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Transcriptional profiles inferring thermal stress responses of the coral *Oculina patagonica* from the Eastern Mediterranean Sea

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Fisiologia Molecolare degli Animali Marini

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Contents

1. Introduction

1.1 The Phylum Cnidaria

The Phylum Cnidaria includes approximately 9000 species (Technau and Steele, 2011) that inhabit aquatic environments, in particular marine habitats. Cnidarians can be found either in the form of a polyp, tied to the substrate, or in the form of a medusa, mostly planktonic. This Phylum comprises four distinct classes: the Anthozoa, the Hydrozoa, the Scyphozoa and the Cubozoa. Anthozoans (sea anemones and corals)can be found solely in the form of solitary or colonial polyps; Hydrozoans (hydroids) are usually present both in the form of a polyp and in the medusa form; Scyphozoans and Cubozoans have primarily only the medusa stage (Nobre Montenegro Ventura, 2018).

Cnidarians share a simple body plan, with a central cavity (the coelenteron) surrounded by two cellular layers, the endoderm and the ectoderm; between these two layers there is an intermediate gel-like region called mesoglea (Fig. 1). The tissues also include nerve cells, interstitial cells, nematocytes and, in some groups, gametes (Galliot and Schmid, 2002).The central cavity (gastrovascular cavity) has only one opening to the environment, and it is responsible for both the digestion of food and the transport of nutrients throughout the body, serving as mouth or anus at different times (Thorp and Covich, 2009).

The feeding aperture of both polyps and jellyfish is typically surrounded by a crown of tentacles (Fig. 1).

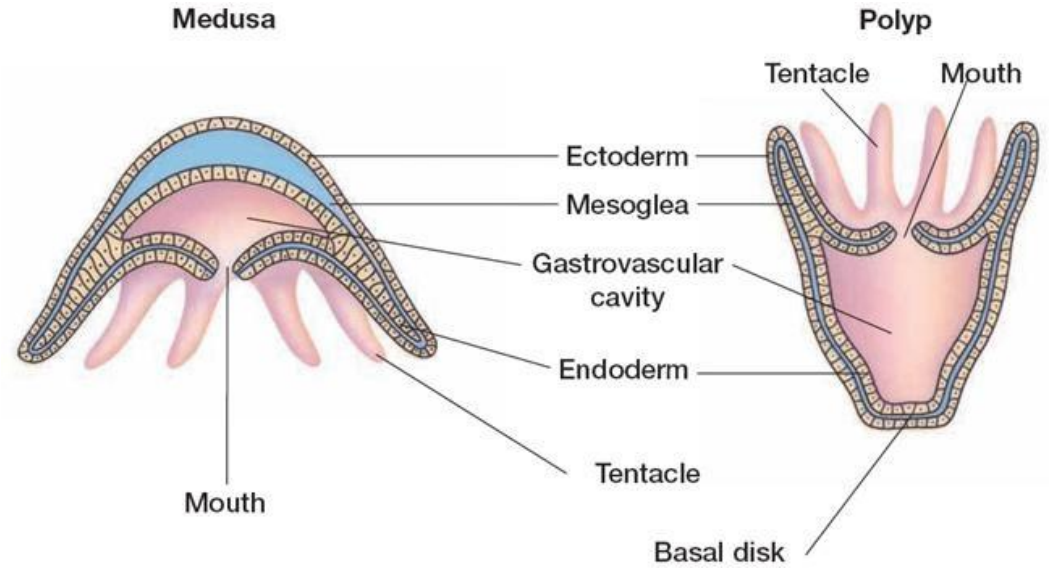

Figure 1. Cnidarian body plan. Two body forms –medusa and polyp- with the same body parts arranged differently. Retrieved from https://www.shapeoflife.org.

In this Phylum reproduction can be both sexual and asexual; during sexual reproduction, the medusa generates gametes that, after fertilization, develop a planktonic larva called planula, that settles on the substrate and metamorphoses into a polyp stage (Nobre Montenegro Ventura, 2018). The polyp can also reproduce asexually by budding or fragmentation either during asexual reproduction or to develop the parental form (the medusa), completing the sexual cycle (Galliot and Schmid, 2002); polyps can also reproduce sexually using gametes that, after fertilization, develop a new polyp.

Anthozoans can only be found in marine environments, with a global distribution from the intertidal zone to over 6,000 meters deep. This Class includes sea anemones, a variety of corals, gorgonians and sea pens, that have polyps with a flower-like appearance (Grzimeck et al., 2004).These cnidarians have solely the polyp form, characterized by a tubular body with tentacles around the mouth, and are predominantly sedentary after the larval stage.

Species might be solitary or colonial: solitary forms attach to a hard substrate or penetrate into soft mud or sand, while the colonial forms build massive skeletons (like the reef-building corals) or thinner fan-like structures (Bridge et al., 1995; Schmidt, 1974).

1.2 The scleractinian corals

The class Anthozoa comprises two groups, that are distinguished by the form and number of the polyp tentacles: the subclass Octocorallia (polyps with eight tentacles), which includes soft corals, sea fans and sea pens, and the subclass Hexacorallia (polyps with six tentacles or primarily multiple of six), which includes sea anemones, stony corals and black corals (Bayer et al., 1983; Berntson et al., 1999; Daly et al., 2007). Stony corals belong to the order Scleractinia and they build themselves a hard skeleton through the deposition of calcium carbonate. Between the scleractinians, the species that contribute with their skeletons to the formation of the stony framework of the reef are called hermatypic corals, whereas the species that do not contribute to the reef development are referred to as ahermatypic (Schuhmacher and Zibrowius, 1985).

Scleractinian corals can be found in the solitary form or colonial form, which is characterized by genetically identical polyps that are interconnected (Van Woesik, 1995). The individual polyp is soft-bodied and has a cylindrical body with a upper oral disk surrounded by a crown of tentacles (Goffredo and Dubinsky, 2016). The lower part of the polyp body secretes layers of calcium carbonate in the form of aragonite, forming a hard cup-shaped skeleton called corallite (Cohen and McConnaughey, 2003). Solitary corals reproduce sexually, whereas in colonial corals the

polyps reproduce asexually by budding, remaining attached to each other and forming a colony with a common skeleton (Van Woesik, 1995).

The majority of the scleractinians are suspension feeders, catching bacteria, small planktonic invertebrates, phytoplankton or suspended organic matter (Grzimeck et al., 2004). Some species are characterized by a symbiotic relationship with dinoflagellate algae, called zooxanthellae, which provide the coral host the products of the photosynthesis, boosting their growth. Zooxanthellae are typically found inside the cnidarian host cells of the gastrodermis, the inner most layer of tissue that surrounds the gastrovascular cavity, and they are enclosed in a symbiosome membrane that isolates the algal cell from the host cytoplasm (Allemand and Furla, 2018; Davy et al., 2012; Wakefield et al., 2000).The dinoflagellates can be acquired by vertical transmission (maternal transmission), through the incorporation of the algae into the released eggs, or by horizontal transmission, through the acquirement of the symbiont from the environment by phagocytosis (Allemand and Furla, 2018; Davy and Turner, 2003).

The cnidarian-zooxanthellae symbiosis provides a partial autotrophy (mixotrophy) to the host; the algae in fact supply their partners with photosynthetic products, in particular lipids and carbohydrates, supporting the host metabolism, survival, growth and reproduction (Allemand and Furla, 2018; Davy et al., 2012; Hoegh-Guldberg, 1999; Meehan and Ostrander, 1997). Furthermore, in the case of stony corals, zooxanthellae can boost the host calcification in a process called “light-enhanced calcification”, supporting coral skeletogenesis (Gattuso et al., 1999; Goreau, 1959; Tambuttè et al., 2011). In return for these benefits, the algae receive nutrients (ammonia and phosphate) from the host waste metabolism and protection from grazers (Allemand and Furla, 2018; Davy et al., 2012; Hoegh-Guldberg, 1999).

1.3 Climate change in marine ecosystems of the Mediterranean Sea

Carbon dioxide, methane and nitrous oxide atmospheric concentrations have incremented conspicuously since 1750 due to anthropogenic activities, in particular fossil fuel use, land-use change and agriculture, exceeding the pre-industrial global values (IPCC, 2007).This increase in heat-trapping greenhouse gases has warmed the low atmosphere and the earth surface of about 0.9°C since the late 19th century, leading to an increment of average ocean temperatures, to the melting of ice sheets and glaciers, and to the rising of global average sea level (Cleugh et al., 2006; IPCC, 2007). Several long-term changes in climate have been detected globally, including variations in ocean salinity, in precipitation and wind patterns, and in the frequency of extreme events like

drought, cyclones and heat waves (IPCC, 2007). Furthermore, the increased carbon dioxide concentration has affected the ocean chemistry: more carbon dioxide reacting with water forms more carbonic acid, that has led to an augmentation of the ocean acidity.

The Mediterranean Sea is located in a transition zone bounded by the dry North Africa climate and the temperate central Europe climate, and it is influenced by even small changes of the circulation patterns of mid-latitude winds and of sub-tropical high-pressure cells, that could cause considerable variations in the Mediterranean climate (Giorgi and Lionello, 2008). Therefore, these features make the Mediterranean a particularly sensible sea to climatic changes, representing one of the most noticeable "Hot-Spots" and most responsive region to global climate change (Diffenbaugh et al., 2007; Giorgi and Lionello, 2008; Shaltout and Omstedt, 2014).

The increased greenhouse gas forcing has caused in particular a large decrease in precipitations, an increase in the inter-annual warm season fluctuations and an increment (maximum in summer) of the surface water temperature (Diffenbaugh et al., 2007; Giorgi and Lionello, 2008).

Concerning future projections, different scenario simulations display a general warming of the Mediterranean Sea during the twenty-first century (Adloff et al., 2015; Gibelin and Dèquè, 2003), with average sea surface temperature increasing nearly up to + 6 °C for the 2071–2100 period compared to the reference period 1986–2015 (Sakalli, 2017) (Fig. 2).

