Margarida Grifo Travesso

Combined effects of light intensity and temperature on molecular and photophysiological responses of *Sarcophyton* cf. *glaucum*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sobre a orientação científica da Doutora Diana Sofia Gusmão Coito Madeira, Investigadora Júnior do Departamento de Biologia da Universidade de Aveiro, e do co-orientador Doutor Ricardo Jorge Guerra Calado, Investigador Principal do Departamento de Biologia da Universidade de Aveiro.

Dedico este trabalho à minha família.
Sem vocês nada disto seria possível.

o júri

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palavras-chave   Holobionte de Coral, Ondas de Calor Marinhas, Fotoaclimatação, Stress Oxidativo, Biomarcadores, Fotofisiologia

resumo

Os recifes de coral são considerados um dos ecossistemas mais bio diversos e produtivos do mundo, fornecendo à Humanidade muitos bens e serviços. As atividades da população humana levaram a um aumento nas emissões de gases de efeito estufa para a atmosfera da Terra, levando a eventos climáticos extremos, como ondas de calor marinhas (MHWs), especialmente em regiões tropicais. Este fenômeno é capaz de levar ao declínio dos recifes de coral associado a eventos de branqueamento, causando mortalidade e perda da estrutura do recife. Em interação com outros fatores de stress, como uma elevada intensidade luminosa, os efeitos da alta temperatura podem ser ainda mais severos sobre os recifes de coral. No presente estudo, avaliámos o desempenho e as respostas moleculares e fotofisiológicas do coral mole *Sarcophyton* cf. *glaucum* sob diferentes cenários de mudanças globais, estudando a influência de dois fatores principais: temperatura e intensidade luminosa. Fragmentos de coral, recolhidos ao redor do mar da Indonésia, foram experimentalmente expostos a diferentes tratamentos: duas intensidades de luz (luz alta, luz baixa, 663 e 253 µmol m^{-2} s^{-1}, respetivamente) e duas temperaturas (26 e 32 °C, representando uma onda de calor) num design fatorial completo, considerando baixa luminosidade e 26 °C como as condições de controlo. Primeiramente, os fragmentos foram fotoaclimatados por 30 dias ("Time-point 2"), e de seguida, uma onda de calor ("Time-point 3") foi simulada por 10 dias, onde a temperatura foi elevada (a 1 ˚C h^{-1}) até 32 °C. Posteriormente, os corais voltaram à temperatura controlo, com 30 dias para recuperação ("Time-point 4"). Estes fragmentos foram amostrados em cada "Time-point" e parâmetros fotofisiológicos - rendimento quântico máximo do fotossistema II (F_v/F_m), uma medição da atividade fotossintética, fluorescência de nível escuro (F_0), como um proxy do conteúdo de clorofila a, número de simbiontes como indicador de branqueamento, e biomarcadores relacionados com o stress (Proteína total, Superóxido dismutase (SOD), Glutationa-S-transferase (GST), Catalase (CAT), Peroxidação lipídica (LPO), Proteína de choque térmico 70kDa (Hsp70), Ubiquitina (Ubi) e Capacidade Antioxidante Total (TAC) - foram avaliados no holobionte. O coral *Sarcophyton* cf. *glaucum* revelou-se fisiologicamente comprometido, uma vez que F_v/F_m, F_0 e o número de endossimbiontes foram significativamente afetados pelos fatores impostos. Especificamente, F_v/F_m foi o parâmetro mais afetado, sendo o único influenciado pelos dois fatores e também pela sua interação, refletindo o dano fotossintético ocorrido no PSII dos simbiontes, responsável por uma diminuição da atividade fotossintética. F_0 e o número de simbiontes foram afetados apenas pela luz e temperatura, respetivamente. Embora estes dois parâmetros estejam correlacionados (uma diminuição num representa uma diminuição no outro), isso não aconteceu: F_0 diminuiu enquanto o número de simbiontes manteve os seus níveis sob luz forte. Esta descoberta pode indicar que os níveis de clorofila *a* por zooxantela mudaram consoante a luz imposta, sem expulsão dos simbiontes pelo hospedeiro. No entanto, a perda de zooxantelas ocorreu sob stress térmico e persistiu mesmo após 30 dias de recuperação. Em relação aos parâmetros moleculares, as enzimas antioxidantes foram o principal mecanismo de ação da resposta celular ao stress. SOD, GST e CAT aumentaram em resposta ao stress térmico, provavelmente devido à alta concentração de ROS nos simbiontes, neutralizando os seus efeitos. A temperatura teve o maior efeito sobre os biomarcadores, enquanto a intensidade da luz afetou apenas a SOD e a CAT. No entanto, a alta intensidade luminosa, em vez de levar a um aumento destas enzimas (esperado uma vez que o stress leva à produção de ROS), levou à sua diminuição. Isto pode ser explicado por um esgotamento do sistema de produção das enzimas antioxidantes em resposta a um alto stress luminoso. No caso dos mecanismos de controlo de qualidade de proteínas, enquanto o aumento de Ubi perante o stress térmico pode representar danos proteicos, onde proteínas desnaturadas são direcionadas para degradação no proteossoma, a falta de alterações na Hsp70 perante o stress pode ser considerada um mecanismo de adaptação de algumas espécies de coral. Após o período de recuperação de 1 mês, a maioria dos biomarcadores voltou aos valores de controlo, enquanto nos parâmetros fotofisiológicos, isto apenas aconteceu com F_0. A principal conclusão é que *S.* cf. *glaucum* foi mais afetado ao nível fisiológico, não tendo os seus simbiontes capacidade de manter o seu desempenho necessitando de mais de 1 mês para se recuperar fotossinteticamente. No entanto, a maioria dos biomarcadores voltou aos níveis controlo, sugerindo algum nível de plasticidade molecular em relação ao stress térmico. O branqueamento ocorreu, comprovado pela diminuição dos parâmetros fotofisiológicos (F_0 e Número de simbiontes).

Este fenômeno foi prevalente em corais expostos à combinação de calor e alta intensidade luminosa (32 °C_HL), mas também em baixa luz sob o regime térmico de 32 ˚C. Estudos futuros sobre alterações climáticas devem concentrar-se mais em corais moles e nos níveis molecular e fotofisiológico para entender melhor como a temperatura e a luz interagem entre si. Além disso, estudos que abordem técnicas e ferramentas multi-ómicas para a conservação de recifes, nomeadamente de evolução assistida, também devem ser desenvolvidos de forma a criar medidas mais eficazes de conservação e recuperação de corais em todo o mundo, contribuindo para a preservação dos oceanos.

abstract

Coral reefs are considered one of the most biodiverse and productive ecosystems in the world, providing Humanity with many goods and services. Human population activities have led to an increase in greenhouse gas emissions to the Earth's atmosphere leading to extreme weather events, like Marine Heatwaves (MHWs), especially in tropical regions. This phenomenon is capable of leading to coral reefs decay associated with bleaching events, causing mortality and loss of reef structure. In interaction with other stress factors, like high light intensity, high temperature effects can be even more severe over coral reefs. In the present study we evaluated the performance and the molecular and photophysiological responses of the soft coral *Sarcophyton* cf. *glaucum* under different global change scenarios, by studying the influence of two main factors: temperature and light intensity. Coral fragments, collected around the Indonesian sea, were experimentally exposed to different treatment scenarios: two light intensities (high light, low light, 663 and 253 µmol m^{-2} s^{-1}, respectively) and two temperatures (26 and 32 °C, representing a heatwave) in a full factorial design, considering low light and 26 °C as control conditions. First, fragments were photoacclimated for 30 days (Time-point 2), and then a heatwave (Time-point 3) scenario was simulated for 10 days, where the temperature was increased (at 1 °C h^{-1}) until 32 °C. Subsequently, corals were returned to control temperature and allowed to recover for 30 days (Time-point 4). Coral fragments were sampled at each time-point and photophysiological parameters - maximum quantum yield of photosystem II (F_v/F_m), a measurement of photosynthetic activity, dark-level fluorescence (F_0), as a proxy of chlorophyll *a* content, number of symbionts as bleaching indicator, and stress-related biomarkers (Total protein, Superoxide dismutase (SOD), Glutathione-S-transferase (GST), Catalase (CAT), Lipid peroxidation (LPO), Heatshock protein 70 kDa (Hsp70), Ubiquitin (Ubi) and Total Antioxidant Capacity (TAC) - were assessed in the holobiont. *Sarcophyton* cf. *glaucum* was physiologically compromised, since F_v/F_m, F_0 and the number of endosymbionts were significantly affected by the factors imposed. Specifically, F_v/F_m was the most affected parameter, being the only one influenced by the two stressors and their interaction, reflecting the photodamage that occurred in symbionts' PSII, responsible for a decrease in the photosynthetic activity. F_0 and the number of symbionts were affected only by light and temperature, respectively. Although these two parameters are correlated (a decrease in one represents a decrease in the other), this did not happen: F_0 decreased while the number of symbionts maintained its levels under high light. This finding could indicate that Chlorophyll *a* levels per zooxanthellae were changing according to light, with no expulsion of the symbionts by the coral host. However, zooxanthellae loss did occur under heat stress and persisted even after 30 days of recovery. Regarding the molecular parameters, antioxidant enzymes were the main mechanism of the Cellular Stress Response to act. SOD, GST, and CAT suffered an increase in response to a heat stress, probably due to a high concentration of ROS in symbionts cells, counteracting its effects. Temperature had the highest effect over the biomarkers, while light intensity only affected SOD and CAT. However, high light intensity, instead of leading to an increase in these enzymes (expected since stress leads to a ROS production), it led to their decrease. This can be explained by an exhaustion of the antioxidant production system in response to a high light stress. For the protein quality control mechanism, while Ubi increase facing heat stress may represent protein damage, where misfolded proteins are tagged for proteasome degradation, the lack of change in Hsp70 facing stress can be considered an adaptation mechanism of some coral species. After the 1-month recovery period, most biomarkers returned to control values, while in the photophysiological parameters only F_0 recovered. The main conclusion is that *S.* cf. *glaucum* was most affected at the physiological level, with their symbionts not being able to maintain their photophysiological performance needing more than 1 month to photosynthetically recover. However, most biomarkers returned to control levels, suggesting some level of molecular plasticity regarding heat stress. Bleaching indeed occurred, seen by the decrease in the photophysiological parameters (F_0 and Number of symbionts). This phenomenon was prevalent in corals exposed to combined heat and high light stress (32 °C_HL), but also on low light under the thermal regime of 32 °C. Future studies of climate change should focus more on soft corals at both the molecular and photophysiological levels to better understand how temperature and light interact with each other. Moreover, studies that address multi-omics techniques and tools for reef conservation, namely assisted evolution ones, should also be developed to create more effective measures of conservation and recovery of corals around the world, contributing to the preservation of the oceans.

