating the signal of ocean global warming. *Geophys. Res. Lett.*, 34, L23610.
- Parikh H. et al. (2008) - Molecular correlates for maximal oxygen uptake and type 1 fibers. *Am J Physiol Endocrinol Metab* 294: E1152–E1159, 2008.
- Peterson N. (2011) - Coral Triangle Initiative on Coral Reefs, Fisheries and Food Security. CTI-CFF Regional Map <http://www.coraltriangleinitiative.org/>
- Petit J.R., Jouzel J., Raynaud D., et al. (1999) - Climate and atmospheric history of the past 420,000 years from the Vostok ice core, Antarctica. *Nature*, 399, 429–36.
- Polato N.R, et al. (2010) - Location-specific responses to thermal stress in larvae of the reef-building coral *Montastraea faveolata*. *PLoS ONE* 5(6):e11221.
- Reyes-Bonilla H., Ketchum-Mejia J. T., Cruz-Piñón G. y Barjau-González E. (2005) - Catálogo de corales pétreos (Anthozoa: Scleractinia) depositados en el Museo de Historia Natural de la Universidad Autónoma de Baja California Sur (MHNUABCS).
- Rhein M., Rintoul S.R., Aoki S., Campos E., Chambers D., Feely R.A., Gulev S., Johnson G.C., Josey S.A., Kostianoy A., Mauritzen C., Roemmich D., Talley L.D., and Wang F. (2013) - Observations: Ocean. In: *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change* [Stocker, T.F., D. Qin, G. -K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.
- Richardson A.J., Schoeman D.S. (2004) - Climate impact on plankton ecosystems in the Northeast Atlantic. *Science* 305:1609–12
- Richter, K., Haslbeck, M., & Buchner, J. (2010) - The heat shock response: life on the verge of death. *Molecular cell*, 40(2), 253-266.
- Rodriguez-Lanetty M, Harii S, Hoegh-Guldberg O. (2009) - Early molecular responses of coral larvae to hyperthermal stress. *Mol Ecol* 18(24):5101–5114.
- Roessig JM., Woodley C.M, Cech JJ., Hansen L.J. (2004) - Effects of global climate change on marine and estuarine fishes. *Rev. Fish Biol. Fish.* 14:215–75
- Rowan R. (1998) - Diversity and ecology of zooxanthellae on coral reefs. *J. Phycol.* 34:407-17
- Sagarin R.D., Barry J.P., Gilman S.E., Baxter C.H. (1999) - Climate-related change in an intertidal community over short and long time scales. *Ecol. Monogr.* 69:465–90
- Schmid D. et al. (1994) - Kinetics of molecular chaperone action. *Science* 263, 971–973
- Senior A.E., Nadanaciva S., Weber J. (2002) - The molecular mechanism of ATP synthesis by F1F0-ATP synthase. *Biochimica et Biophysica Acta* 1553 (2002) 188^211
- Sharp, V. A., Brown, B. E., & Miller, D. (1997) - Heat shock protein (hsp 70) expression in the tropical reef coral *Goniopora djiboutiensis*. *Journal of Thermal Biology*, 22(1), 11-19.

- Sheppard C.R.C., Spalding<sup>2</sup> M., Bradshaw<sup>4</sup> C. and Wilson<sup>3</sup> S. (1998) - Erosion vs. Recovery of Coral Reefs after 1998 El Niño: Chagos Reefs, Indian Ocean. *AMBIO: A Journal of the Human Environment* 31(1):40-48. 2002 doi: <http://dx.doi.org/10.1579/0044-7447-31.1.40>
- Siegenthaler U., Stocker T.F., Monnin E., et al. (2005) - Stable carbon cycle-climate relationship during the late Pleistocene. *Science*, 310, 1313–7.
- Smith D. M., and Murphy J. M., (2007) - An objective ocean temperature and salinity analysis using covariances from a global climate model. *J. Geophys. Res. Oceans*, 112, C02022.