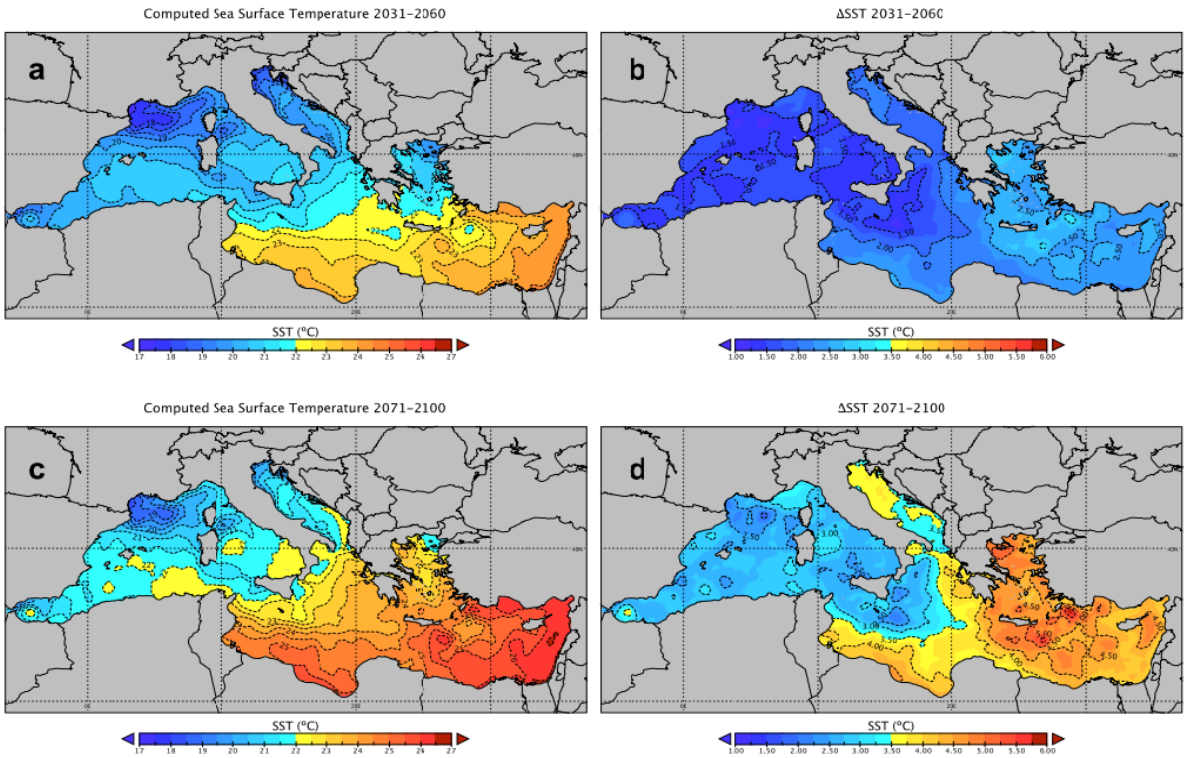

Figure 2. Scenario simulations of the predicted 30-year a) 2031-2060 and c) 2071-2100 average sea surface temperature (SST) in the Mediterranean Sea, and the relative differences (b and d) to the 30-years reference time period (1986-2015), respectively. Modified from Sakalli (2017).

1.4 Effects of climate change in corals

Climate change is expected to have major effects on the Earth ecological systems, leading to shifts in animal and plants distributions, loss of diversity, changes in the timing of seasonal activities, incremented frequency of diseases and diffusion of invasive species (Harvell et al., 2002; Lovejoy, 2006; Parmesan and Yohe, 2003; Walther et al., 2002). Corals are especially sensitive to rapid climate changes, since the exposure to seawater temperatures that are just a few degrees over the long-term average could lead to coral stress, bleaching and eventually death (Hoegh-Guldberg, 1999).

Coral bleaching takes place when corals lose or expel their symbiotic zooxanthellae, when the algae themselves lose pigmentation, or when a combination of these episodes occurs; after this loss, the hard corals calcium carbonate skeletons get visible and the corals result bleached (Allemand and Furla, 2018; Brown, 1997; Glynn, 1991; Hoegh-Guldberg, 1999; Meehan and Ostrander, 1997; Weis et al., 2008). This phenomenon is not restricted to scleractinian corals, but it can also take place in many other organisms that have a symbiotic relationship with the zooxanthellae, like hydrocorals, soft corals and bivalves (Glynn, 1991).

The loss of dinoflagellates has a pronounced effect on the coral host, since the photosynthesis products from the algae represent a great portion of the energy requirements of the coral and since they are a major enhancement for calcification; if corals are able to gain their zooxanthellae back in a short amount of time they often recover, but if the bleaching event is too intense and it lasts for too long corals usually die (Fitt and Warner, 1995; Glynn, 1991; Lang et al., 1992).

Bleaching can be caused by several biological and physical elements, like increased levels of ultraviolet radiation (Gleason and Wellington, 1993), salinity fluctuations (Goreau, 1964) and increased sedimentation (Stafford-Smith,1993), but more commonly it is driven by a higher than average sea water temperature, which induces cellular damages and the deterioration of the algae photosystem, that leads to an increase of reactive oxygen molecules and to the subsequent degradation and/or expulsion of the zooxanthellae through several potential mechanisms (Allemand and Furla, 2018; Brown, 1997; Coles and Brown, 2003; Glynn, 1991; Weis et al., 2008).

Furthermore, temperature variations may induce transcriptional modifications, lipid and protein deterioration and cell death induction in both the corals and their symbiotic partners (Dunn et al., 2004; Moya et al., 2012; Richier et al., 2006; Tchernov et al., 2004; Warner et al., 1999).

There is a high variability connected to bleaching events, that is not always correlated to sea water temperature variations; coral colonies in fact usually display a spectrum of bleaching intensity

within the colony itself, and the tendency to bleach can also be dissimilar between colonies positioned next to each other (Hoegh-Guldberg, 1999). Since the water temperature unlikely varies between different part of the same coral colony or between adjacent colonies, the propensity to bleach must be correlated to other factors, like the genotype of the corals and their acclimation capacity (Hoegh-Guldberg, 1999).

Another major threat for corals is ocean acidification, which is caused by the increasing concentration in the Earth atmosphere of carbon dioxide, that is absorbed by the surface of the ocean resulting in more hydrogen ions, which increase the acidity of the water.

Ocean acidification has dramatic impacts on corals, limiting their growth by corroding the aragonite skeletons and by slowing their growth rate, weakening the symbiotic relationship with the zooxanthellae and negatively impacting coral larvae physiology (Hoegh-Guldberg et al., 2007; Nakamura et al., 2011).

1.5 Molecular mechanisms of physiological responses to stress: induction of the heat shock proteins

Environmental acclimatization is the mechanism by which organisms tune their physiology and molecular machinery within their lifetime in order to cope with mutable environments, and it is also addressed to as physiological plasticity (Gates and Edmunds, 1999; Hofmann and Todgham, 2010).

Over the last few decades there has been a significant increase in the number of investigations on the molecular mechanisms that drive the coral acclimatization processes, since they may provide useful insights into the possible future distributional shifts of these organisms, and also they may contribute to characterize the relative vulnerabilities of the species to climate change (Louis et al., 2017; Seneca and Palumbi, 2015). In this regard, the variation of gene transcription depicts one of the most important tools used by organisms to cope with environmental stress, which is defined as an exogenous factor that causes modifications in a biological system and that can be potentially dangerous, impacting the organism fitness (Evans and Hofmann, 2012; Hofmann and Parsons, 1991). Monitoring changes of mRNA expression profiles provides early-warning molecular markers of physiological plasticity and represents an important source of information on the mechanisms underlying the differences in coral physiological plasticity (Dixon et al., 2015; Poli et al., 2017; Seneca and Palumbi, 2015).

The induction of heat shock proteins (Hsps; Feder and Hofmann, 1999) is one of the most conserved and ubiquitous physiological mechanism associated with environmental acclimatization (Kültz, 2005). Hsps are members of the multi-protein family of molecular chaperones, whose physiological role is to secure the correct protein folding during protein synthesis and aid in intracellular protein trafficking (Hartl et al., 2011). Under stress conditions, they act to stabilize and refold denatured proteins, or they are also involved in the degradation of severely damaged proteins (Benarroch, 2011; Ellison et al., 2017; Fabbri et al., 2008; Louis et al., 2017; Tomanek, 2010). Hsps are induced by several stressors; however, they are greatest distinguished by their essential role in cellular level thermal tolerance (Gates and Edmunds, 1999); in fact, temperature is one of the primary stressors in marine environments, and just a few degrees variations could lead to an imbalance of protein homeostasis, damages to cellular structures and disrupted cellular functions (Richter et al., 2010).

Hsps are classified into several families according to their molecular weight: hsp110, hsp100, hsp90, hsp70, hsp60, hsp40, hsp10 and small hsps (Feder and Hofmann, 1999).The best studied and most abundant family is that of the 70-kDa Hsps (Hsp70), that are highly conserved in almost all organisms studied thus far and are characterized by a peculiar flexibility in terms of activity range (Fabbri et al., 2008; Morris et al., 2013; Richter et al., 2010). Indeed, the Hsp70s can interact through their protein binding domain with various peptides, and their chaperoning activity and affinity for the substrates is regulated by the binding and hydrolysis of ATP in the ATPase domain and by the presence of several cofactors (Richter et al., 2010). These proteins represent the most frequently up-regulated amongst all Hsps in response to stress in the majority of the organisms (Morris et al., 2013).

Generally speaking, members of the HSP70 multigene family can be distinguished by three different expression patterns: (a) strictly stress-inducible HSP70 (Voellmy et al., 1985), (b) cell-cycle-dependent, stress-inducible HSP70 (Hunt and Morimoto, 1985; Milner and Campbell, 1990), and (c) constitutively expressed, less stress-dependent HSP70 genes, e.g., heat shock cognate or HSC70 (Ali et al., 1996). The Hsps biosynthesis and chaperoning activities are energetically expensive, therefore it is generally observed that the expression of inducible Hsp isoforms occurs solely above a determined threshold temperature (Dong et al., 2008). Nevertheless, many marine organisms display deviations from this general scheme as a consequence of their peculiar adaptive features (Morris et al., 2013). In particular, enhanced thermotolerance in intertidal invertebrates as limpets, mussels, oysters, sea cucumbers, and amphipods, is linked to higher physiological

expression of stress-inducible Hsp70 (Bedulina et al., 2010; Dong et al., 2010; Franzellitti and Fabbri, 2005; Piano et al., 2005), a phenomenon known as “gene frontloading” (Barshis et al., 2013).

1.5.1 Hsps as molecular markers of environmental stress in corals

The evolution of transcriptomics and molecular tools for corals has emphasized the opportunity to generate molecular markers as tools to assess and monitor coral stress, to detect the possible causes and target resilient individuals for restoration purposes (Louis et al., 2017).

A molecular marker is a DNA or RNA sequence that can be used to predict an organism’s risk of disease and to identify a disorder at its early stage or even before the symptom manifestation; therefore a transcriptional biomarker denotes the expression, regulation and function of that particular gene chosen as biomarker (Louis et al., 2017).