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List of abbreviations

A

AOI, Area of Interest

Abs, Absorbance

ABTS, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)

B

BSA, Bovine Serum Albumin

C

CV, Coefficient of Variation

CTRL, Control

CO_2, Carbon Dioxide

CSR, Cellular Stress Response

CAT, Catalase

CT_{max}, Critical Thermal Maximum

CT_{min}, Critical Thermal Minimum

Cl a, Chlorophyll a

CNDB, Chloro-2,4-dinitrobenzene

D

DNA, Deoxyribonucleic Acid

E

EDTA, Ethylenediaminetetraacetic acid

ELISA, Enzyme-Linked Immunosorbent Assay

F

F_v/F_m, Maximum Quantum Yield of Photosystem II

F0, Amount of Cl a

F_m, Maximum Fluorescence

Fc, Fragment Constant Region

Fab, Fragment Antibody-binding Region

G

GST, Gluthation-s-transferase

H

Hsp70, Heat Shock Protein 70 kDa

Hsps, Heat Shock Proteins

HL, High Light

HW, Heat Wave

HCL, Hydrochloric Acid

H_2O_2, Hydrogen Peroxide

I

IBR, Integrated Biomarker Response

IgG, Immunoglobulin G

IPCC, Intergovernmental Panel on Climate Change

K

kDa, Kilodalton

KOH, Potassium Hydroxide

L

LL, Low Light

LPO, Lipid Peroxidation

L:D, Light:Dark

L, Light

L x T, Interaction between Light and Temperature

M

MDA, Malondialdehyde bis (dimethylacetal)

N

NBT, Nitroblue Tetrazolium

P

P, p-value

PBS, Phosphate-buffered Saline

PERMANOVA, Permutational Analyses of Variance

PCA, Principal Component Analysis

PWC, Partial Water Changes

PAM, Pulse Amplitude Modulated Fluorimetry

PSII, Photosystem II

PAR, Photosynthetic Active Radiation

PUFAs, Polyunsaturated Fatty Acids

R

ROS, Reactive Oxygen Species

S

SST, Sea Surface Temperature

SOD, Superoxide Dismutase

SD, Standard Deviation

SDS, Sodium Dodecyl Sulphate

T

TAC, Total Antioxidant Capacity

TBARS, Thiobarbituric Acid Reactive Substances

TCA, Trichloroacetic Acid

TBA, Thiobarbituric Acid

T_{opt}, Optimum Temperature

T, Temperature

U

UBI, Total Ubiquitin

UV, Ultraviolet

UN, United Nations

W

W, Watts

X

XOD, Xanthine Oxidade

1. Introduction

1.1 Coral reef ecosystems under global climate change

Coral reefs are considered one of the most biodiverse and productive ecosystems in the world, harboring at least 25% of all known marine species (Hoegh-Guldberg et al., 2019). Reef ecosystems provide Humankind with many goods and services, such as food security, coastal protection (for both Humans and organisms that use them as shelter), medical resources and economic benefits that arise from tourism activities (Bruckner, 2002). Corals are divided into two categories: stony (hard) and soft corals. The first ones are known for building reefs and producing a rigid skeleton made of calcium carbonate, while the second ones lack a skeleton but contain structural sclerites that contribute to the sustention of the polyp structure. The accumulation of these skeletons and cemented sclerites throughout time creates a complex reef structure that can be colonized by other species (Hoegh-Guldberg et al., 2019; Jeng et al., 2011).

Despite the importance of coral reefs for the livelihoods of over 500 million people worldwide, these ecosystems continue to face numerous global and local threats (Hoegh-Guldberg et al., 2019). Human population activities, since the industrial revolution, have led to an increase in greenhouse gas emissions to the Earth's atmosphere, resulting in the rise of air and ocean temperatures for the past century. In fact, global surface temperatures in the period of 2001-2020 were 0.99 ˚C higher than in 1850-1900 (IPCC, 2021). Moreover, the ocean acts as a CO_2 sink, which has resulted in a reduction of its pH, a phenomenon known as ocean acidification. Human-induced climate change is also leading to shifts in weather and climate extremes, including heavy rainfall, heatwaves (HWs), cold spells, droughts and storms (IPCC, 2021). In addition to these global threats, local human activities can also have substantial impacts on coral reefs, such as overharvesting, over-, and destructive fishing, anchor damage, ship groundings and pollution. These activities create ecological shifts, declines in biodiversity and ultimately species extinctions in multiple ecosystems (Parmesan, 2006), including coral reefs (Burke et al., 2011; Hoegh-Guldberg et al., 2019)

Among the many threats faced by coral reefs, HWs have far-reaching consequences across taxa, affecting a wide range of biological and ecological processes (Smale et al., 2019). Four mass coral bleaching events have already impacted tropical

coral reefs around the world in the past two decades associated with extreme heating conditions (Bleuel et al., 2021). Climate models predict that almost all of coral reef ecosystems will experience extreme temperatures annually before the end of the century (Van Hooidonk et al., 2016) due to the rise of ocean temperatures (Hoegh-Guldberg & Jones, 1999). It is foreseen that coral reefs will face real threats in places where tropical seas will suffer a rise of about 2 °C to 4 °C in temperature by the end of the century (Donner et al., 2005). Also, bleaching events are expected to occur annually or biannually for corals around the world in the next 30-50 years (Donner et al., 2005). In fact, extreme weather events, such as HWs, are predicted to happen more regularly , for longer periods and to be more intense, consequently causing severe damage and substantial mortality of corals (Figure 1), negatively affecting all the benefits they provide (Dias et al., 2019b; Genevier et al., 2019; Smale et al., 2019; Stillman, 2019). These issues are especially relevant if one considers that the number of years between severe bleaching events has already reduced about fivefold in the past 40 years, shifting from an occurrence of once every 25-30 years in the 1980s to once every 5.9 years in 2016 (Hughes et al., 2018). Considering the short time intervals between bleaching events, coral assemblages do not have enough time to fully recover, as this usually takes around 10 to 15 years (Hughes et al., 2018). In fact, recent reports by Leggat et al. (2019) and Fordyce et al. (2019) state that severe marine heatwaves (MHWs) (Figure 2) lead to coral reef decay associated with coral bleaching and widespread mortality, rapid skeletal dissolution, and the loss of reef structure. Despite the widespread impacts of thermal stress events in coral reefs, bleaching patterns can vary spatially and temporally. In a recent study synthesizing field observations of coral bleaching across 81 countries from 1998 to 2017, researchers concluded that bleaching events are more likely to occur at tropical mid-latitudes in comparison to equatorial sites, despite similar thermal stress (Sully et al., 2019). The authors state that this could be due to geographical differences in species composition and thermal tolerance thresholds, the higher genotypic diversity at low latitudes and some level of pre-adaptation to thermal stress at warmer equatorial sites (Sully et al., 2019). Moreover, sites with greater temperature variability may harbor more resistant corals, reducing the probability of bleaching. In any case, a higher thermal threshold for

bleaching was observed in the last decade, occurring at a significantly higher SST, suggesting some level of adaptation of corals to high thermal stress (Sully et al., 2019).
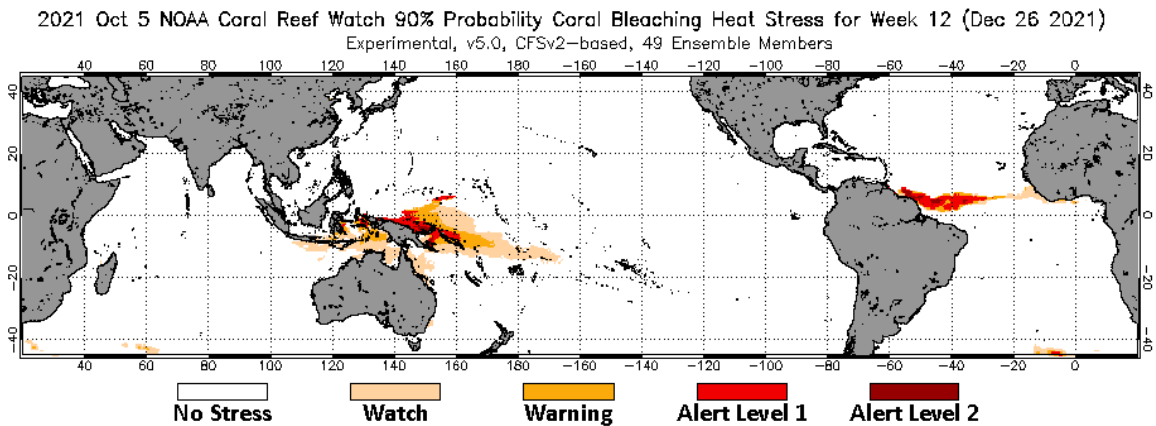
Figure 1 - Areas with a 90% probability of bleaching by the end of 2021 (December 26th, 2021). Bleaching probability increases with the increase in color intensity (source: NOAA, Coral Reef Watch, accessed at 19/10/2021).
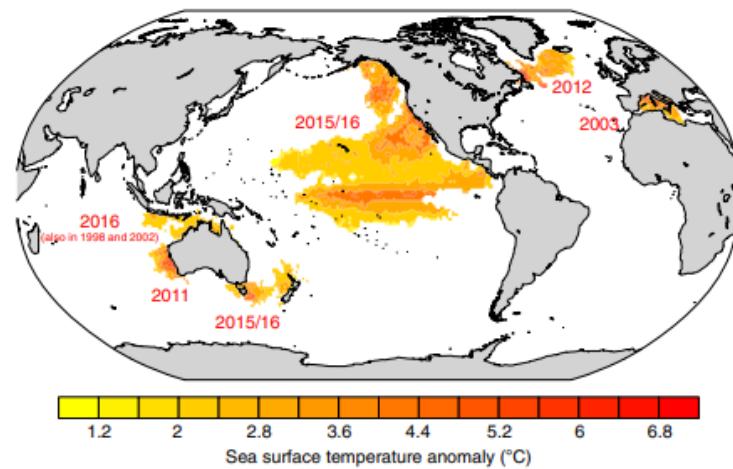
Figure 2 – Recent MHWs, documented and analyzed in literature, showing the maximum SST in regions where temperature exceeds the 99th percentile. MHW intensity increases with the increase in color intensity (Frölicher & Laufkötter, 2018).

Given the intense human-induced degradation of reef ecosystems, with coral cover losses of about 50% in the Indo-Pacific region and up to 80% in the Caribbean over the past 3 decades (reviewed by Hoegh-Guldberg et al., 2019), it is urgent to conserve and restore degraded coral reefs. However, to secure the future of these tropical

ecosystems, restoration actions need to be combined with global and local threat reduction actions and should be monitored in the long-term to provide evidence of success   (e.g. Hein et al., 2020, 2021). As UN declared 2021-2030 the Decade on Ecosystem Restoration and the Decade of Ocean Science for Sustainable Development, common frameworks for action need to be developed aiming for science-informed management and mitigation actions. Up to date, coral conservation and restoration has been focused on several types of actions including proactive (climate change mitigation, predator and disease management, marine protected areas, water quality management, fisheries control) and reactive (coral gardening and transplantation, enhancement of coral adaptation capacity, substrate addition/manipulation, algae control and larval propagation) (Hein et al., 2021). These actions are implemented aiming to return coral reef ecosystems to a pre-disturbance state. However, sometimes, ecosystems can suffer such a severe change that it is no longer practical, or even impossible, to restore them back to a healthy state (Perring et al., 2015). Considering climate change scenarios, all reef management actions should be climate-smart (*sensu* Stein et al., 2014, West et al., 2017), consider ecosystem state and biodiversity goals, and address socio-economic needs of local populations. Involving all stakeholders in conservation processes, as early as possible, will be key to ensure their success and secure the future of tropical coral reefs (e.g. Hein et al., 2020, 2021).

1.2   Impacts of thermal stress in corals

According to Kordas et al. (2011), the performance and metabolism of marine organisms depend mainly on the direct and indirect effects of temperature. Most marine organisms are ectothermic and hence do not regulate body temperature. Consequently, environmental temperature determines the rate of biochemical reactions, directly impacting metabolic rates, as theorized by Gillooly et al. (2001) (but see also Clarke, 2004). Hence, animal physiology, growth rates and developmental times are strongly impacted by temperature (Assan et al., 2020; Madeira et al., 2017; Martell et al., 2005), including in corals (Anderson et al., 2017a; Crabbe, 2008; Dias et al., 2018, 2019c; Hoadley

et al., 2015; Miller, 1995). Moreover, reproductive (e.g. Paxton et al., 2016), and foraging activities (e.g. Nowicki et al., 2012) of marine organisms are also influenced by temperature variation. However, not all species are equally affected by this environmental parameter. For example, species that live in warmer or more stable habitats tend to be more affected by rising temperature because they live closer to their upper acute thermal limit and have a lower acclimation capacity (Madeira et al., 2012b; Somero, 2010), as is the case of tropical marine organisms (Glynn, 1993). Thus, understanding physiological and molecular responses to temperature in diverse taxonomic groups is paramount to be able to predict how species will respond to future global climate change.