- Smith, D.J., Suggett, D.J., Baker, N.R., (2005) - Is photoinhibition of zooxanthellae photosynthesis the primary cause of thermal bleaching in corals? *Global Change Biol* 11: 1–11.
- Smith, L.W., Wirshing, H., Baker A.C., Birkeland C. (2008) - Environmental versus Genetic Influences on Growth Rates of the Corals *Pocillopora eydouxi* and *Porites lobata* (Anthozoa: Scleractinia). *Pac Sci* 62(1):57–69.
- Southward A.J., Langmead O., Hardman-Mountford N.J., Aiken J., Boalch G.T., et al. (2005) - Long-term oceanographic and ecological research in the western English Channel. *Adv. Mar. Biol.* 47:1–105
- Spalding, M.D., Ravilious D, and Green E.P. (2001) - United Nations Environment Programme, World Conservation Monitoring Centre. *World Atlas of Coral Reefs*. University of California Press: Berkeley. 416 pp.
- St-Cyr J, Derome N, Bernatchez L (2008) - The transcriptomics of life-history trade-offs in whitefish species pairs (*Coregonus* sp.). *Molecular Ecology*, 17, 1850–1870.
- Storey, K.B., Storey J.M., (2004) - Metabolic rate depression in animals: transcriptional and translational controls. *Biological Reviews*, 79, 207–233.
- Swanson, R., and Hoegh-Guldberg, O. (1998) - The assimilation of ammonium by the symbiotic sea anemone *Aiptasia pulchella*. *Marine Biology* 131, 83-93.
- Taylor D L. (1974) - Symbiotic marine algae: Taxonomy and biological fitness. In *Symbiosis and the Sea*, ed. CBW Vernberg, pp. 245-62. Columbia: Univ.S . C. Press
- Thompson, D.M., van Woesik, R., (2009) - Corals escape bleaching in regions that recently and historically experienced frequent thermal stress. *Proc R Soc Lond, Ser B: Biol Sci* 276: 2893–2901.
- Todgham AE, Schulte PM, Iwama GK (2005) - Cross-tolerance in the tidepool sculpin: The role of heat shock proteins. *Physiol Biochem Zool* 78(2):133–144.
- Tomanek L (2011) - Environmental Proteomics: Changes in the Proteome of Marine Organisms in Response to Environmental Stress, Pollutants, Infection, Symbiosis, and Development. *Annual Review of Marine Science* 3: 373–399.

- Trenberth K.E., Stepaniak D.P., and Caron J.M. (2002). - Interannual variations in the atmospheric heat budget. *Journal of Geophysical Research -Oceans*, 107C, 4066, doi: 10.1029/2000JD000297.
- Trench R.K. (1979) - The cell biology of plant-animal symbiosis. *Annual Reviews of Plant Physiology* 30, 485-31.
- Trench R.K. (1993) - Microalgal-invertebrate symbiosis-a review. *Endocytobiosis Cell Res.* 9:135-75
- Turak ., DeVantier L. (2010) - Biodiversity and conservation priorities of reef-building corals in the Papuan Bird's Head Seascape. A Rapid Marine Biodiversity Assessment of Teluk Cendrawasih and the FakFak-Kaimana Coastline of the Papuan Bird's Head Seascape, Indonesia. R.A.P. Bulletin of Biological Assessment. Conservation International, Washington, DC
- Turak E. Souhoka J. (2003) - Coral diversity and the status of coral reefs in the Raja Ampat Islands. Report on a rapid ecological assessment of the Raja Ampat Islands, Papua, Eastern Indonesia, held October 30–November 22. The Nature Conservancy Southeast Asia Center for Marine Protected Areas, Sanur, Bali, Indonesia
- VanGuilder, H. D., Vrana, K. E., & Freeman, W. M. (2008). Twenty-five years of quantitative PCR for gene expression analysis. *Biotechniques* 44:619–626.