In the last few years many studies have focused on the application of transcriptional analyses to identify stress in corals, especially thermal stress and bleaching (Barshis et al., 2013; Dixon et al., 2015; Meyer et al., 2009; Seneca and Palumbi, 2015). In particular, the focus has been on those transcripts that can appraise the stress promptly, through their immediate up-regulation after the exposure of corals to a stress factor. Transcripts encoding the Hsp70 are amongst the primary early responders to stress in corals, hence suitable candidates as coral molecular stress markers (Louis et al., 2017; Traylor-Knowles et al., 2017).

The phylogenetic analysis reported by Poli et al. (2017) pointed out the conserved phylogenetic and structural features of the coral Hsp70 multi protein family (Fig. 3).

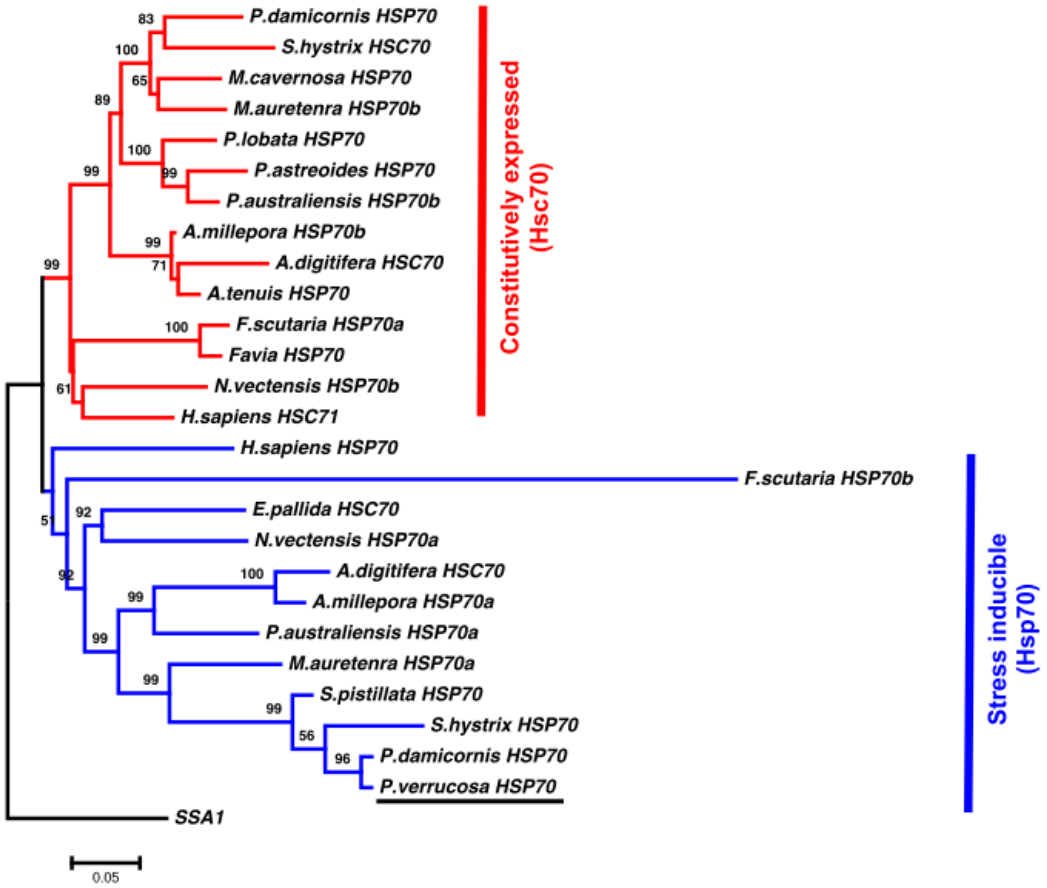

Figure 3. Phylogenetic relationships among Hsp70 deduced amino acid sequences of corals. Multiple sequence alignment was generated with the MEGA software ver 6 and the MUSCLE algorithm. The tree was constructed by the Maximum Likelihood algorithm using PAUP*. Bootstrap confidence values for the sequence groupings are indicated in the tree (N = 1000). The appropriate model parameters for the maximum-likelihood analysis were determined using a likelihood-ratio test with jModelTest. The human Hsp70 (Human_HSP70, GenBank Ac. Numb. P17066) and Hsc70 (Human_HSC71, GenBank Ac. Numb. AAH19816) sequences were included in the alignment for comparison. SSA1 from *Saccharomyces cerevisae* was used as outgroup (GenBank Ac. Numb. P10591). Modified from Poli et al. (2017).

In particular, under a structural point of view, corals retain the same classification between HSC70 and HSP70 proteins. Nevertheless, this putative inducible *hsp70* transcript was found expressed at well detectable levels under physiological conditions (Poli et al., 2017, Franzellitti et al., 2018) in agreement with previous studies demonstrating *hsp70* frontloading in intertidal organisms (Fabbri et al. 2008). Furthermore, many studies showed induction of *hsp70* expression in corals under different abiotic and biotic cues, including temperature (Maor-Landaw et al., 2014; Seveso et al., 2014; Zhang et al., 2018), pH/pCO_2 (Moya et al., 2015), salinity (Ellison et al., 2017), pollutants (Jovanović and Guzmán, 2014; Overmans et al., 2018; Venn et al., 2009), and bacterial infections (Brown et al., 2013; Seveso et al., 2016). On the whole, the above evidence suggests that cnidarian

hsp70 gene expression and function is similar across multiple stressors and comparable to vertebrates (Kvitt et al., 2016). Furthermore, since hsp70s are under diel cycle (Levy et al. 2011), their expression patterns may be affected by internal processes related to the regulation of the metabolic machinery and host-symbiont interactions (Rosic et al., 2014b). The prompt up-regulation of *hsp70* transcripts at the initial stage of the stress response appears to be a protective mechanism loaded by corals to avoid the onset of severe pathological conditions or to postpone bleaching (Maor-Landaw et al., 2014; Rosic et al., 2014a). To this regard, hsp70s may be considered as a marker for holobiont homeostasis (Levy et al., 2011; Rosic et al., 2014b).

1.6 *Oculina patagonica*

Oculina patagonica (De Angelis, 1908) is a zooxanthellate scleractinian coral supposedly originated from the Southwest Atlantic (Zibrowius, 1974). *O. patagonica* is a facultative symbiotic species that is capable of heterotrophic feeding, capturing plankton as well as dissolved and particulate organic matter (Armoza-Zvuloni et al., 2011; Tremblay et al., 2011).

This species is an opportunistic dominant settler, that grows over the hard structures of barnacles, serpulids, etc. (Fig. 4), and that eradicates other soft organisms living around its growing boundary (Fine et al., 2001; Sartoretto et al., 2008). *O. patagonica* forms encrusting colonies at a depth range of 0.5-10m (Rodolfo-Metalpa et al., 2006), and it is able to grow in diverse littoral habitats, in unspoiled as well as polluted areas, such as ports and industrial zones. It has both sexual and asexual reproduction, a high growth rate and an early reproductive age, characteristics that favor this species proliferation and dissemination (Fine et al., 2001; Sartoretto et al., 2008).

O. patagonica has the ability to thrive under a wide range of environmental conditions in terms of temperatures, salinity, UV radiation, turbidity, and wave energy (Fine et al., 2001). This species is capable of developing gametogenesis even in polluted waters (Armorza-Zvuloni et al., 2011) and can resist to repeated bleaching events (Ainsworth et al., 2008). Indeed, it thrives in areas where summer water temperatures may reach 40°C in tidal pools (Fine et al., 2001), and about 26°-28°C in open seas along the eastern Mediterranean coasts (Rodolfo-Metalpa et al., 2006; Sartoretto et al., 2008). Bleaching tolerance in this species appears tightly related to its most noticeable feature of facultative symbiosis, which is the ability of some temperate corals to occur in both symbiotic and asymbiotic forms, depending on the environmental conditions in which they live (Dimond and Carrington, 2008; Piniak, 2002). Unlike obligate zooxanthellates, facultative zooxanthellate corals can persist in a healthy azooxanthellate state by switching from a prevalent autotrophic to an

exclusive heterotrophic nutrition, i.e. the capture of dissolved organic matter, particulate organic matter and zooplankton predation (Houlbrèque and Ferrier-Pagès, 2009).

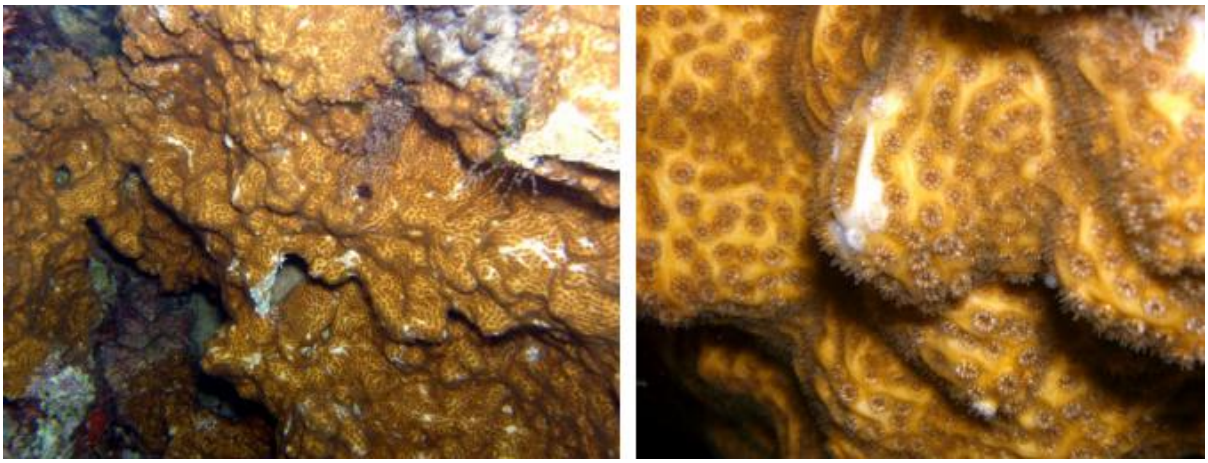

Figure 4. Colonies of *Oculina patagonica.* Photographed along the Turkish coast of the Mediterreanean by Melih Ertan Çinar (Çinar et al., 2006).

The first sighting of this coral in the Mediterranean was due to the diver Luigi Morra, who observed a wide colony of *O. patagonica* on the Ligurian coast in the 1960s (Sartoretto et al., 2008). Several years after its discovery, the species was found in France, Spain, Algeria, Tunisia, Egypt, Israel and Lebanon (Bitar and Zibrowius, 1997; Çinar et al., 2006; Sartoretto et al., 2008) (Fig. 5).