Previous studies have shown that temperature has a strong effect on the different components of the coral holobiont (Berkelmans & Van Oppen, 2006; Gates et al., 1992; Pootakham et al., 2019; Takahashi et al., 2009). Older studies in corals defined the coral holobiont as the association of a coral host and endosymbiotic algae from family Symbiodiniaceae, commonly known as zooxanthellae (Muscatine & Porter, 1977). However, scientists have recently recognized the importance of the microbiome to corals, leading to a new conceptualization of the coral as a meta-organism/holobiont composed of three parts: coral host, zooxanthellae and microbiome (Goulet et al., 2020). Focusing on the coral-algal mutualism, zooxanthellae are known to be the main source of energy for the coral host. These algae are capable of fixing carbon through photosynthesis, along with sugars and other products that help the growth and survival of the coral species (Muscatine & Porter, 1977). While the coral provides basic nutrients to the algae, such as phosphate, calcium, nitrate and carbon dioxide, zooxanthellae supply coral polyps with carbohydrates, amino acids and glycerin (Göltenboth et al., 2006). However, this relationship is threatened by multiple global and local stressors, being the increase in global sea surface temperature the most significant one (Logan et al., 2014).

Thermal stress can occur in the form of short-term stress (7 days or less) (McLachlan et al., 2020), which includes MHWs, defined as "a period in which water temperature is above the 90th percentile for that area´s historical conditions for five or more days", being one of the most extreme forms of thermal stress that living organisms

may endure in the wild (Hobday et al., 2016). Temperature anomalies can also occur as long-term stress (exposures of more than 30 days) where ocean warming events are included (McLachlan et al., 2020). Overall, heat stress can cause various effects in coral reefs, including coral growth impairment, disease outbreaks and phase shifts that affect reproduction (Chaves-Fonnegra et al., 2018). Severe temperature perturbations can also lead to the loss of symbiotic dinoflagellates from host coral tissues, resulting in a white appearance color (Figure 3) (Lesser, 2011). However, this white color can also come from the degradation of the symbiont's pigments, making the coral tissues transparent. This is a phenomenon known around the world as coral bleaching (Hoegh-Guldberg & Jones, 1999). The molecular mechanism underlying coral bleaching is related to an increased production of Reactive Oxygen Species (ROS), especially because of damage caused to endosymbionts' photosystem II (PSII) (Cziesielski et al., 2019). This increase in ROS compromises the integrity of host cells, not only due to direct damages on cellular components, but also because ROS trigger nitric oxide production leading to cell death (reviewed by Cziesielski et al., 2019).

Bleaching Process
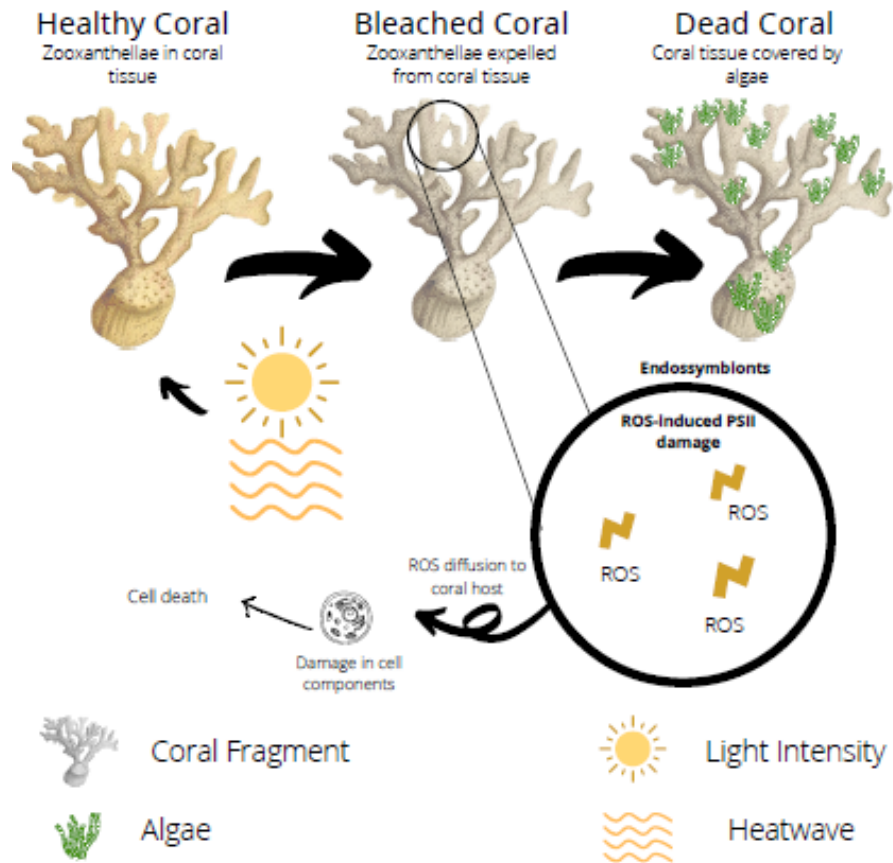
Figure 3 - Bleaching Process in coral reefs from the point of view of a coral fragment with two stress factors involved: a HW and light intensity (image by the author).

Heat stress is known to destabilize nutrient cycling in corals due to increased energy demands, shifting metabolism from a nitrogen- to a carbon-limited state (Rädecker et al., 2021). Thus, bleached corals are nutritionally compromised (Hughes et al., 2018) and need to find other sources of energy to fulfill nutritional needs: they can predate on plankton and ingest particulate organic matter or uptake dissolved organic carbon as alternatives. This ability for heterotrophic plasticity may improve the resilience of some symbiotic cnidarians to thermal stress (Levas et al., 2016). However, when heat stress is very prolonged in time, corals may lose the ability to physiologically recover. When this happens, corals tend to die, since they cannot survive without their symbiotic

partners (Fitt et al., 2001). However, corals can also engage in a recovery process, where they regain zooxanthellae, although this capacity depends on the characteristics of the holobiont, more specifically the host and the taxon of the endosymbiont (Slattery et al., 2019). In the case of zooxanthellate corals, the duration of this recovery can vary, ranging from 3 months to more than 2 years (Brown & Suharsono, 1990; Glynn & D'Croz, 1990).

Both hard and soft corals can have a symbiotic relationship with zooxanthellae. Bleaching events impacting soft corals are usually characterized by the loss of the coral's dinoflagellate symbionts, the death of the colony and its disintegration (Fabricius, 1999), while in the case of hard corals, their calcium carbonate skeletons persist (Mollica et al., 2018). Bleaching temperatures (e.g., 30 °C – 31 °C) are, in general, identical for both coral categories (Marshall & Baird, 2000; McClanahan et al., 2007). However, according to Fabricius (1999), in a same location, soft corals tend to be more susceptible to bleaching events, although sensitivity to this damage depends mainly on the species and genera exposed (Kayanne et al., 2002). Also, corals that display higher rates of recruitment and a quicker growth tend to be more predisposed to thermal stress and its deleterious consequences (Jokiel & Coles, 1990). Information regarding soft corals and bleaching events is scarce when compared to information on scleractinian corals (i.e. stony/hard corals). Still, records of mass bleaching in soft corals such as *Sarcophyton* cf. *glaucum* (Chavanich et al., 2009) and *Sinularia* spp. (Slattery et al., 2019) have already been documented.

The overall response and tolerance of corals to thermal stress is dependent on coral species (e.g. Hoadley et al., 2015). This suggests that coral holobionts may have some ability to adapt, acclimate/acclimatize (i.e. be phenotypically plastic) to rising temperatures (Hughes et al., 2003), or even associate with stress-resistant endosymbionts (Carballo-Bolaños et al., 2020). While the process of adaptation can be defined as "changes in the genetic composition of a population that are passed onto the next generation through natural selection" (Brown, 1997b), phenotypic plasticity is the "ability of one genotype to express varying phenotypes when exposed to different environmental conditions" (Fox et al., 2019). The resulting increase of tolerance toward stressful environmental conditions is known as acclimation or acclimatization (Yampolsky

et al., 2014). In more detail, acclimation refers to "short-term phenotypic changes under manipulative experimental conditions in the laboratory", while acclimatization relates to "phenotypic changes of corals in their natural environment" (Carballo-Bolaños et al., 2020). Overall, these changes can be reversible (organism goes back to its homeostatic state) or not (organism stays in its new homeostatic state) (Figure 4), being both these mechanisms able to confer an improvement of the individual fitness (Carballo-Bolaños et al., 2020; Whitman & Ananthakrishnan, 2009).
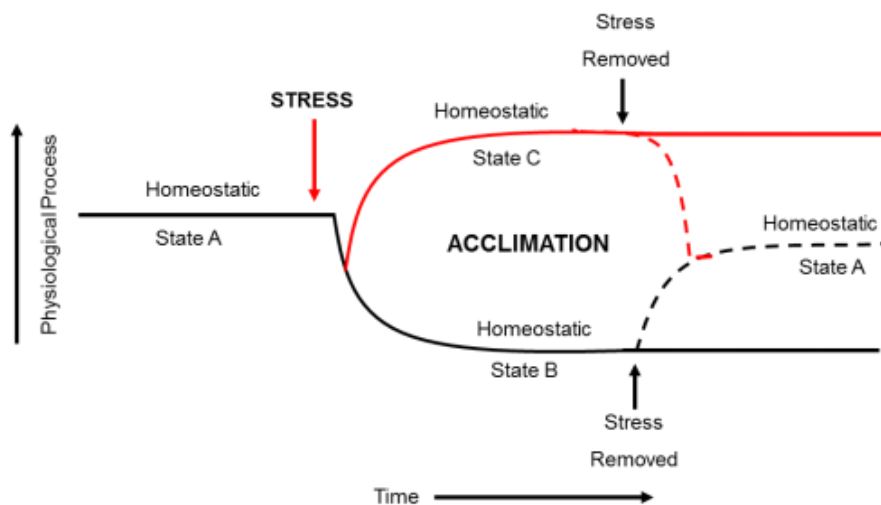
Figure 4 - Acclimation process of an organism when a stress is induced (Whitman,2009).

According to Carballo-Bolaños et al. (2020), acclimation/acclimatization of corals to local conditions provides a higher thermal tolerance during stressful events (bleaching). In fact, several studies have identified a correlation between thermal preconditioning and bleaching susceptibility, for example, in a thermal stress experiment where corals were subjected to 31 ˚C for 8 days only the preconditioned ones were able to resist the bleaching events (Bellantuono et al., 2012). The ability of corals to withstand thermal stress events after preconditioning may be related to changes at the molecular and cellular level, as elevated temperatures induce variation on gene expression, which include an "upregulation of genes involved in oxidative stress responses and carbon metabolism" (carbon substrates increase to be used in coral respiration) (Leggat et al., 2011). A comparison of gene expression in corals preconditioned to thermal stress

showed differences in the number of expressed genes compared to non-preconditioned ones (Leggat et al., 2011).