- Veron JEN. (1995) - Corals in space and time: the biogeography and evolution of the scleractinia. Cornell University Press, Ithaca
- Veron JEN. et al. (2000) - Corals of the world (3 vols) Australian Institute of Marine Science, Townsville
- Veron JEN. Et al. (2009) - Delineating the Coral Triangle. *Galaxea, Journal of Coral Reef Studies* 11: 91–100
- Veron JEN. et al. (2011) – The Coral Triangle. *Coral Reefs: An Ecosystem in Transition*, 47-55
- Vidal-Dupiol, J., Adjeroud, M., Roger, E., Foure, L., Duval, D., Mone, Y., Ferrier-Pages, C., Tambutte, E., Tambutte, S., Zoccola, D., Allemand, D., Mitta, G., (2009) -Coral bleaching under thermal stress: putative involvement of host/symbiont recognition mechanisms. *BMC Physiology* 9, 14. Yang, R.T., 1985. Coral communities in Nan-wan Bay.
- Voolstra C.R., et al. (2009) - Effects of temperature on gene expression in embryos of the coral *Montastraea faveolata*. *BMC Genomics* 10:627.
- Wallace A.R. (1869) - The Malay Archipelago (2 vols). Macmillan, London
- Wallace, C.C. and Wolstenholme, J. (1998) - Revision of the coral genus *Acropora* (Scleractinia: Astrocoeniina: Acroporidae) in Indonesia. *Zoological Journal of the Linnean Society*(1998), 123: 199-384



- Warner, M.E., Fitt W.K., and Schmidt G. W. (1999) - Damage to photosystem II in symbiotic dinoflagellates: a determinant of coral bleaching. *Proceedings of the National Academy of Sciences* 96:8007–8012.
- Weis V.M. (2010) - The susceptibility and resilience of corals to thermal stress: adaptation, acclimatization or both?. *Molecular Ecology* 19: 1515–1517
- Weis, V.M., (2008) - Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. *J Exp Biol* 211: 3059–3066.
- Wells F.E. (2002) - Centres of species richness and endemism of shallow water marine molluscs in the tropical Indo-West Pacific. *Proc Ninth Intern Coral Reef Symp Bali* 2000(2):941–945
- West J.M., Salm R.V. (2003) - Resistance and resilience to coral bleaching: Implications for coral reef conservation and management. *Conserv Biol* 17(4):956–967.
- Wielicki B.A., Barkstrom B.R., Harrison E.F., Lee R.B., Smith and J. E. Cooper G.L., 1996: Clouds and the Earth's radiant energy system (CERES): An Earth observing system experiment. *Bull. Am. Meteor. Soc.*, 77, 853–868.
- Wild M., Folini D., Schär C., Loeb N., Dutton E.G., and G. König-Langlo, (2013) - The global energy balance from a surface perspective. *Clim. Dyn.*, 40, 3107-3134.
- Wilkinson, C. (2008) - Status of the Coral Reefs of the World: 2008. Townsville, Australia. Global Coral Reef Monitoring Network and Reef and Rainforest Research Centre.
- Wilkinson, C. (2004) - Status of coral reefs of the World: 2004. Volume 2. Status of coral reefs of the world.
- Williams, G.J., Knapp, I.S., Maragos, J.E., Davy, S.K. (2010) - Modeling patterns of coral bleaching at a remote Central Pacific atoll. *Mar Pollut Bull* 60: 1467–1476.
- Wooldridge S.A and Done T.J. (2009) - Improved water quality can ameliorate effects of climate change on corals. *Ecological Applications*, 19(6), pp. 1492–1499.
- Wooldridge S. A. (2009) - A new conceptual model for the warm-water breakdown of the coral–algae endosymbiosis. *Marine and Freshwater Research*, in press.
- Yoshimune K, Yoshimura T, Nakayama T, Nishino T, Esaki N (2002) - Hsc62, Hsc56, and GrpE, the third Hsp70 chaperone system of *Escherichia coli*. *Biochem Biophys Res Commun*. 293:1389-95