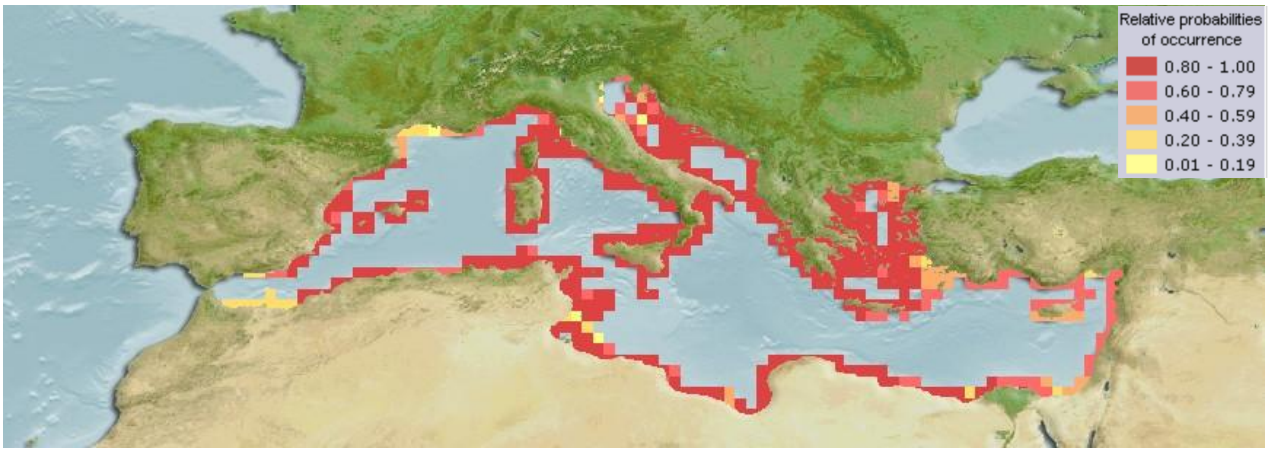

Figure 5. Distribution of *O. patagonica* along the Mediterranean coasts (data source: http://www.aquamaps.org). Distribution range colors indicate degree of suitability of habitat which can be interpreted as probabilities of occurrence.

Initially, this non-lessepsian species was thought to have been accidentally brought into the Mediterranean by transoceanic transport as a fouling organism through the strait of Gibraltar, and

that from the western Mediterranean it was dispersed towards the eastern basin by the maritime traffic (Bitar and Zibrowius, 1997; Çinar et al., 2006; Fine et al., 2001; Zibrowius, 1974). However, the study by Leydet and Hellberg (2015) has brought to light new genetic evidence on this species origin, according to which Mediterranean populations of *O. patagonica* are closely related to populations from the western Atlantic, but they are genetically distinguished from them and have not been brought into the Mediterranean from that region in anthropogenic times. It is likely that *O. patagonica* has long been present in the eastern Atlantic and only recently it has been expanding at "invasive levels" in the Mediterranean due to environmental change and anthropogenic modifications of coastal habitats (Leydet and Hellberg, 2015). Invasive species are usually considered to have been introduced from elsewhere; nevertheless, species can also turn invasive within their native range (Carey et al., 2012; Simberloff and Rejmánek, 2011). Therefore, *O. patagonica* might have already been present in the Mediterranean without been detected, but only recently it reached invasive levels due to human-mediated disturbances that favored its disclosure (Leydet and Hellberg, 2015).

2. Objectives

Critical to forecast how coral populations will cope with the effects of climate change is understanding the role of natural variation of gene expression in determining phenotypic plasticity. In particular, it is essential to understand the physiological susceptibility of corals to the physical drivers of climate change, and the physiological ability of these organisms to buffer further environmental modifications. Different coral species show diverse sensibilities to environmental stress (Barshis et al., 2013; Marshall and Baird, 2000; Meehan and Ostrander, 1997); hence, in order to assess the importance of response patterns to a stress source, it is crucial to expand the physiological studies to more comprehensive investigations between different species likely having different stress sensitivities. During mass bleaching events caused by the anomalous sea water temperature increases, the survival of sparse coral colonies indicates that some species may have an intrinsic thermal tolerance (Marshall and Baird, 2000). Furthermore, some naturally warm environments retain healthy coral populations that show elevated bleaching tolerances (Oliver and Palumbi, 2011). These inherently thermotolerant corals have most likely the strongest ability to cope with future climate change (West, 2003); therefore, they constitute a remarkable source of information on the mechanisms that govern coral physiological resilience (i.e. the capacity of an organism to resist or rapidly recover from an environmental stress). *Oculina patagonica* is one of these thermotolerant coral species (Fine et al., 2001; Rodolfo-Metalpa et al., 2006b) widely distributed in the Mediterranean Sea, and that can eventually adapt to life without zooxanthellae by virtue of a facultative symbiotic partnership. *O. patagonica* has been employed in this study to investigate the influence of its peculiar physiological traits on stress responsiveness by assessing baseline *hsp70* expression and its temporal dynamics of induction after an acute heat stress. Furthermore, data collected were further integrated and discussed within a comparative analysis with similar findings reported in 5 temperate corals of the Mediterranean Sea living in different thermal environments (Franzellitti et al., 2018). The whole set of addressed species is representative of different growth modes (colonial vs solitary) and trophic strategies (obligate/facultative zooxanthellate vs azooxanthellate). This comparison allowed addressing whether the *hsp70* expression varies in accordance with the stress sensitivity of coral populations inhabiting different thermal environments and possessing different physiological traits. The different capabilities of species to promptly increase *hsp70* transcription within a short-term intense heat stress exposure may forecast their relative stress susceptibility.

The interplay between stress response and cell death mechanisms is believed to be at the basis of coral sensitivity to bleaching (Granados-Cifuentes et al., 2013). The relative temporal dynamics of different cytoprotective, metabolic or cell-cycle regulator mechanisms may be used to predict bleaching events and monitoring subsequent coral recovery (Granados-Cifuentes et al., 2013; Maor-Landaw et al., 2014). Therefore, a further stage of the investigation reported in this study is addressed to the analysis of post heat-stress coordinated transcriptional regulation of transcripts related to energy metabolism, redox regulation, and DNA damage to disclose a potential time line of the events occurring in *O. patagonica* in response to an acute heat stress.

Coral collection, thermal stress experiments and protocol setup for transcriptional analyses were performed at the Leon H. Charney School of Marine Science of the University of Haifa (Israel).

3.Materials and Methods

3.1 Coral sampling and experimental design

During May 2018, 8 colonies of *Oculina patagonica* were collected by SCUBA diving under a special permit from the Israeli Natural Parks Authority from Sdot-Yam, Israel (32°29'25.3"N 34°53'17.6"E). Colonies were sampled at depths of 4-6 meters, and they were transported to a closed-circuit aquarium system at the Leon H. Charney School of Marine Science at the University of Haifa (Israel) (Fig. 6).

Figure 6. Closed-circuit aquarium systems of the Leon H. Charney School of Marine Science at the University of Haifa (Israel). Image courtesy of Tali Mass.

Each colony was split into fragments of about 2.5 cm^2 in size; the fragments were placed into two aquaria and acclimatized for 2 months at a constant water temperature of 24°C and a photoperiod of 16h:8h light:dark, matching the average conditions at the collection sites. The fragmentation of

a single colony provided replicates to account for the biological variability related to different morphologies and life history traits of corals native to diverse colonies.

Thermal stress experiments were performed employing a 2-hours heat shock treatment at a temperature of 35°C, followed by 0 to 24 h (0h, 2h, 12h and 24h) of post-stress recovery at the coral acclimatization temperature (24°C).

After the acclimatization period 8 coral fragments, one per each colony, were randomly collected to account for dissimilarities in coral physiological status at the beginning of the experimental exposure to thermal stress, hence representing the experiment control conditions.

The remaining samples were divided randomly on top of a moving tray, to facilitate their manipulation during the experiment (Fig. 7A) and they were moved to an experimental aquarium were the water temperature was increased to 35°C.

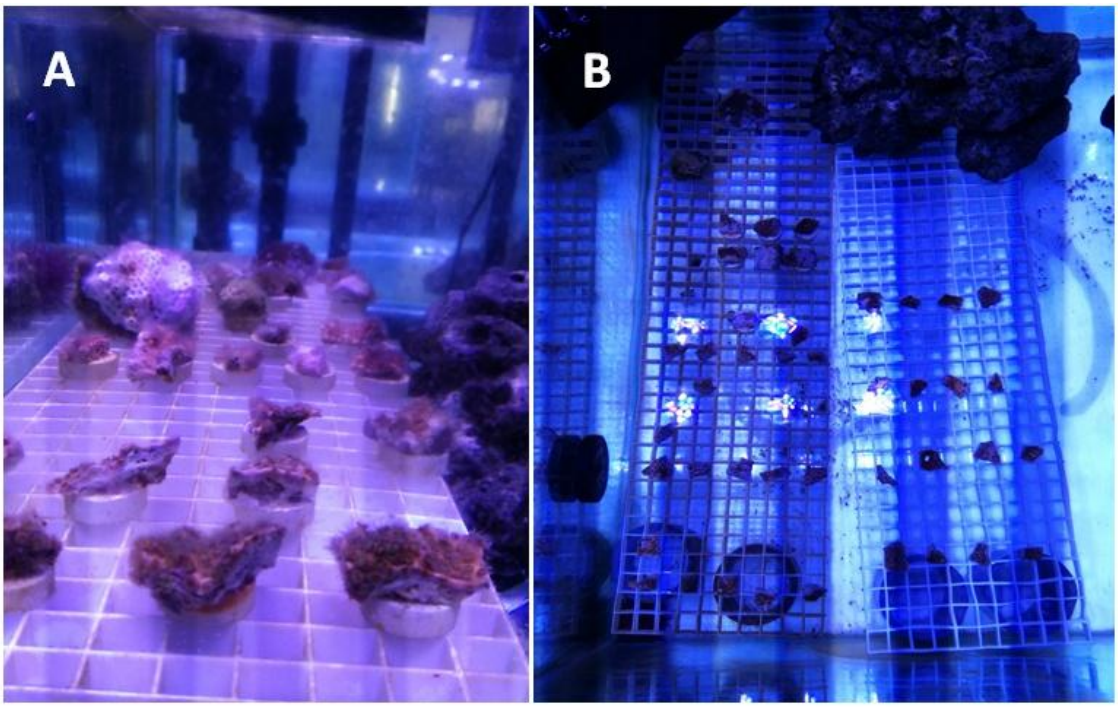

Figure 7. Pictures of the experimental setting. (A) *O. patagonica* samples in the 24°C aquarium after the acclimatization period and before the beginning of the experiment. (B) Treatment group in the 24°C aquarium at 2 hours post-stress recovery; some samples show signs of a mild bleaching. Pictures taken at the Leon H. Charney School of Marine Sciences.