1.3   Mechanisms of thermal tolerance in corals

The thermal tolerance window (Figure 5) can be defined as "the favorable range of temperature or performance breadth of a species" (Angilletta & Angilletta, 2009). When environmental fluctuations occur towards a species limits or outside of this range, the performance of an individual can be negatively affected, possibly leading to its death (Angilletta & Angilletta, 2009).
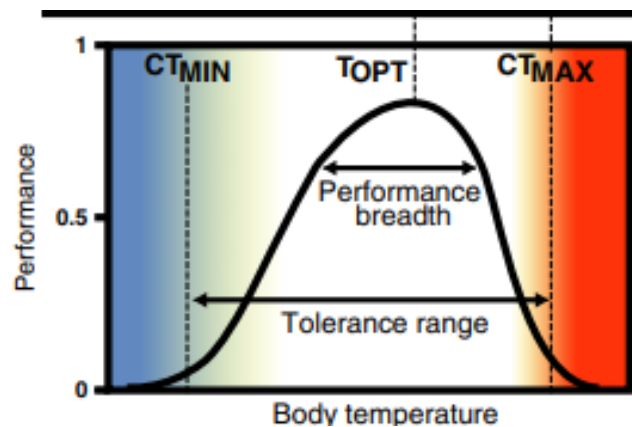
Figure 5 - Thermal Tolerance Window describing the relationship between an organism's body temperature and its physiological performance. CT_{Min} represents the organism's critical thermal minimum, CT_{Max} the organism's critical thermal maximum and T_{opt} it's optimal body temperature where the performance breadth resides (Tuff et al., 2016).

Still, when temperature conditions are within the sub-optimal range, organisms can shift their physiology and biochemistry to maintain performance. One way to shift heat tolerance is to modulate gene expression. In fact, previous studies have suggested that there is a stereotyped transcriptional response of corals to stress, namely the Coral Environmental Stress Response, observed under all types of high-intensity stress (Dixon et al., 2020). This response involves a down-regulation of cell growth genes, induction of apoptosis, response to oxidative stress, protein folding and degradation and induction of immune response (Dixon et al. 2020). In accordance with this idea, Cziesielski et al. (2018)

also refer the occurrence of a common core cnidarian response to heat stress, involving cellular functions related to protein folding and response to oxidative stress. However, Dixon et al. (2020) state that under low-intensity stress, corals may show variable responses with the involvement of different cellular functions.

The described Coral Environmental Stress Response is a version of the well-studied Cellular Stress Response (CSR), which is ubiquitous in all domains of life and involves the complex rewiring of cellular metabolism to cope with homeostasis-disrupting stress (Kültz, 2020). The CSR has four major tasks in the cell, namely: (i) prevent and repair macromolecular damage, (ii) regulate the cell cycle via the activation of cell cycle checkpoints, (c) regulate metabolic energy via mobilization or reallocation, and (d) initiate programmed cell death (apoptosis) when cell repair mechanisms are not enough to avoid extreme cellular damage (Kültz, 2020). Coral adaptation to thermal stress has been associated with a high constitutive expression of genes involved in the CSR, such as heat shock proteins (Hsps) and antioxidant enzymes (Barshis et al., 2013; Cleves et al., 2020), possibly as a result of genetically based local adaptation (see Palumbi et al. 2014). Additionally, Hsps and chaperonin proteins are also involved in coral heat acclimatization processes, as the expression of their genes is indeed modulated when corals are exposed to heat stress (Desalvo et al., 2010; Kenkel et al., 2011; Palumbi et al., 2014) . Heat shock proteins are critical in proteostasis (protein homeostasis) (Kim et al., 2013), and are responsible for maintaining the integrity of proteins during environmental stress, particularly when this is caused by temperature (Whitley et al., 1999). They can refold denatured proteins and prevent the aggregation of non-native proteins through degradation (Whitley et al., 1999), contributing to the success of an organism survival by allowing them to temporarily thrive in environmental extremes (Hofmann, 2005). Ubiquitin tagging and proteolysis modulation are also key in cellular proteostasis, being responsible for the elimination of irreversible misfolded proteins that, due to stress, loose their native function and form cytotoxic aggregations (Hofmann, 2005). Overall, in more detail, the CSR leads to the production of molecules involved in various cellular functions, which include the heat shock response and antioxidant defense (Barshis et al., 2013), proteolysis (Somero, 2020), lipid metabolism (Mayfield et al., 2021), apoptosis regulation

(Somero, 2020) and immune function (Palareti et al., 2016). Accordingly, the importance of innate immune responses and apoptosis regulation in thermal tolerance of corals has also been highlighted in previous studies (Barshis et al., 2013). All of these pathways are critical for organisms to be able to cope with thermal-induced damage to cellular components, such as proteins, nucleic acids and membranes (Somero, 2020), leading to an enhanced fitness and increased survival (Angilletta & Angilletta, 2009). Recent studies have also shown that long-term exposure of hard corals to elevated temperature results on oxidative damage, leading to an increase in antioxidant enzymes (Dias et al., 2019b, 2020), thus confirming the involvement of the antioxidant function in responses to heat stress.

During heat-stress induced coral bleaching, the photoinhibition of the photosynthetic apparatus of the symbionts and photosystem degradation results in the overproduction of ROS, decreasing photosynthetic efficiency and inducing oxidative stress in the holobiont (Oakley & Davy, 2018). This oxidative stress may be exacerbated by ROS produced from other sources (e.g. mitochondria of the host and symbiont), resulting in disturbances in cell homeostasis, DNA degradation, lipid peroxidation (LPO), protein carbonylation, reductions of photosynthetic activity (Weis, 2008) and, ultimately, cell death (Abele & Puntarulo, 2004). To prevent these oxidative damages, cells resort to antioxidant agents that transform these ROS into forms of oxygen that are less harmful to the cell (Abele & Puntarulo, 2004). The elimination of these free radicals is critical to restore homeostasis and is a major part of the CSR. The CSR is not exclusively induced by heat stress, it can also be induced by many other factors, including pollution and UV radiation (Lesser, 2006). However, most of the information on coral CSR comes from hard corals, meaning that there is an overall lack of knowledge on the molecular mechanisms underlying soft corals' responses to environmental stressors.

In addition to these shifts in molecular pathways, the thermal tolerance of corals may be related to genetic differences of their endosymbionts (van Oppen & Oakeshott, 2020). This may be related to differential thermotolerance and physiology of the symbionts, as some phylotypes are much more tolerant to heat (e.g. D1, C15, A3) than others (e.g. C3, B17, A13) (see Swain et al. 2017). Interestingly, some coral species can

associate with heat-resistant endosymbionts, having the ability to shift the abundance of their dominant symbionts: background ones can represent <10% of the symbiont community and can become dominant, conferring thermal tolerance to the coral host (Carballo-Bolaños et al., 2020).

1.4   Light intensity and its relevance to coral health

Light refers to the "part of the electromagnetic spectrum visible for the human eye" and PAR (Photosynthetic Active Radiation) is the "portion of this light that can be used for photosynthesis by photosynthetic organisms, quantified in µmol photons m^{-2} s^{-1}" (Osinga et al., 2008). As light penetrates seawater, PAR decreases with depth (Osinga et al., 2008).

The light energy reaching a photosynthetic organism can follow three pathways:

1-   Light can be used in photosynthesis via photochemical reactions in the reaction center of PSII.

2-   It can dissipate from PSII reaction center in the form of small quantities of heat, kwon as "non-photochemical quenching".

3-   It can be reduced through fluorescence, the pathway directly measured in fluorometry. By this process, it is possible to determine the amount of light energy used in photochemical and non-photochemical pathways (usually functioning at the same time) (Schreiber & Bilger, 1993).

Fluctuations in light intensity (in space and time) have consequences for light-dependent corals, affecting their physiology by stimulating or inhibiting the photosynthetic activity of symbionts (Legendre et al., 1986; Osinga et al., 2008). The coral holobiont must be able to balance the absorbance of light to be processed via photochemistry without inducing tissue damage, thus maintaining primary productivity (e.g. Roth, 2014). Therefore, light intensity is a major factor determining zooxanthellate coral distribution, productivity and growth (Camp et al., 2018).

As the coral host depends on photosynthetic zooxanthellae to fulfill its energy requirements, light conditions strongly affect the overall health and nutritional status of

corals (Osinga et al., 2008; Osinga et al., 2011). Still, the coral holobiont has evolved an adaptative response, termed photoacclimation, to cope with variations in light intensity. Overall, this can occur through photoprotective processes or through an adaptation mechanism of the photosynthetic complex to maximize light harvest (Titlyanov & Titlyanova, 2002). In more detail, different coral photoacclimation strategies have been documented, including physiological, metabolic, morphological and behavioral shifts of the coral host, as well as physiological and behavioral changes of their photosynthetic endosymbionts (such as migration in the host tissue) (e.g. Cohen & Dubinsky, 2015; Eyal et al., 2019; Kuguru et al., 2010; Lesser et al., 2010; Lohr et al., 2019; Roth, 2014). Photoacclimation responses (Figure 6) may also depend on the timeframe and duration of the exposure to shifts in the light regime.
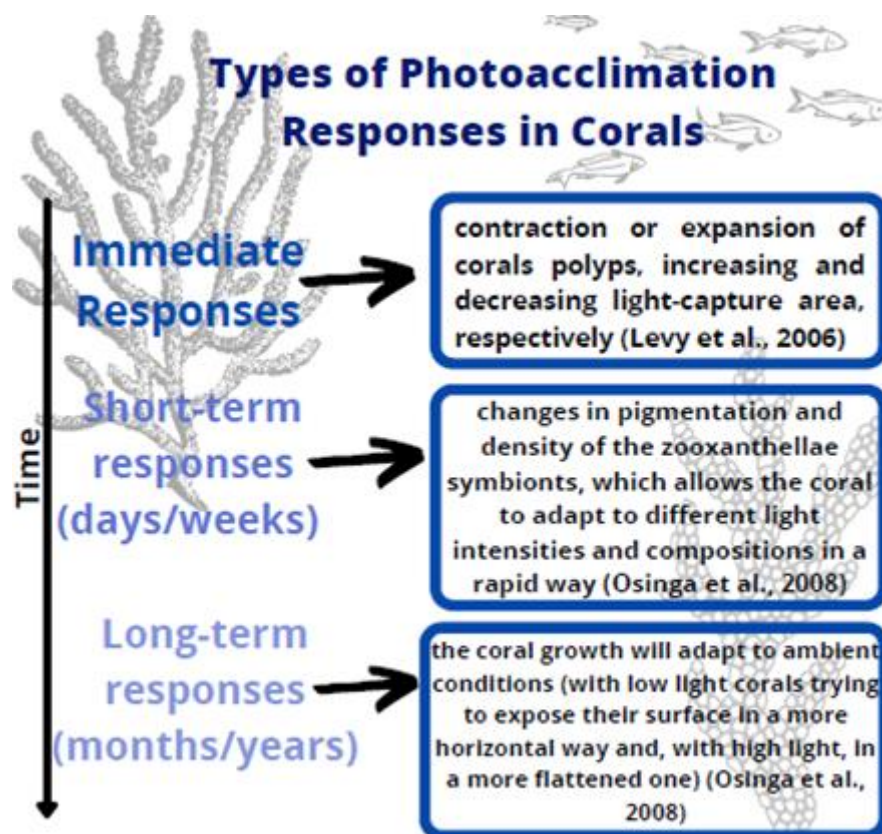
Figure 6 - Types of Photoacclimation Responses in corals according to the duration of the stress being imposed (image by the author).

Short-term responses, such as changes in zooxanthellae's density, photosynthetic pigment concentration and photosynthetic efficiency, as well as their consequences in coral physiology and response to stress, have been widely studied in reef-building corals (e.g. see Lesser et al., 2010; Roth, 2014; Venn et al., 2008). However, this has not been the case for soft coral species, with these issues still remaining largely unknown. According to available literature, corals can adjust the density of zooxanthellae within 15 days of exposure to new light conditions (see review by Roth, 2014). The density of zooxanthellae varies inversely with light levels, decreasing under excess light conditions and increasing under low light conditions (Roth, 2014). This photoacclimation mechanism is hypothesized to optimize photosynthesis while regulating the amount of oxygen produced within the coral host to avoid ROS damage (Roth, 2014). With regards to light-harvesting pigments, they allow the capture of light so its energy can be used in reaction centers of PSI and PSII. Pigments can be diverse, each one with specific capacities concerning light capture, matching the light environment of the organism (Roth, 2014). The main pigments of zooxanthellae (Symbiodiniaceae) are chlorophyll pigments, in particular chlorophyll *a* (Cl *a*) and *c_2*, and also peridinin (Roth, 2014). Cl *a* absorbs blue light at wavelengths of 400-500 and red light at 630-700 nm. Since different pigments have different absorption spectra and different organisms contain different pigments, photosynthetic organisms can be found in various areas and depths, under different light conditions. This is the case of several zooxanthellate corals, like *S.* cf. *glaucum* (Titlyanov & Titlyanova, 2002). The adjustment in pigment concentration, as a photoacclimation strategy, helps the coral holobiont to control light absorption, with pigmentation usually increasing under low light and decreasing under high light conditions (e.g. Iglesias-prieto & Trench, 1994) or extended photoperiods (Meireles, 2017). Photosynthetic efficiency (often measured via the maximum quantum yield of PSII) has also been shown to vary in corals exposed to different light conditions in laboratory experiments and in natural reef conditions (Roth 2014). Under high light, PSII reaction centers close and may undergo photodamage, leading to a photoinhibition process, resulting in a decrease in photosynthetic activity (e.g. Karim et al., 2015b). Such conditions can disrupt the mutualistic relationship between the zooxanthellae and their coral host, leading to coral

bleaching (Karim et al. 2015b, Roth 2014). In fact, bleaching and mortality have already been reported for corals exposed to high irradiance in intertidal areas (Anthony & Kerswell, 2007; Brown et al., 1994). However, it should be noted that Symbiodiniaceae clades may differ in their photo-sensitivity (Karim et al., 2015b). Therefore, corals may change endosymbiont genetic type or show enhanced heterotrophy when exposed to sub-optimal light conditions (reviewed by Camp et al., 2018). In fact, the "Adaptive Bleaching Hypothesis" states that corals can expel all their zooxanthellae as a response to changes in the environment, subsequently choosing another strain of symbionts that are better adapted to the new environmental conditions. Although this is a more common mechanism to cope with thermal shifts, it can also occur with light variations (Osinga et al., 2008).

This phenotypic plasticity (i.e. photoacclimation capacity) of corals with respect to the use of light (see also Osinga et al., 2008) is crucial to allow these organisms to colonize different bathymetries (Cohen & Dubinsky, 2015). Light conditions (both intensity and quality) vary along depth gradients, potentially explaining differences in physiology and bleaching susceptibility of corals (intertidal/shallow water vs subtidal/deeper water acclimatized corals) (Legendre et al., 1986; Osinga et al., 2008). Despite the many studies addressing photoacclimation mechanisms in hard corals, much less is known for soft ones, highlighting the need to address mechanisms of phenotypic plasticity in this understudied, yet highly abundant group of corals.

1.5 Multiple-stressor responses in corals: combined effect of thermal and light stress

With increasing awareness on the impacts of elevated temperatures on coral reefs, understanding how this factor interacts with other stressors becomes highly relevant, particularly in the face of aggravating global change trends. A coral natural environment consists of various physical, chemical, and biological factors that interact with each other to produce "the ecological framework within which the organism must survive and reproduce" (Coles & Jokiel, 1978). The impacts of stressor interactions on

organisms' physiology and biology are especially important near the species limits of tolerance for a given parameter (Coles & Jokiel, 1978). Several authors have highlighted the relevance of addressing multiple-stressor interactions in global change biology (Todgham & Stillman, 2013) and more recently in conservation and ecosystem management (Côte et al., 2016). There are several types of interactions between stressors (Figure 7), namely (i) antagonistic, when the interaction results in a smaller combined effect than predicted by the null model (i.e. the null model being an additive effect, thus an antagonistic effect is inferior than the sum of individual stressor effects), (ii) synergistic, when the interaction results in a greater combined effect than predicted by the null model (Orr et al., 2020, see also Côte et al. (2016)).
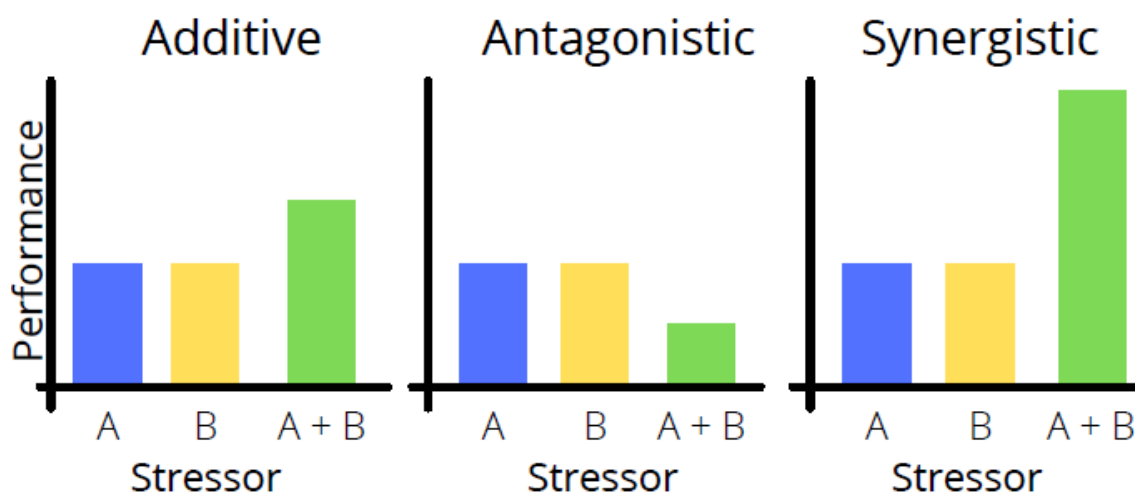
Figure 7 - Possible additive and interactive effects of two stressors (A and B) on the mortality of an organism. Multiple stressors can influence mortality independently (Additive), interact reducing the mortality (Antagonistic) or interact increasing the mortality (Synergistic) (adapted from Todgham & Stillman, 2013).

Evidence of multiple-stressor interactions has already been reported in coral reefs (reviewed by Ban et al., 2014). The top three stressors exerting an influence on other stressors in these ecosystems are temperature, sedimentation and storms, with temperature boosting the effects of ultraviolet radiation, pathogen growth/virulence and low salinity stress (Ban et al., 2014). The interaction between temperature and irradiance has been the one most studied, addressing bleaching responses and quantitative

measures of photosynthesis (Ban et al., 2014). In most studies, a synergistic deleterious effect has been reported for the interaction of temperature and irradiance on corals, although antagonistic effects have been reported when corals were preconditioned to stress (see review by Ban et al., 2014). Nevertheless, most studies are focused on photosynthetic parameters, while molecular assessments are still lacking.

In response to high temperatures, high intensity light tends to aggravate the effects experienced by corals (Coles & Jokiel, 1978). The combination of these stressors is known to enhance the production of ROS, which can disrupt cellular components and induce physiological malfunction, culminating in the expulsion of endosymbionts from its host (Wicks et al., 2010). Also, it causes substantial loss of zooxanthellae's pigments, higher mortality rates, reduced carbon fixation and lowered growth rates (Coles & Jokiel, 1978). In fact, most bleaching events are caused by the combination of high temperatures and elevated irradiance, both contributing to the impairment of PSII by a loss of functional PSII centers, especially protein D1 (Lesser & Farrell, 2004). Moreover, high temperature and irradiance increase the damage to photosystems and could lead to an increased turnover rate of D1 protein, resulting in an injured photosynthetic apparatus that cannot be repaired at a proper rate to maintain photosynthesis (see Warner et al. (1999)). Concomitant decreases in the activity of the carboxylating enzyme Rubisco can also contribute to the decrease in photosynthesis and breakdown of the algal-coral symbiosis (Lesser, 1996). However, in short-term studies, there is a lack of information on the interaction between these two factors (McLachlan et al., 2020), which highlights the need for more studies evaluating the joint effect of more than one environmental stressor; indeed, overlooking interactions may result in wrong estimations of threats to biodiversity (e.g. Simmons et al., 2021). Over the next century, coral species can lose as much as 50% of their current habitat, depending on species-specific preferences and sensitivity to temperature and irradiance (Cacciapaglia & van Woesik, 2015). Given the importance of the combined effects of these environmental factors on the onset of coral bleaching, scientists need to develop new tools, that incorporate the effects of light along with heat stress, to improve the prediction and monitoring of large-scale bleaching events on tropical reefs (e.g. Skirving et al., 2018).

1.6 Integrative health assessments in corals under stress

As already mentioned above, when facing a multiple-stressor scenario, such as combined changes in light intensity and temperature, cellular damage imposed to marine organisms can be magnified (Carballo-Bolaños et al., 2020). As the global future climate change scenario is not likely to be altered without an effort in reducing greenhouse emissions, this problem will likely be aggravated. It is therefore necessary to monitor and understand the status of coral reefs around the world through the definition of parameters that can help to better understand their conditions in the face of climate change if we want to avoid coral bleaching events and, thereby, contribute to their conservation (West & Salm, 2003). In this context, stress factors are known to have direct and indirect effects on organism's physiology, metabolism and biochemistry, eventually scaling up to changes in the marine ecosystem (Figure 8) (Hollowed et al., 2013). There are several ways to test these effects on zooxanthellate corals, but multi-biomarker approaches and the various levels of biological complexity are of special relevance to help scientists assess the impacts of anthropogenic stressors on coral reefs.
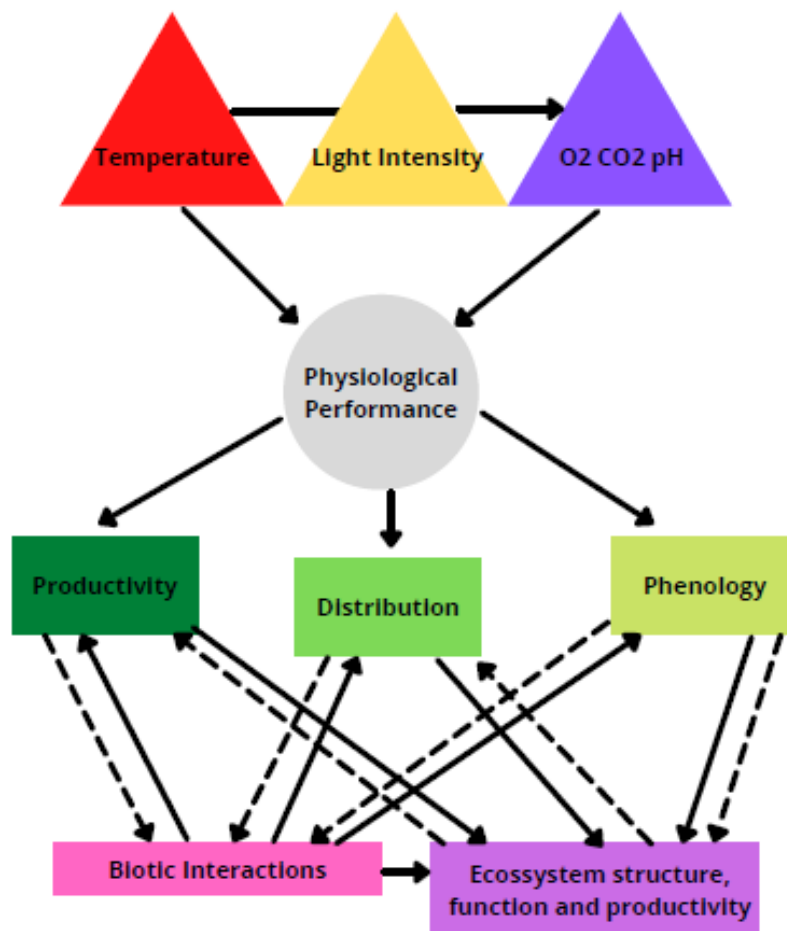
Figure 8 - Direct and indirect effects of multiple stressors on organism's physiology and implications in marine ecosystems. Solid arrows represent direct consequences and dashed ones represent indirect ones (adapted from Hollowed et al., 2013).