After 2 hours at 35°C, 8 coral fragments (one per each colony) were randomly collected, immediately preserved in liquid nitrogen and stored at – 80°C until analyzed. The remaining samples were transferred back at 24°C and allowed to recover for 2-, 12- or 24-h post-stress periods (Fig. 7B). For each time point 1 fragment per colony (8 fragments per time point) was

randomly collected, immediately preserved in liquid nitrogen and stored at – 80°C until analyzed (Fig. 8).

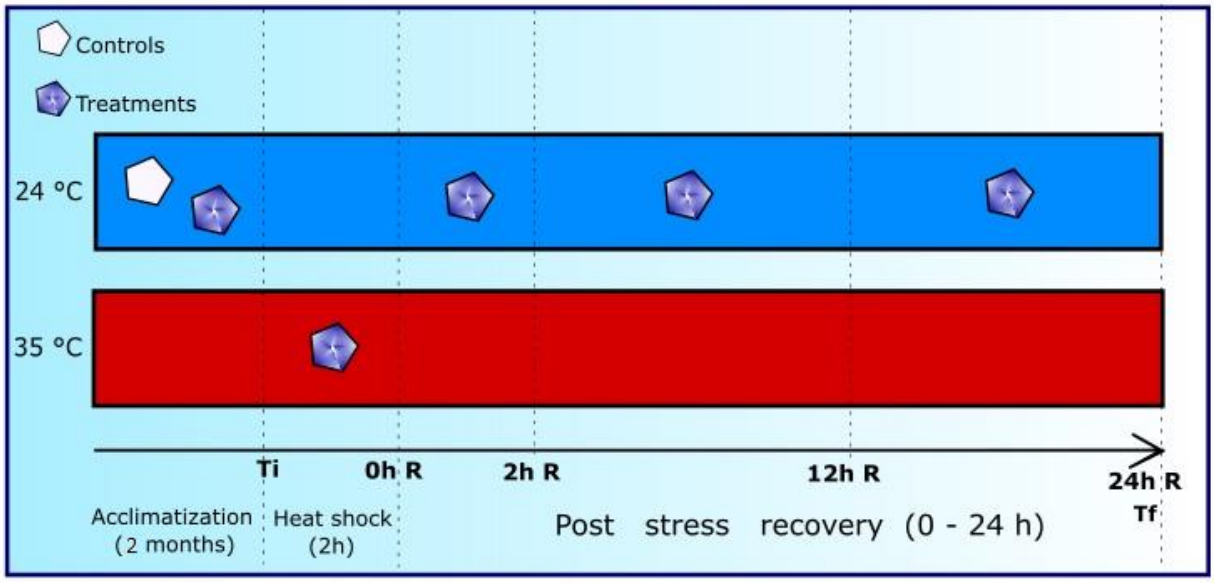

Figure 8. Scheme of the experimental design. Controls and treatments were both acclimatized at 24°C; Ti: beginning of the experiment, the controls were collected and the treatments were exposed to the heat stress for 2 hours. 0-,2-,12- and 24-h post-stress recovery periods (R) representing the treatments sampling time-points. Tf: termination of the experiment.

3.2 RNA extraction and cDNA preparation

Coral fragments were mechanically homogenized in 2ml tubes with 600µL of TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). The tubes were placed in the bead bitter for 3 minutes; the supernatant was transferred to a QIAshredder tube (QIAGEN, Hilden, Germany) and it was centrifuged for 3 minutes at maximum speed. 10% of BCP was added to the tube, that was afterwards shaken with a vortex and left to stand for 10 minutes. Total RNA was extracted using the Invitrogen PureLink RNA micro kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturers' protocol. DNAase I treatment was performed within the RNA extraction procedures according to the manufacturers' instructions (ThermoFisher Scientific, Waltham, MA, USA).

RNA concentration and quality were confirmed using a NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA) and electrophoresis using a 1.5% agarose gel under denaturing conditions.

The RNA integrity was assessed based on clear 28S and 18S ribosomal RNA bands in the electrophoresis.

First strand cDNA for each sample was synthesized from 77 ng total RNA using the Thermo Scientific RevertAid First Strand cDNA Syntesis kit following the manufacturers' protocol (ThermoFisher Scientific, Waltham, MA, USA).

3.3 Real Time quantitative PCR expression analysis

Target-specific primer pairs for the genes Bcl-2, SOD, Gadph and Bax were designed with the IDT's PrimerQuest tool (https://eu.idtdna.com/Primerquest/home/Index): homologous nucleotide sequences retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) from other coral species (Table 1) were searched on the *O. patagonica* transcriptome using the NCBI BLAST+ software (ver. 2.7.1); the resulting sequences were then used to design the primers. The target-specific primer pair for Hsp70 employed in this study was designed using the reference transcriptome of *O. patagonica* (Zaquin, 2019). The primer pairs for the reference transcripts 28S and 18S were designed using *Balanophyllia europaea* sequences; these primers were used in this study after being validated in *O. patagonica.*

Table 1. Investigated genes names, functions and homologous sequences from GenBank.

Gene name	Homologous sequence	Function
HSP 70	/	Molecular chaperone
SOD (Cu/Zn)	XM_015919888	Redox regulation
BCL-2	XM_015899700.1	Apoptosis regulation
BAX	EU161958.1	Apoptosis regulation
GADPH	XM_015919552.1	Energy metabolism

Preliminary endpoint PCR tests were performed for each gene to ensure the specificity of the selected primers; the products were assessed by electrophoresis on a 2.5 % wide range agarose gels (Sigma Aldrich, Milan, Italy), and by analysis with the Gel Doc™ EZ System and the Image Lab software (Bio-Rad Laboratories, Milan, Italy). A single PCR product of the expected size was obtained for each gene of interest; no PCR product was detected from negative controls (reactions without reverse transcriptase added).

To set the protocols for qPCR analyses, the amplification efficiency for each primer was first calculated using a 1:4 dilution of controls cDNAs. qPCR reactions were performed in a final volume of 10 µL containing 5 µL iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy), 2

µL diluted cDNA and 0.2 µM specific primers. Minus-reverse transcriptase (no-RT) controls were included in the qPCR analysis to ensure the specificity of the amplification. Amplification was detected with a StepOne real time PCR system (Life Technologies, Milan, Italy) using a Comparative Ct "fast mode" thermal protocol. Reactions were developed in a 10 µL volume containing 5 µL iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Milan, Italy), 2 µL of samples cDNA, 0.2 µM specific primers and RNase-free H_2O; values within each run were normalized to the endogenous controls 18S and 28S. Melting curves were generated for each sample to confirm that a single product was amplified.

Data were expressed as means ± standard error of the relative variation (fold changes) between each treatment (0h, 2h, 12h and 24h) and the control sample.

qPCR standards for the target transcript were prepared by serial dilution of the PCR purified product containing the Hsp70 transcript to obtain a standard curve of C_t values *vs* the logarithmic DNA amount. Absolute mRNA abundance was estimated from the standard curves and plotted as copy number over nanograms of total RNA (mean ± SEM). All reactions were performed in a final volume of 10 µL containing 5 µL TaqPath™ ProAmp™ Master Mix (Life Technologies Corporation, Austin, TX, USA), 1 µL diluted cDNA and 0.2 µM specific primers. Technical replicates were performed within each run/plate (samples in duplicate) and between different plates (standards and samples replicated on different plates). Equal loadings within each qPCR reaction were assured by verifying the amounts of each standard and cDNA sample using the Qubit system with Qubit® dsDNA HS (High Sensitivity) assay kit (Thermo Scientific, Milan, Italy).

3.4 Data analysis

Univariate comparisons of qPCR data from the heat stress experiment were performed using the non-parametric one-way ANOVA (Kruskal-Wallis test), followed by the Mann-Whitney U-test ($P<0.05$), after deviations from parametric ANOVA assumptions being verified (Normality: Shapiro-Wilk's test; equal variance: Bartlett's test and Brown-Forsythe test). The GraphPad Prism 8 software (GraphPad Inc.) was used to perform all the statistical tests and to create the graphs. Data are presented as means (fold changes) ± standard errors.

4. Results

4.1. Baseline expression and heat stress induction of *hsp70* in *Oculina patagonica*

Baseline expression of a *hsp70* transcript was assessed in *O. patagonica* using an absolute quantitative real-time PCR assay protocol, obtaining the average value of 382.6 ± 104.5 copy number/ng RNA (mean ± sem). The results obtained were compared with those reported in five Mediterranean coral species by Franzellitti et al. (2018). This comparison is showed by the box plot reported in Figure 9. *O. patagonica* showed a higher variability compared to the other species, while mean *hsp70* levels were similar to those of *B. europea* and *C. caespitosa,* and significantly higher compared to *A. calycularis* and *L. pruvoti*.

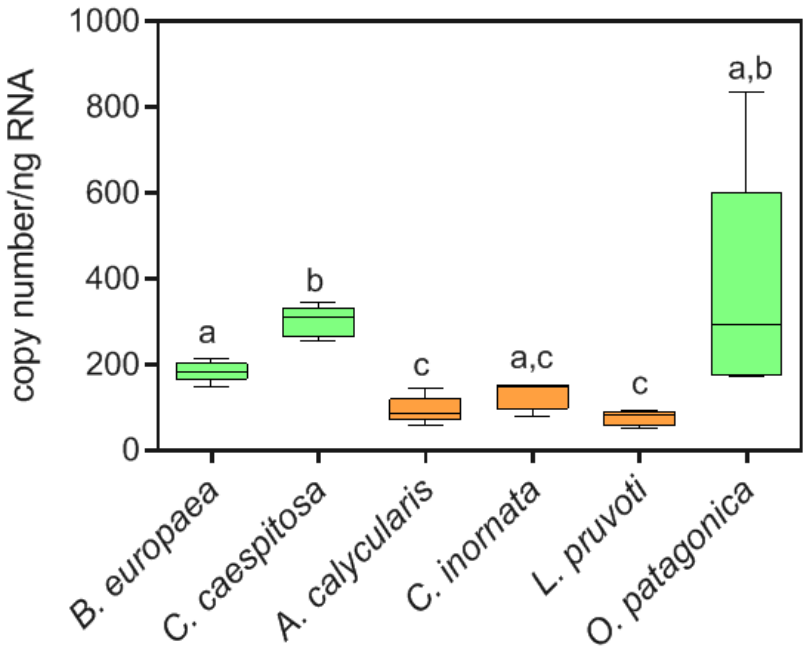

Figure 9. Comparison of *hsp70* baseline expression in *O. patagonic*a with that of *B. europaea, A. calycularis, C. inornata, C. caespitosa, and L. pruvoti* reported by Franzellitti et al. (2018). Box-and-whisker plots represent median, upper and lower quartiles (N = 6) of *Hsp70* copy number normalized over nanograms of total RNA employed in each PCR reaction. Different letters (a, b, c) indicate statistical differences ($P < 0.05$, Mann-Whytney U-test). Species in green = *O. patagonica*, *B. europaea* and *C. caespitosa* (zooxanthellate); species in orange= *A. calycularis, C.inornata,* and *L. pruvoti* (azooxanthellate).

Changes of *hsp70* expression were further evaluated following an acute heat shock (2 h at 35°C) and several periods of post-stress recovery (0 h, 2 h, 12 h and 24 h) at the control temperature of

24°C (Fig. 8). Data are expressed as fold change compared to mRNA levels at the beginning of the experimental exposure to thermal stress, representing the experiment control conditions (control samples, ctr). *O. patagonica* showed significantly increased *hsp70* expression at 0 h post-stress recovery (3.5-fold up-regulation), with levels decreasing back towards the control levels at 2 h post-stress. No significant subsequent increase was detected (Fig. 10).

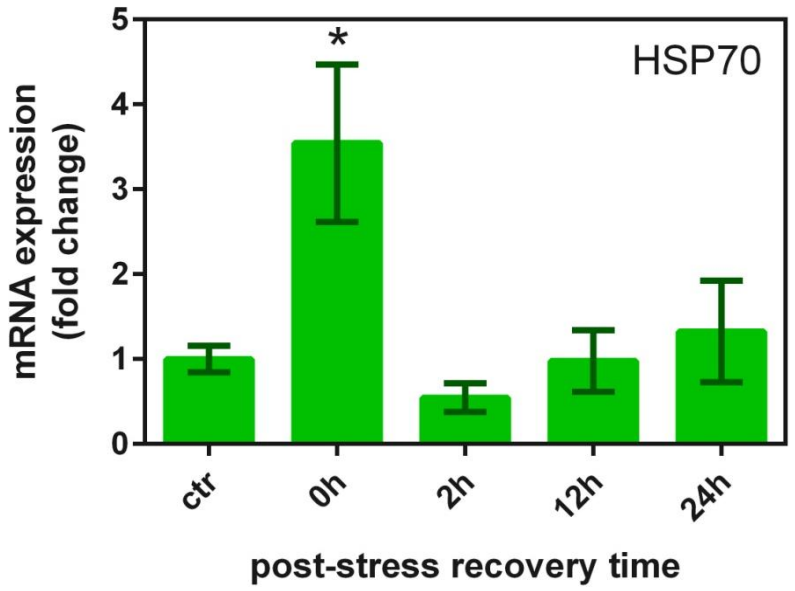

Figure 10. Expression of *hsp70* in *O. patagonica* following an acute heat shock treatment (2 h at 35°C) and different post-stress recovery periods (0 – 24 h) at 24°C. Levels of *hsp70* are expressed as relative variation (fold change) to controls (means ± SEMs; N = 8). Ctr: control samples. *P < 0.05 vs ctr (Mann-Whytney U-test).

4.2. Heat stress regulation of energy metabolism, DNA damage and redox regulation transcripts in *O. patagonica*

Sod transcript levels significantly increased at 12 h post-stress recovery (2-fold up-regulation), with levels going back towards the control conditions at 24 h (Fig. 11A). *Bcl-2* expression levels significantly rose at 12 h post-stress recovery (3.7-fold up-regulation), decreasing back to the control levels at 24 h (Fig. 11B). *Bax* levels were significantly increased compared to controls at 24 h post stress recovery (Fig. 11C). *Gadph* showed a significant fold-change increase at 2 h post-stress recovery (4.5-fold up-regulation), with levels remaining significantly higher than the control

conditions at both 12 h (2.6-fold up-regulation) and 24 h (3.3-fold up-regulation) post-stress recovery (Fig. 11D).

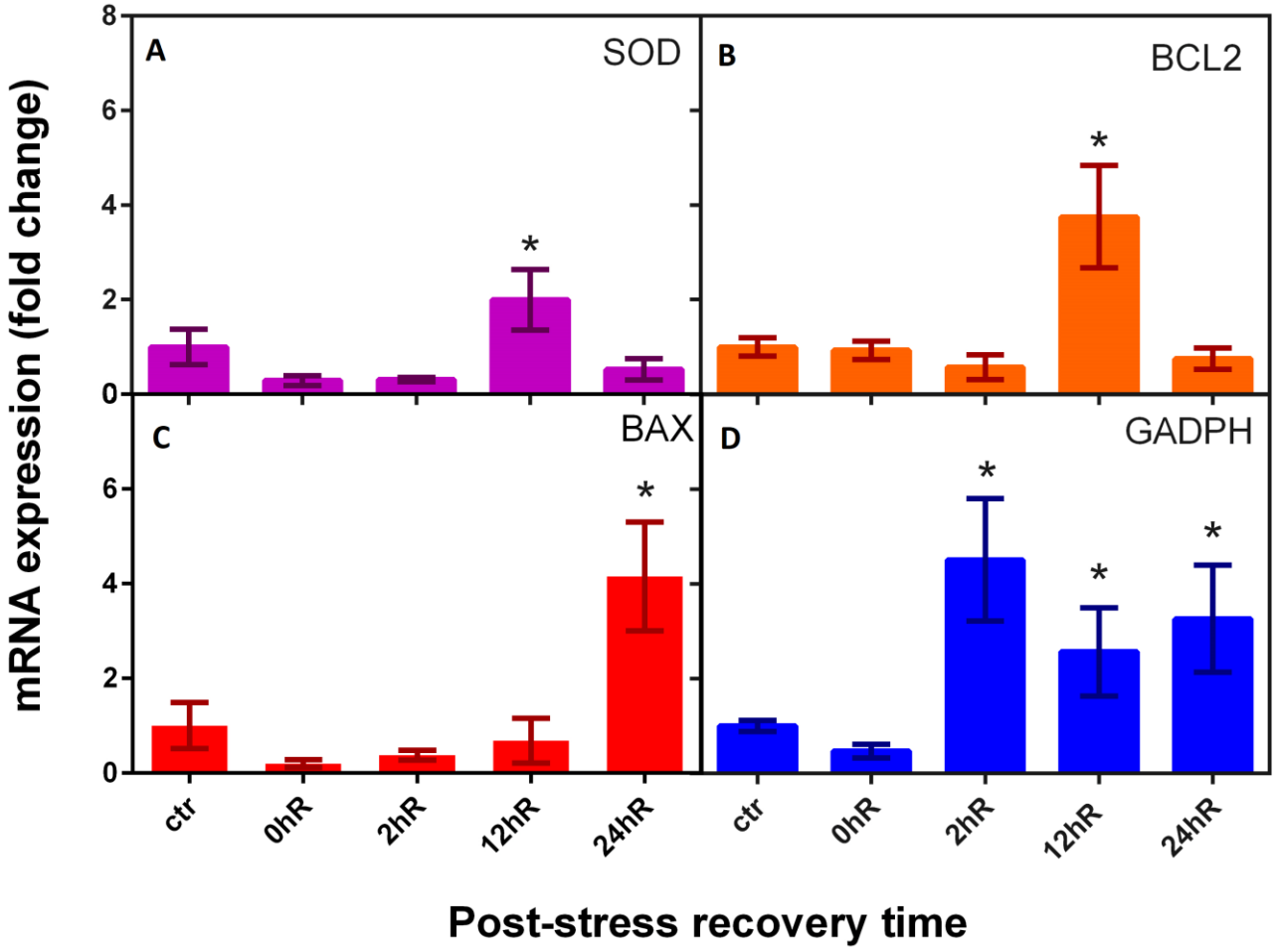

Figure 11. (A-D) Expression of *Bcl-2, Gadph, Sod* and *Bax* in *O. patagonica* following an acute heat shock treatment (2 h at 35°C) and different post-stress recovery periods (0 – 24 h) at 24°C. Levels of the four genes are expressed as relative variation (fold change) to controls (means ± SEMs; N = 8). Ctr: control samples. *P < 0.05 vs ctr (Mann-Whytney U-test).

Right after the heat shock, all coral samples showed signs of differential degrees of bleaching; samples started to recover and regain their coloration after 2 hours of post-stress recovery (Fig. 7B).

5. Discussion

5.1 The Hsp70 response in *Oculina patagonica*

Progressively more studies are exploring the transcriptome responses of corals to environmental stress, in the light of an era of rapidly changing climate, the main purpose being understanding the mechanisms that might provide enhanced tolerance to mutating environmental conditions (Somero, 2012; Walther et al, 2002), or, alternatively, those that may forecast the onset of pathological outcomes (Louis et al., 2017). In the first stage of this study, expression of *hsp70* under non-stressful physiological conditions (baseline levels) and its induction after a heat shock and different periods of post-stress recovery (0 h to 24 h) were assessed in *O. patagonica.* Stress-inducible HSP70s participate to the short-term stress response, after which subsequent protective mechanisms may be activated (Maor-Landaw and Levy, 2016; Morris, 2013; Poli et al., 2017). This could lead to the loss of the *hsp70* expression changes and/or to their underestimation with protracted exposure (Franzellitti et al., 2010). Therefore, the experimental scheme employed in this study considered the regulatory characteristics of Hsp70s and the above described temporal pattern of gene transcription, favoring a fitter analysis of the ability of marine invertebrates to rapidly boost *hsp70* transcription as a fast response to environmental stress (Franzellitti et al., 2018; Franzellitti and Fabbri, 2005; Piano et al., 2005, 2004), which may forecast their stress susceptibility.

Corals living in shallow-water warm environments constitutively express inducible Hsp70 isoforms under baseline conditions, in accordance with a phenomenon known as “constitutive gene frontloading”, an acclimatory priming defense to better respond to stress factors or to elude them (Barshis et al., 2013; Dixon et al., 2015; Franzellitti et al., 2018; Poli et al., 2017; Seneca and Palumbi, 2015). This mechanism, also displayed by other intertidal invertebrates (Dong et al., 2008; Fabbri et al., 2008), is thought to have a crucial role in coral stress tolerance, physiological plasticity, and resilience. Under natural conditions, a relatively higher potential for protein denaturation by thermal stress exists in thermally challenging environments; therefore, high constitutive levels of inducible *hsp70* might serve as a preparatory protective function to readily cope with the effects of erratic and frequently experienced stress periods (Dong et al., 2008; Morris et al., 2013).