1.6.1 *in vivo* Chlorophyll fluorescence

In vivo Chlorophyll Fluorescence can be studied using Pulse Amplitude Modulated Fluorimetry (PAM) (Osinga et al., 2008). Overall, the light energy that is reduced through fluorescence in a photosynthetic organism can help scientists to study the relationship between PAR intensity and photosynthesis, and one way to achieve this is by PAM (Osinga et al., 2008). The conclusion that can be taken is that, at first, there is a positive correlation between these two parameters, as a higher PAR intensity results in a higher photosynthetic activity. However, as the maximum photosynthetic capacity is reached, a

higher PAR makes photosynthesis rate decrease due to photoinhibition, a light-induced decrease in the photosynthetic capacity of a coral in response to a high intensity light (Figure 9) (Osinga et al., 2008).
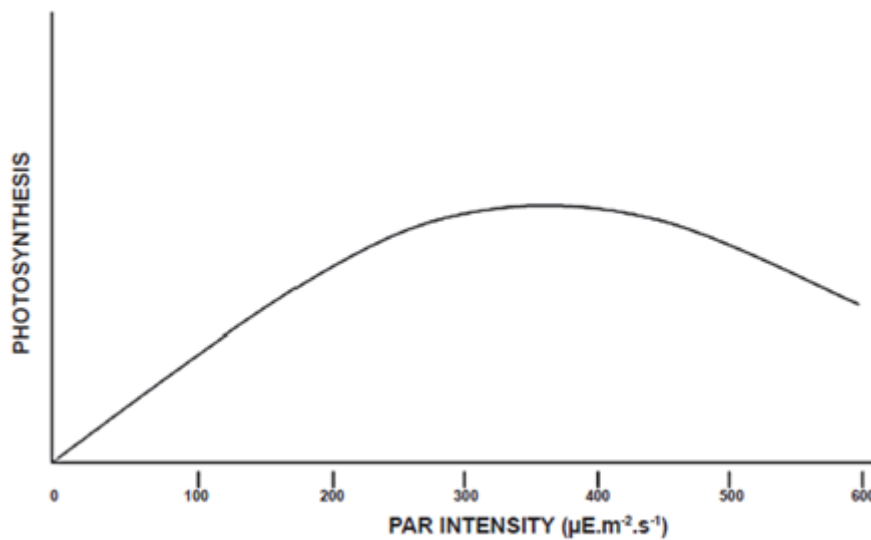
Figure 9 - Theoretical relationship between photosynthesis and irradiance (Osinga et al., 2008).

Thus, PAM helps scientists to measure the rate of photosynthesis of a coral or, in other words, the maximum quantum efficiency (hereafter referred as yield (F_v/F_m)) of PSII. Yield can be calculated by the following equation: $F_v/F_m = ((F_m - F_0)/F_m)$, where F_m is the maximum fluorescence, measured when the PSII reaction centers are closed and plastoquinone is reduced and F_0 is the minimum fluorescence, measured when the PSII reaction centers are fully open and the plastoquinone is in the ground state (Cima et al., 2013). This last parameter is expected to correlate with the amount of Cl a in an organism (Serôdio et al., 2001).

Following a study by Rocha et al. (2013a), coral fragments under lower PAR treatments, have higher F_v/F_m values than corals under higher PAR. Similar results were obtained in a study by Rocha et al. (2013b), additionally being noticed that Cl a content (F_0) was significantly higher in coral fragments under low light regimes.

With regards to the influence of sea temperature on maximum quantum efficiency of a coral, there is evidence of a decline in F_v/F_m of PSII as temperature increases (Gardner et al., 2017a; Hoadley et al., 2019; Hoegh-Guldberg & Jones, 1999;

Jones et al., 1998; Jones et al., 2000; Karim et al., 2015a; Yakovleva et al., 2004). Also, when high irradiance intensity is combined with elevated temperature the same result is observed (Coles & Jokiel, 1978; Hueerkamp et al., 2001; Jones et al., 1998; Karim et al., 2015a; Nielsen et al., 2018; Wicks et al., 2010).

1.6.2 The relevance of zooxanthellae density

Most cnidarians contain high densities of zooxanthellae (around 1-5 (x10^6) cells cm^{-2} of host live surface tissue in stony corals). These endosymbionts are located in vacuoles within the endoderm cells of their cnidarian host (Muller-Parker & Davy, 2001).

As mentioned above, PAM results can indicate the relative Cl a content (F$_0$) of a coral, which can be correlated with the content of zooxanthellae in a coral fragment (Fitt et al., 1993; Hoegh-Guldberg & Smith, 1989). However, in some cases, after a bleaching event, a loss of zooxanthellae can occur without a decrease in Cl a content, as pigment concentration can actually increase (Fitt et al., 2001; Hoegh-Guldbergl & Smith, 1989; Jones, 1997) masking this relationship. According to Le Tissier & Brown (1996), this increase can be explained by: 1) a great loss of zooxanthellae from the apical tissues, which leaves dark-adapted zooxanthellae in the lower tissues, 2) products of chlorophyll and other pigments capable of interfering with the absorption peak that is used to compute the Cl a content and 3) a relation to the nutrient status of these symbionts (by increasing Cl a content, the zooxanthellae that remain are able to increase the nutrient availability through decreased competition). Therefore, zooxanthellae quantification through proper methods within known samples of coral tissue is the best way for measuring changes in their densities, providing a clear vision of the extent and severity of bleaching events (Fitt et al., 2001), which are a real threat to coral reefs around the world (Hughes et al., 2018; Hughes et al., 2017) . There are many ways to do this quantification, including through the use of Neubauer or Neubauer improved counting chambers, both being commonly employed to count coral zooxanthellae (Burtscher et al., 2015).

Recently, studies have shown that zooxanthellae density within the coral host can vary in relation to exogenous factors, such as increases in seawater temperature and

PAR/light intensity (Pillayl et al., 2005). According to Gardner et al. (2017a), Hueerkamp et al. (2001), Karim et al. (2015a) and Nielsen et al. (2018) corals exposed to high seawater temperatures (≈30 °C) bleached and had reduced population densities of zooxanthellae.

Titlyanov et al. (2001) described that under low light intensities corals tend to display higher zooxanthellae densities than conspecifics exposed to high light intensities. This increase in symbiont density is thought to be an adaptative reaction of corals to low light to maximize their light harvesting capacity. The increase in the concentration of photosynthetic pigments by zooxanthellae can also be an adaptative measure.

1.6.3 Biomarkers

When it comes to molecular biomarkers, several molecules have been successfully used in aquatic environmental monitoring and ecosystem health assessments to estimate the degree of biological change and damage in response to stressors (Quintero & Zafra, 2016). The potential use of such molecular biomarkers to assist coral reef conservation and restoration science has also been highlighted (Parkinson et al., 2019). Protein biomarkers are useful to characterize phenotypes of interest (Parkinson et al., 2019), as proteins are the functional units of the cell, mediating biological responses to environmental stress factors (e.g. López, 2007). The most frequently tested biomarkers of stress in marine organisms are proteins involved in the CSR, which is one of the main molecular pathways influenced by fluctuations in temperature, among others (e.g., energy related pathways, cytoskeleton dynamics, cell signaling) and provide information on how an organism responds to stress at a molecular level (Madeira et al., 2017).

Biomarkers can be defined as "any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological; the measured response may be functional and physiological, biochemical, at cellular level, or a molecular interaction" (World Health Orgnization, 1993) and they are used to detect stress signals in organisms. Biomarkers can be divided into three categories: biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility

(World Health Orgnization, 1993). Biomarkers of exposure include exogenous substances, their metabolites or the interaction between a substance and a target molecule within an organism. Biomarkers of effect are measurable alterations within an organism, including biochemical, physiological, and behavioral changes that indicate health impairment. Biomarkers of susceptibility are indicators of the ability of the organism to respond to environmental challenges, serving as early warning signals of a negative biological response towards an environmental factor, such as temperature and light intensity (Bucheli & Fent, 1995).

Considering all stress response mechanisms mentioned above, the most used biomarkers to detect stress levels in marine organisms are Hsps, ubiquitin (Ubi), and antioxidant enzymes (examples in corals, Bromage et al., 2009; Dias et al., 2019a; Dias et al., 2020; Gardner et al., 2017a; Putnam et al., 2013; Sharp et al., 1997). To complement these biomarkers, oxidative damage products, like LPO, are also commonly measured, to assess the level of cellular injury experienced by corals (e.g. Cziesielski et al., 2019; Dias et al., 2020). These biomarkers can be useful in both field and laboratory experiments, being important tools for the health assessment of corals (Dias et al., 2019a; Dias et al., 2020; Fang et al., 1987; Kenkel et al., 2014; Louis et al., 2017; Madeira et al., 2015; Seveso et al., 2020; Teixeira et al., 2013).

1.6.3.1 Heat shock proteins (Hsp70) and ubiquitin

Upon stressful conditions, the activation of CSR leads to the production of Hsps, which can maintain the integrity of the protein pool during environmental stress, functioning as good detectors of damage (Whitley et al., 1999). These proteins are chaperones and play a primary role in intracellular defense (Csermely & Yahara, 2005).

There are several stressors that can induce the production of Hsps: temperature (Kregel, 2002; Madeira et al., 2012a), hypoxia (Hall et al., 1999), ROS (Hall et al., 1999), pollution (Köhler et al., 2001), UV radiation (Downs et al., 2013), viral and bacterial infections (Das et al., 2015), and osmotic stress (Tine et al., 2010). In the face of stressing events, protein misfolding, aggregation or disruption of regulation and disassembly can

occur, leading to the activation of Hsps's activity and subsequent upregulation and rise in corals (e.g. Csermely & Yahara, 2005; Downs et al., 2000; Hofmann, 2005; Louis et al., 2017; Pirkkala et al., 2001; Prahlad & Morimoto, 2009; Robbart et al., 2004; Whitley et al., 1999). Hsp70 kDa is one of the most studied Hsps, alongside with Hsp90 kDa (Hofmann, 2005). Both can occur in several cellular organelles, such as the nucleus, peroxisome, lysosome, mitochondria, and endoplasmic reticulum, and also in the cytosol (Hofmann, 2005). Furthermore, Hsp70 is also important for the targeting of proteins for the ubiquitin-proteasome pathway (Figure 10) (Kriegenburg et al., 2012). This pathway is carried out by the regulatory protein Ubi, where it tags misfolded proteins (due to stress events) and translocates them to the proteasome for degradation. Besides maintaining cellular homeostasis, this process is highly important in various cellular processes (e.g cell cycle regulation) (Hershko, 1996). By these means, Ubi can act as a biomarker of irreversible protein damage, whose activity and abundance can increase with stressful environmental conditions (Hofmann, 2005; Madeira et al., 2020; Madeira et al., 2014a), including in corals (e.g. Cziesielski et al., 2019; Downs et al., 2000; Woo et al., 2006; Yum, 2006).
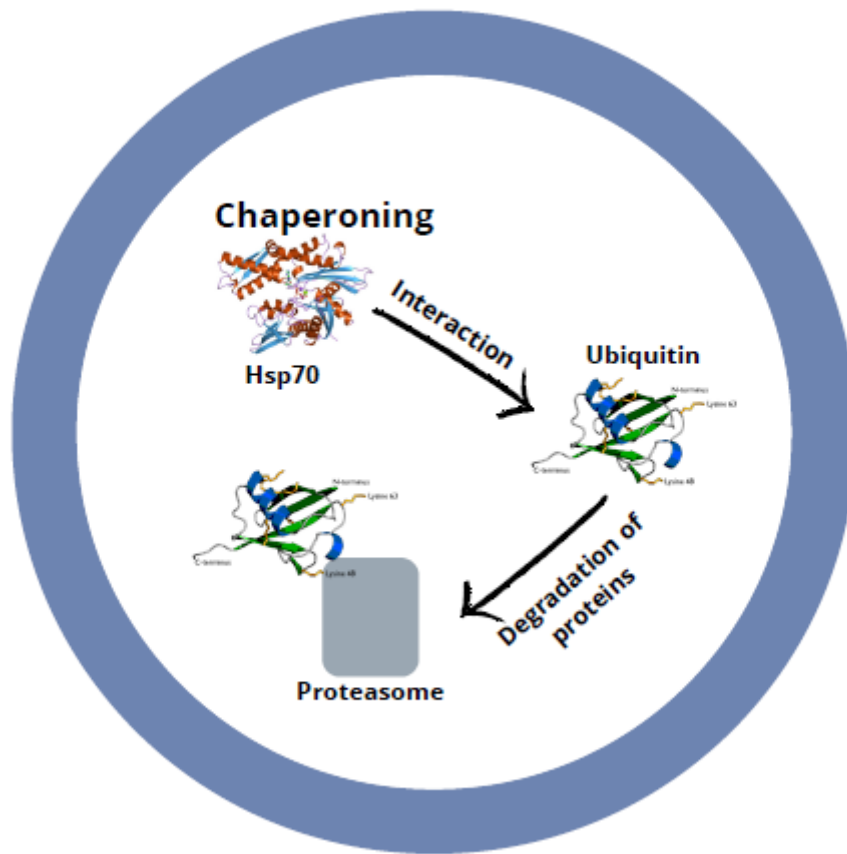
Figure 10 - Scheme of interaction between Hsp70 and Ubi inside the cell and the ubiquitin-proteasome pathway where misfolded proteins are degraded (adapted from Madeira, 2016).

1.6.3.2 Lipid peroxidation

As mentioned above, the elimination of free radicals, such as ROS, is another important step in the CSR. One method to assess the impacts of these ROS in cells is through the quantification of LPO, a form of oxidative damage, where LPO concentrations increase due to the enhanced production of ROS associated to higher metabolic rates (Richier et al., 2006) and destabilization of zooxanthellae's photosynthetic apparatus upon exposure to stress (Lesser et al., 2010; Roth, 2014; Venn et al., 2008). ROS perform an oxidative attack on certain unsaturated lipids of cell membranes, which can culminate in their disruption and the formation of lipid peroxides (Halliwell, 2006). Lipid peroxides are considered to be destructive, as they can compromise cellular functions (Olsen et al., 2013).

1.6.3.3 Antioxidant enzymes and total antioxidant capacity

Antioxidant agent's activity (Figure 11) is an indicator of oxidative stress and can prevent or reduce the damages caused by ROS. These agents can be of two types: enzymatic and non-enzymatic (Abele & Puntarulo, 2004; Chainy et al., 2016). These molecules are referred to as "Oxidative Stress Biomarkers" since they allow us to understand the metabolic state and health of an organism (Abele & Puntarulo, 2004; Chainy et al., 2016). Enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST) and glutathione peroxidase (GSH)) are the most important ones (Kültz, 2020). These enzymatic antioxidants quench ROS through the catalyzation of reactions that transform toxic ROS into non-toxic products. The first enzymes to act are SOD and CAT, with SOD converting the superoxide radical to H_2O_2 and water. Subsequently, CAT regulates the concentration of these compounds in the cells by catalyzing the conversion of H_2O_2 into water and oxygen. GST acts on membrane lipids (PUFAs) by transforming lipid peroxides into lipids-OH (Kültz, 2020).

Non-enzymatic biomarkers, which are constituted by vitamins, carotenoids, tocopherol and glutathione, can be measured through the total antioxidant capacity method (TAC) (e.g. Bartosz, 2010). This assay can indicate the capacity of an organism to counteract oxidative-stress in cells (Chainy et al., 2016) and has been applied to study marine organisms' responses to environmental changes (e.g. Khan et al., 2021; Madeira et al., 2021), including corals (Strahl et al., 2016).
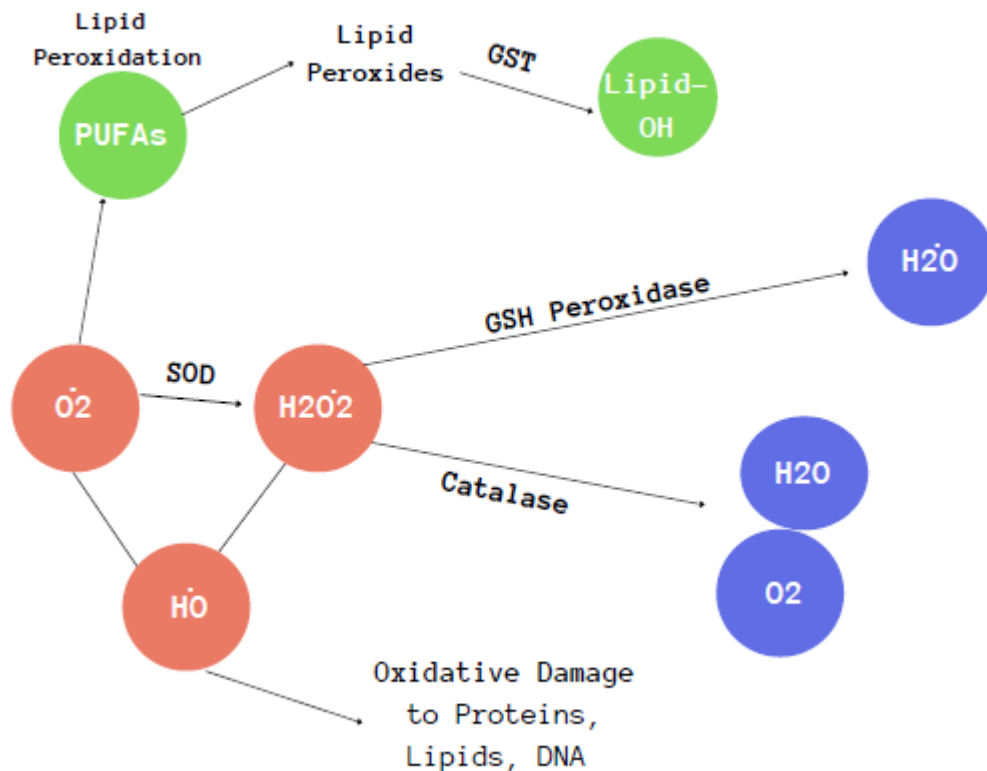
Figure 11 - Oxidative Stress Response pathway regarding the action of antioxidant enzymes (SOD, CAT, GSH, GST) (adapted from Tisthammer, 2020).

1.6.3.4 Total protein content

Protein content is an important component of corals' energy reserves and essential for the maintenance of metabolic functioning under stress (see Buerger et al., 2015; Grottoli et al., 2004). Thus, bleached corals tend to rely on protein reserves to survive and recover from bleaching events, with protein content showing a general tendency to decrease under stress conditions (Fitt et al., 1993), including heat stress (e.g. Ezzat et al., 2019). Protein content is commonly used as an index of the physiologically active biomass of cnidarians regarding skeletal and cellular growth, the interaction between host and symbionts (Tentori et al., 2004), and the expression of certain proteins (Phelan et al., 2006). As such, this parameter can function as a health indicator. Usually, total protein concentration decreases as zooxanthellae density decreases, indicating that

the loss of endosymbiots leads to a loss of energy reserves (as the coral can no longer rely on photosynthates), proving the correlation between protein loss and bleaching events (Knochel, 2017). Nonetheless, it is necessary to consider that some corals may have the capacity to maintain their energy reserves (including protein concentration) by increasing heterotrophy, as already documented for bleached *Montipora capitata* (Rodrigues & Grottoli, 2007)*.

1.7   *Sarcophyton* cf. *glaucum* as a soft coral model species

Species from genus *Sarcophyton*, popularly termed as mushroom or leather corals, are important members of shallow-water reef communities in the Indo-West Pacific and in the Red Sea. They can be found in high energy areas (surge zones and tide pools) and in deep waters, down to 30 meters (Ellis, 1999). According to Aratake et al. (2012) this is a genus with an overall lack of knowledge regarding all of its features due to a very small amount of experimental work performed using *Sarcophyton* as a target species for taxonomy and ecology studies. These corals are also often studied due to their production of important bioactive compounds, such as diterpenes and sesquiterpenes, which display antitumor activity (McFadden et al., 2006).

The species *Sarcophyton glaucum* (Figure 12) (see Figure 13 for taxonomy) is a sessile, subtropical and colony forming soft coral. It can be found mostly in intertidal waters, but also on subtidal habitats, occurring in soft bottom substrates on muddy coastal areas, as well as offshore locations (Fabricius & Alderslade, 2001). The preferred temperature of this coral species ranges from 25.7 to 29.3 °C (Kaschner et al., 2016). It is native from the Western Pacific (New Caledonia, Taiwan, Ryukyu Island and Palau) (Figure 14) (Benayahu et al., 2004).

The taxonomic status of *S. glaucum* is not consensual (Aratake et al., 2012) and, according to McFadden et al. (2006), this species can be divided into six different clades, based on sequence analyses of mitochondrial proteins. Following this argument, the corals used in this experiment will be termed as *Sarcophyton* cf. *glaucum*.
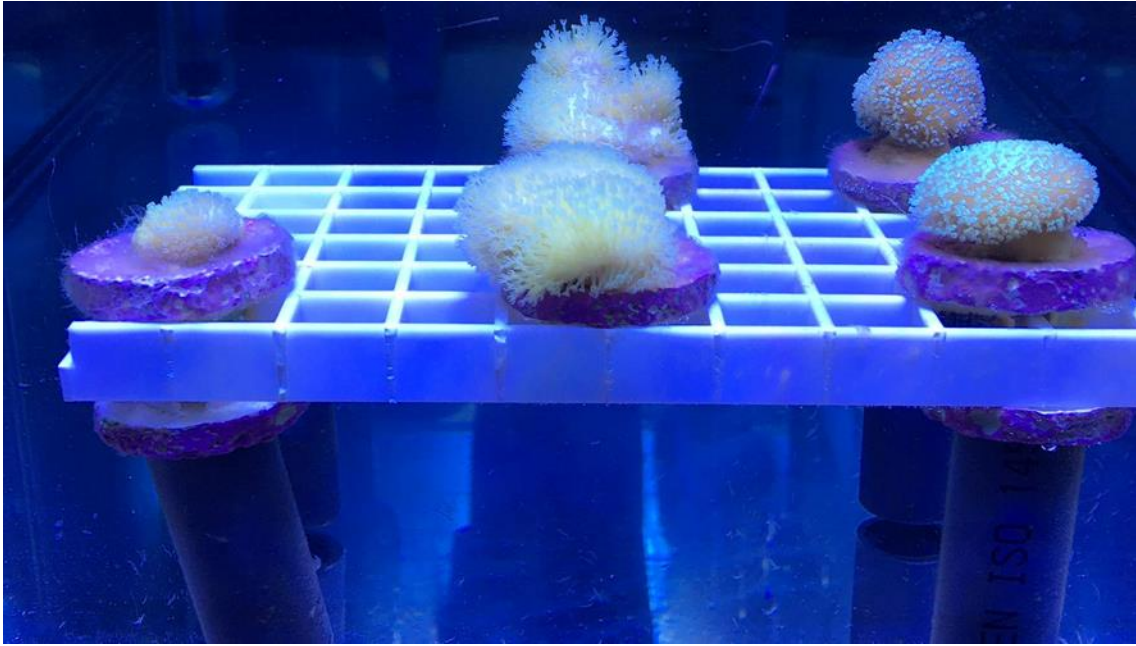
Figure 12 – *Sarcophyton* cf. *glaucum* fragments under experimental conditions.