Results of this study show that *O. patagonica* does express stress inducible *hsp70* under baseline conditions, in agreement with the constitutive gene frontloading mechanism. Furthermore, *O.*

patagonica is able to induce a robust *hsp70* response right after the heat stress exposure (0 h post stress recovery; 3.5 fold change), and to quickly recover to baseline expression levels (within a 2h post-stress recovery time). Previous studies have shown that the prompt return of transcription to physiological levels, known as transcriptional resilience, is a crucial part of resilience to thermal stress (Seneca and Palumbi, 2015; Traylor-Knowles et al., 2017), and that the rapidity and magnitude of the Hsp70 expression is related to the survival response to thermal stress and to the molecular adaptation to transitional environments of corals (Rodriguez-Lanetty et al., 2009) and of other marine invertebrates (Fabbri et al., 2008). For example, Traylor-Knowles et al. (2017) showed that the distinctly heat tolerant *Acropora hyacinthus* promptly induced the *hsp70* expression after a heat stress exposure, as a primary early response that plays a critical role in the organisms' development of thermotolerance. Moreover, Rodriguez-Lanetty et al. (2009) individuated a rapid up-regulation of the Hsp70 gene after a hyperthermal stress exposure in the coral *A. millepora,* as a key component of thermal tolerance in scleractinian corals. Therefore, the fast *hsp70* induction at 0 h post-stress recovery and the prompt recovery may reflect a high resilience of *O. patagonica* to thermal stress. This finding is line with the observation that *O. patagonica* inhabits shallow and light exposed environments of the Eastern Mediterranean, frequently subjected to abrupt and intense temperature variations, thus being adapted to harsh physical conditions. This species shows a higher physiological plasticity (Armoza-Zvuloni et al., 2011; Rodolfo-Metalpa et al., 2006; Rubio-Portillo, 2014), and the baseline *hsp70* expression levels together with their fast reaction may be part of its acclimatory mechanisms to cope with the frequently experienced prolonged periods of thermal stress.

5.2. Comparative analysis of the hsp70 response in scleractinian corals from the Mediterranean Sea

The inclusion of *O. patagonica* in the comparative analysis of the *hsp70* response in coral species from the Mediterranean Sea carried out previously (Franzellitti et al., 2018) does corroborate and strengthens the observation that the *hsp70* responses align with relative differences in stress susceptibility and physiological plasticity of the coral species investigated. All three zooxanthellate corals (*O. patagonica, C. caespitosa* and *B. europea*) showed a higher *hsp70* baseline expression compared to the azooxanthellate ones (*A. calycularis, C. inornata* and *L. pruvoti*), suggesting that trophic strategy (zooxanthellate vs azooxanthellate) may play an important role in shaping coral

resilience and physiological plasticity, as previously hypothesized (Seveso et al., 2018). Considering the zooxanthellate species, the different responses and degrees of thermotolerance of *O. patagonica* compared to *C. caespitosa* and *B. europea* from the study of Franzellitti et al. (2018) could be explained by the zooxanthellae (*Symbiodinium* spp.) clade composition hosted by the corals. *O. patagonica* was found to harbor clade B2 in the Mediterranean Sea (LaJeunesse et al., 2012; Rodolfo-Metalpa et al., 2014) as well as the other two species, while a "temperate" clade A was found only in *B. europaea* (Barbrook et al., 2006; Meron et al., 2012; Leydet and Hellberg, 2016). In general, clade B seems to be flexible and well adapted to the temperature range encountered in seawater throughout its wide global distribution (Karako-Lampert et al., 2005). This clade is also resistant to short-term exposure to elevated temperatures (Rodolfo-Metalpa et al. 2006; Shenkar et al. 2006; Rodolfo-Metalpa et al. 2008). These studies suggest that type B2 is able to endure a wide range of temperatures, which may facilitate the ability of *O. patagonica* to thrive in a broad spectrum of temperatures, including extreme ones. Pending data on the actual *Symbiodinium* composition in *O. patagonica* colonies at the selected sampling sites, it is worth noting that Savage et al. (2002) found significant variation in photosynthesis versus irradiance among *Symbiodinium* isolated from corals at different geographical locations. These differences were not correlated with clade type, and demonstrate the potential for acclimation within each genotype (Karako-Lampert et al., 2005), thus suggesting the influence of the environment.

Levin et al. (2016) demonstrated that transcriptional responses by thermo-tolerant *Symbiodinium* may allow maintaining symbiosis with their coral host at elevated temperature, while stress effects (mainly increased ROS cell leakage and decreased photosynthetic efficiency) induced in thermo-sensitive *Symbiodinium* may cause oxidative damage to the coral host, resulting in bleaching. Therefore, the interspecific variation observed in coral transcriptional profiles and stress responsiveness may be the result of the combined responses to heat stress of the corals and their specific *Symbiodinium* clades (Fitt et al., 2009; Franzellitti et al., 2018; Gates and Edmunds, 1999; Louis et al., 2017; Rodolfo-Metalpa et al., 2006a). However, we should consider that facultative zooxanthellate corals as *O. patagonica* are less dependent on their algal partners compared to obligate zooxanthellates; nevertheless, the *Symbiodinium* communities that they harbor may still reflect environment suitability amongst different habitats.

Aside from the supposed influence of symbiosis, peculiar adaptive features and life history traits of the species may have a role in determining their observed *hsp70* responses. Species living in environments characterized by frequent temperature changes and higher maximum temperatures

are suggested to have higher thermal tolerances (Dong et al., 2008; Lockwood et al., 2015; Oliver and Palumbi, 2011; Poli et al., 2017), and the different levels of thermotolerance are intimately related to the levels of hsp70 expression (Dong et al., 2008; Lockwood et al., 2015; Rodriguez-Lanetty et al., 2009). These relationships could explain the differences between *O. patagonica* and *B. europaea* and *C. caespitosa*. The map reported in Figure 12 show that these species occupy different thermal regimes at the selected sampling areas, which may have shaped their physiological responses to thermal stress. The comparatively high(er) baseline hsp70 levels displayed by *C. caespitosa* and *O. patagonica* compared to *B. europaea* may render this species less responsive to additional stress(ors). Indeed, transient (*O. patagonica*) or no further (*C. caespitosa) hsp70* up-regulation during post-stress recovery was observed, suggesting a limited ability or need of these species to further adjust its physiology to increasing temperatures. Previous physiological studies (Rodolfo-Metalpa et al., 2006b, 2006a) suggest that *O. patagonica* may be more able than *C. caespitosa* to resist high temperature conditions because of its rapid bleaching capacity. As we will specifically address in the second part of this study (Paragraph 5.3), the interplay between the hsp70 response and primary cellular protective mechanisms is thought to determine the temporal dynamics of bleaching induction and recovery (Maor-Landaw et al., 2014).

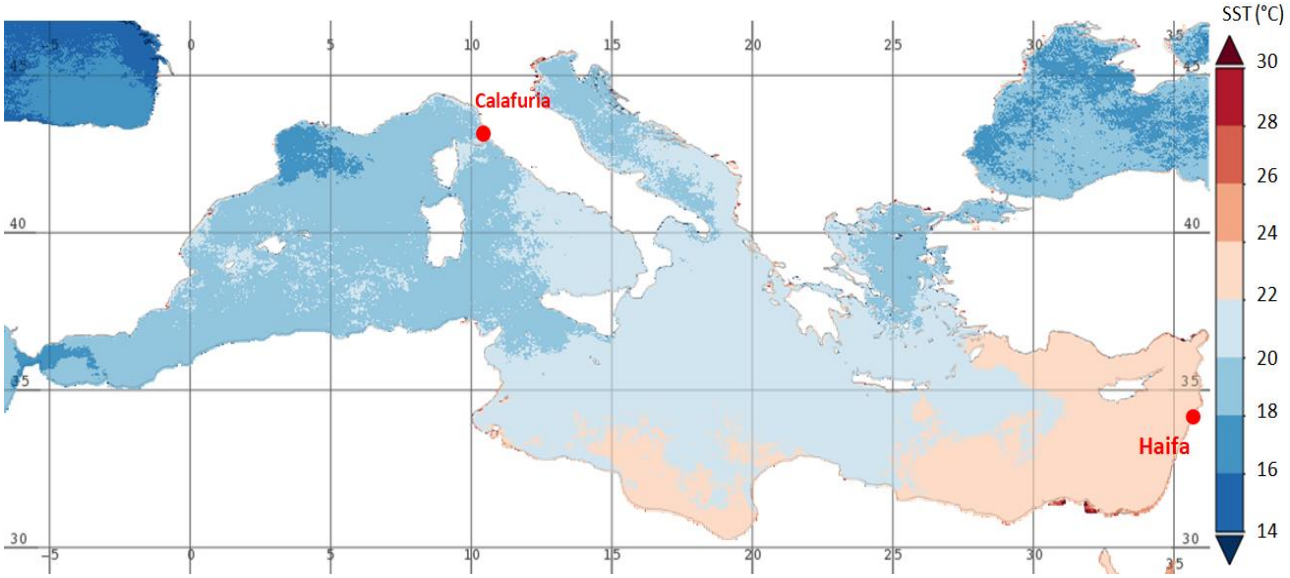

Figure 12. Mean annual surface temperatures of the Mediterranean Sea and study sampling site. Sampling site relative to Franzellitti et al (2018): Calafuria. Sampling site for *O. patagonica*: Haifa. Data are satellite-derived and mapped with the Giovanni-NASA web tool (https://giovanni.gsfc.nasa.gov/giovanni/).

5.3 *Hsp70* expression as molecular marker of stress and the time-line of the cellular stress response in *O. patagonica*

In the present study, *hsp70* up-regulation was observed in *O. patagonica* right after the heat shock treatment, likely serving as a primary and fast response to stress (Gates and Edmunds, 1999; Louis et al., 2017; Robbart et al., 2004). Many studies exploring the dynamics of the stress response in corals clearly pointed out that this is a common pattern to the species analyzed thus far (Barshis et al., 2013; Bellantuono et al., 2012; Fitt et al., 2009; Granados-Cifuentes et al., 2013; Kitchen, 2016; Maor-Landaw and Levy, 2016; Meehan and Ostrander, 1997; Traylor-Knowles, 2017; Franzellitti et al. 2018). This recurrent scheme provides a sound basis for the view of *hsp70s* as molecular markers of stress in corals, as already theorized by several works (Louis et al., 2017 and reference therein). Beyond Hsps, proteins involved in cellular processes like cell death, oxidative stress, cell signaling and immunity are thought to play a role in shifting the set points at which corals undergo bleaching (Barshis et al., 2013). This study explored the transcriptional regulation of genes underpinning redox regulation, DNA damage response, and energy metabolism, and integrated their response patterns (by including also *hsp70*) to disclose a possible time line of the molecular processes occurring in *O. patagonica* following heat stress. Taking advantage of the development of a reference transcriptome for *O. patagonica* available at the School of Marine Science of the University of Haifa (Zaquin, 2019), the mRNA sequences of candidate genes were identified and specific protocols (primer sequences and reaction settings) were designed for targeted qPCR analyses of:

- Superoxide dismutase (sod), involved in redox regulation;
- Bcl-2-associated X (bax) and the B-cell lymphoma 2 (bcl-2), mediating pro-apoptotic and anti-apoptotic pathways, respectively;
- Glyceraldehyde 3-phosphate dehydrogenase (gadph), involved in carbon metabolism

In case of elevated seawater temperatures, scleractinian corals tend to lose their zooxanthellae, gaining a bleached appearance and subsequently die, if this condition endures for a prolonged period of time. Several mechanisms of coral bleaching have been hypothesized, and one of these involves the elevated production of reactive oxygen species (ROS) by the endosymbiotic dinoflagellates due to photosynthetic dysfunction, that causes cellular damages and the consequent expulsion of the algae (Lesser, 2006; Weis, 2008). The diffusion of ROS into the coral tissues leads to oxidative stress, denaturing proteins, damaging nucleic acids and oxidizing

membranes (Louis et al., 2017). ROS could be implicated in the first stages of apoptosis, representing one of the trigger signals for programmed cell death (Kvitt et al., 2011). Increased *sod* expression as that observed in this study is reported in several coral species subjected to heat stress treatments, and addressed to as a direct evidence that the ROS production had increased (Downs et al., 2000; Gates and Edmunds, 1999; Lesser and Farrell, 2004; Souter et al., 2011). In particular, Barshis et al. (2013) found that thermally tolerant populations of *Acropora hyacinthus* showed lower *Cu-Zn sod* expression compared to more thermally sensitive populations, result that was indicated by the authors as a reduced need for this protein activity in tolerant corals. In the case of *O. patagonica*, the late *sod* up-regulation (from 12 h post-stress recovery) may be related to a higher thermotolerance of this coral species, that might have already had enough *sod* baseline expression to cope with the immediate effects of heat stress, incrementing this gene transcription well after the heat treatment, when the enzyme had to be replaced.

As a result of increased ROS production after a thermal stress event, programmed cell death, or apoptosis, of dysfunctional and damaged cells is initiated in corals (Kvitt et al., 2011). Apoptosis has received attention in coral physiology, given that it is one possible mechanism by which corals undergo bleaching (reviewed by Weis, 2008). The study of Granados-Cifuentes et al. (2013) addressing the main pathways related to bleaching susceptibility in *A. millepora* showed the differential regulation of *bcl-2* and *bax* transcripts, where bcl-2 promotes cell survival by suppressing the activity of bax. The authors hypothesized that corals repress apoptosis under normal physiological conditions (Bcl-2 up-regulation), while colonies of *A. millepora* undergoing heat stress showed evidence of induction of apoptosis during thermal stress with a delayed up-regulation in Bcl-2 (anti-apoptosis) of surviving cells. This is addressed as a protective mechanism whose temporal dynamics determine the onset of bleaching (Pernice et al., 2011). The mechanism described above may explain the relative timing of *bcl-2* and *bax* transcriptional regulation, with the (anti-apoptotic) *bcl-2* from 12 h post-stress recovery and the (apoptotic) *bax* being up-regulated only at 24 h post stress, when *bcl-2* recovered basal expression levels.

Results of the relative variation of *gadph* show that *O. patagonica* significantly increased this gene transcription at 2 hours post-stress recovery (4.5-fold up-regulation), with elevated levels persisting up to 24 h post-stress recovery (3.3-fold up-regulation).

Gadph is a protein involved in carbon metabolism whose over-expression following a heat stress is reported in other coral species (Leggat et al., 2011). The heightened expression of this transcript indicates that there was an intensified need for carbon substrates to be used for cellular

respiration after the temperature increase, as previously shown by other studies on coral respiration rates following a heat treatment (Leggat et al., 2011). Furthermore, the prolonged *gadph* up-regulation until 24 h post-stress recovery suggests the occurrence of increased metabolic needs to satisfy the enhanced energetic demands to cope with stress conditions. For example, the molecular chaperone activity of Hsp70s is ATP-dependent; therefore, the incremented production of chemical energy through cellular respiration might have provided the required elements for Hsp70 proteins to carry out their cytoprotective role. In fact, the early up-regulation of the Hsp70 gene observed after the heath shock (0 h post-recovery) indicates that considerable levels of Hsp70 proteins, encoded by that gene, were synthesized starting from that time point; as a result, the subsequent increased expression of *gadph* (from 2 h post-stress compared to control levels) is consistent with the incremented energy demand of more Hsp70 proteins existing in the cells.

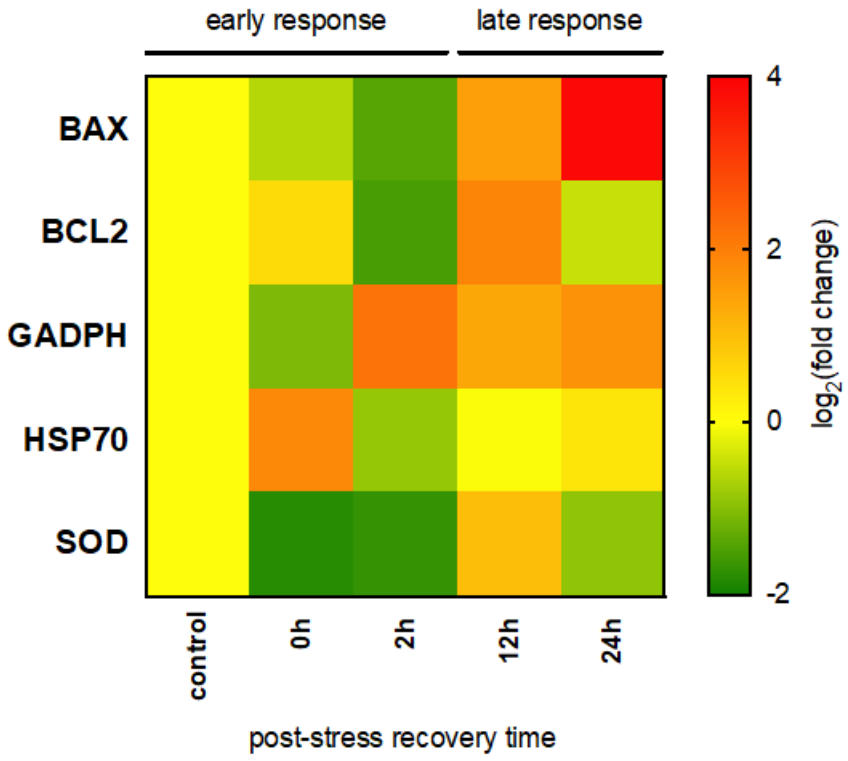

Figure 13. Heatmap comparing the expression patterns of molecular chaperone (hsp70), redox regulation (sod) , apoptosis related (bcl-2, bax) and metabolic (gadph) transcripts in *O. patagonica* following a short-term heat stress. Significant genes upregulation relative to controls indicated as an early (up to 2 h post- stress recovery) or late (after 2 h post-stress recovery) response. Colors represent relative expression levels with respect to control (log_2-transformed fold changes).

Based on these results, a potential time line was derived for the events occurring in *O. patagonica* in response to an acute heat stress, which largely match that reported previously for the tropical coral *Stylophora pistillata* (Maor-Landaw et al., 2014) (Fig. 13). The "early response" (< 2 h post heat stress) is sustained by molecular chaperones (in this study *hsp70*), which assist in maintaining protein homeostasis. The expression of genes related to energy metabolism (in this study gadph) increases to satisfy the energetic demands of processes occurring within the cell, such as protein degradation, protein refolding (chaperoning) and (later on) DNA repair. The "late response" (> 2 h post heat stress) is sustained by transcripts responsible for sensing and repairing DNA damage (in this study *bcl-2* and *bax*). A remarkable difference between *O. patagonica* (this study) and *S. pistillata* (Maor-Landaw et al., 2014) is the position of antioxidant responses, here represented by *sod*, that in *O. patagonica* is listed as a late response, whereas in *S. pistillata* accounted for the "early warning" response. Being aware that our findings are based only on one component of the antioxidant response and that a more comprehensive analysis is advisable, we can take advantage of the whole transcriptome comparative analysis between *S. pistillata* and *B. europaea* (here considered as a model for temperate shallow water Mediterranean corals) showing that an elevation of only 2 °C was sufficient to change the pattern of gene expression in *S. pistillata*, but an increase of 9 °C was needed to induce consistent temperature related transcriptional changes in *B. europaea* (Maor-Landaw et al., 2017). Furthermore, the observed enrichment of metabolic processes are proposed to enable *B. europaea* to postpone temperature-induced protein degradation and cell death (Maor-Landaw et al., 2017). These findings show that temperate Mediterranean corals are used to higher annual temperature fluctuations than those faced by tropical species showing different temporal dynamics of stress response at the transcriptional level. A future direct comparison between *O. patagonia* and *B. europaea* would clarify whether the result of this study on *sod* regulation may be, at least partially, ascribed to the peculiar physiological features and life history traits of *O. patagonica* living in the Eastern Mediterranean Sea.

6. Conclusions

Data presented in this study show that *O. patagonica* transcriptional response relative to the Hsp70 gene aligns with the formerly observed high tolerance for elevated sea water temperatures reported for this species (Rodolfo-Metalpa et al., 2006; Rubio-Portillo et al., 2014).
Results show that *O. patagonica* does express high levels of stress inducible *hsp70* under baseline conditions, in agreement with the constitutive gene frontloading mechanism, which may serve as a preparatory protective function that contributes to a limited need of this species to further adjust its physiology to increasing temperatures. This allows *O. patagonica* to readily cope with the effects of erratic and frequently experienced stress periods (Dong et al., 2008; Morris et al., 2013), elevating this species thermal tolerance and providing the molecular basis for the capacity of the coral host to live without the symbiont in the case of extended periods of bleaching (Zaquin, 2019). To this regards, the comparative multispecies analysis presented in this study also suggests that trophic strategy (i.e. presence/absence of the symbiotic partnership) may play an important role in shaping coral resilience and physiological plasticity. More extensive temporal studies on both coral host and *Symbiodinium* transcriptional responses would be necessary to identify the precise molecular events underlying *O. patagonica* stress responses observed in this study.

Tracking the differential expression of transcripts related to energy metabolism, redox regulation, and DNA damage along with *hsp70* provided a more in-depth analysis of the coral heat stress status and allowed to set the potential time line for the events occurring in *O. patagonica* in response to an acute heat stress. These findings indicate that higher annual temperatures fluctuations faced by the Mediterranean Sea endow corals with a higher resilience through a more responsive molecular machinery for the environmental stress response, compared to species living in tropical environments (Maor-Landaw et al. 2014). Understanding these molecular processes is particularly demanding for corals inhabiting the Mediterranean, which is considered a biodiversity "hot spot", in light of projected scenarios of anthropogenic global change.

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