Figure 13 - Taxonomy of the species *Sarcophyton* cf. *glaucum.*
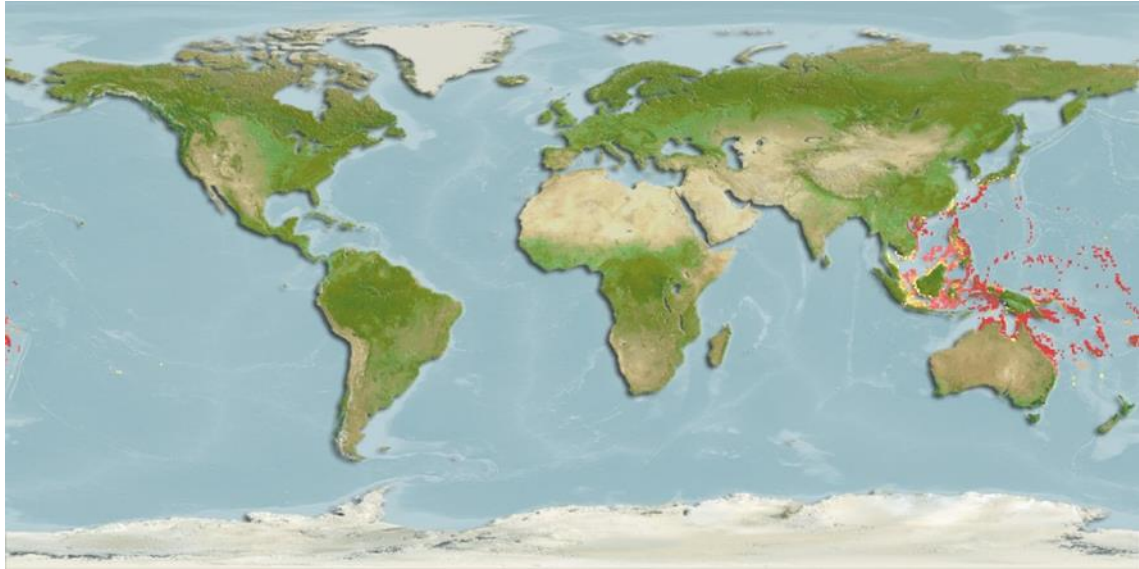
Figure 14 - Map of the native distribution of *Sarcophyton* cf. *glaucum* (source: SeaLife base, accessed 25/01/2021).

Like any other soft coral, *Sarcophyton* cf. *glaucum* also has a symbiotic relationship with dinoflagellate algae of the family Symbiodiniaceae (Fitt et al., 2001). The symbiotic relationship between soft coral species and zooxanthellae is maintained within a temperature range from 18 to 33 ˚C, with optimal temperatures being in the range of 25 to 29 ˚C (Farag et al., 2021). According to Floros et al. (2004) species from genus *Sarcophyton* are very susceptible to bleaching events. Despite scarce information on bleaching in *Sarcophyton* spp., there have already been reports on the mass bleaching of this species (Chavanich et al., 2009).

The reproduction of *S.* cf. *glaucum* consists in the shed of mature gametes into the coelenteron (a central body cavity) of the coral, which are subsequently spawned through their mouth. The zygote then transforms into a planktonic planula larva, which initiates the metamorphosis process: first the morphogenesis of the tentacles, followed by the septa, and then the pharynx. The process ends with larval settlement (Ruppert et al., 2004).

Given that most knowledge on corals comes from scleractinian corals, more studies are needed to unravel phenotypic plasticity mechanisms in soft corals in response to environmental stress. Important physiological and ecological differences between stony and soft corals have already been highlighted, namely (i) their contrasting ability to

create multiple symbiotic partnerships (Baker & Romanski, 2007), with soft corals presenting less symbiont diversity (Goulet et al., 2008), and higher stability in symbiont communities over space and time (Goulet & Coffroth, 2003). In addition, soft corals seem to rely much more on mixotrophy than stony corals (Fabricius & Klumpp, 1995). Such differences highlight the potential for different sensitivities of hard vs soft corals to thermal and light stress, warranting further studies. Moreover, *S.* cf. *glaucum* is not considered a well-studied species (McLachlan et al., 2020), thus relevant issues on its ecology, reproduction, and physiology are yet to be unraveled.

1.8   Aims, scope and hypotheses

The present study aims to evaluate the performance and molecular and physiological responses of the soft coral *Sarcophyton* cf. *glaucum* under different global change scenarios. To fulfill this objective, the species was maintained under controlled conditions with the effect of two main factors being experimentally tested: temperature and light intensity.

With this purpose, the main objectives of this thesis were:

1- To assess the bleaching susceptibility of *S.* cf. *glaucum* exposed to a MHW scenario under different light regimes (low light and high light), simulating their distribution across different depths (intertidal vs subtidal).

Within this objective, the null and alternative hypotheses tested were as follows:

H_0: HWs and light intensity do not cause stress on the soft coral *Sarcophyton* cf. *glaucum* that can lead to the disruption of the symbiotic relationship between the coral host and zooxanthellae (bleaching).

H_1: HWs and light intensity cause stress on the soft coral *Sarcophyton* cf. *glaucum* that can lead to the disruption of the symbiotic relationship between the coral host and zooxanthellae (bleaching).

2- To identify changes in the photophysiology of *S.* cf. *glaucum*, namely on the maximum quantum yield of PSII and Cl *a* content of specimens exposed to different light intensities and a MHW scenario to infer their ability to maintain photosynthetic activity when exposed to these stressors.

Within this objective, the null and alternative hypotheses tested were as follows:

H_0: Maximum quantum yield of PSII and Cl *a* content of the soft coral *Sarcophyton* cf. *glaucum* are not influenced by heat stress and different light intensities, as these stressors do not cause damages to PSII, not leading to ROS formation and subsequent bleaching. Temperature and light stress do not interact to produce a synergistic effect on the coral, thus no substantial decline in photosynthetic parameters should be observed.

H_1: Maximum quantum yield of PSII and Cl *a* content of the soft coral *Sarcophyton* cf. *glaucum* are influenced by heat stress and different light intensities, as these stressors cause damage to PSII, as well as the formation of ROS and lead to bleaching. Temperature and light stress combined promote exacerbated effects given the presence of a synergistic interaction, leading to a substantial decline in photosynthetic parameters.

3- To quantify the number of zooxanthellae within *Sarcophyton* cf. *glaucum* exposed to different light intensities and a HW scenario, using this parameter as a proxy for the level of bleaching.

Within this objective, the null and alternative hypotheses tested were as follows:

H_0: The number of zooxanthellae within the soft coral *Sarcophyton* cf. *glaucum* does not change under heat stress and the addition of light intensity as another stressor does not enhance the loss of these endosymbionts.

H_1: The number of zooxanthellae within the soft coral Sarcophyton cf. *glaucum* can change under heat stress and the addition of light intensity as another stressor can enhance the loss of these endosymbionts.

4- To uncover the molecular mechanisms underlying *S.* cf. *glaucum*'s physiological changes and bleaching susceptibility/tolerance by quantifying selected biomarkers

that indicate how this species reacts to temperature and light intensity shifts over time.

Within this objective, the null and alternative hypotheses tested were as follows:

H_0: Heat stress does not affect the CSR, not leading to macromolecular damage on the soft coral *Sarcophyton* cf. *glaucum* and different light intensities do not interact with temperature, thus not leading to a synergistic effect on the CSR and oxidative damage resulting from enhanced ROS.

H_1: Heat stress affects the CSR and leads to macromolecular damage on the soft coral *Sarcophyton* cf. *glaucum* and different light intensities can interact with temperature, leading to a synergistic effect on the CSR and oxidative damage resulting from enhanced ROS.

2. Materials and Methods

2.1 Coral husbandry and fragmentation

Coral fragments of *Sarcophyton* cf. *glaucum* were obtained from three distinct mother colonies living between a 5 and 15 m depth, originating from Indonesia. Mother colonies were farmed in the Indonesian sea and imported to Portugal by Tropical Marine Centre Iberia, an aquarium wholesaler promoting the sustainable trade of marine ornamental species. These mother colonies were then transported overnight to CEPAM-ECOMARE in plastics bags with 1/3 seawater and 2/3 oxygen in Styrofoam boxes to secure thermal insulation and protection from mechanic shock. Upon arrival, the three mother colonies of *Sarcophyton* cf. *glaucum* were stocked in a recirculated water system (600 L) for 1 month under the following conditions: (i) lighting was provided by a 150 W metal halide lamp (BLV, Germany) with a photoperiod of 12 hours and a PAR intensity of 120 μmol quanta m^{-2} s^{-1}, (ii) temperature was maintained at 26 ˚C using heaters equipped with a thermostat (EHEIM 3616, 300W, Germany), iii) salinity was kept at 35-36 using an osmoregulator, and (iv) pH was kept at around 8.1 through weekly partial water changes. After the acclimation period, the fragmentation process was carried out as follows: the three mother colonies were cut using sterilized scalpels to produce 96 similar sized fragments (about 30 mm diameter). These fragments were then secured individually to labelled coral plugs using rubber bands.

Until the beginning of the experiment, all fragments were maintained in a tank (350 mm x 500 mm x 1500 mm, 260 L) illuminated from above with white light T5 fluorescent lamps (HAILEA Sunshine Tube 4 x 80 W, China) with a photoperiod of 14 L:10 D (14 hours of light and 10 hours of dark). PAR values were measured at the level of coral fragments using a Spherical Micro Quantum Sensor US-SQS/L (ULM-500, WALZ, Germany) and were ~190 μmol m^{-2} s^{-1}. A circulation pump (Turbelle nanostream 6015 3.5 W, Germany), two heaters equipped with thermostats (EHEIM 3616 300 W, Germany) and a protein skimmer (Deltec Skimmer 3000ix, Germany) were used to keep water parameters within optimal values, at a salinity of 35/36 and a temperature of 26 °C.

2.2 Experimental design

A full factorial design was applied, to test the effect of two independent variables, Temperature and Light Intensity on corals' photophysiology and molecular responses. Each independent variable had 2 levels: 26 and 32 ˚C for temperature and High Light and Low Light for light Intensity (PAR values of 663 µmol m^{-2} s^{-1} ± 35.9 and 253 µmol m^{-2} s^{-1} ± 17.4, respectively, measured using a Spherical Micro Quantum Sensor US-SQS/L (ULM-500, WALZ, Germany)), for a total of 4 treatments. The experimental system consisted of 8 glass tanks (345 mm x 490 mm x 370 mm, 62.5 L), with the number of replicate coral fragments per tank per treatment being n= 6 (Figure 15). Each tank was divided into two parts: one received a High Light (HL) intensity and the other one a Low Light (LL) intensity mimicking the light regimes of possible habitats occupied by these corals in the wild: intertidal and subtidal areas, respectively. Low light intensity was achieved by placing a shading frame (Figure 16) on top of each tank. Of the 8 tanks, 4 were kept under a control temperature (CTRL), 26 ˚C, throughout the whole experimental period, while the other four were used to simulate a HW scenario, where temperature was risen from 26 to 32 ˚C for 10 days (please see below for further details).

Figure 15 - Setup of the life support system: panoramic view (on the left) and front view (on the right).

Figure 16 - Shading frame used to achieve a LL intensity in one part of the tanks.

Each tank was numbered (from 1 to 8) and each temperature treatment was randomly assigned to a tank (Figure 17). All LL treatments were performed on the back

side of each tank for technical reasons related with maintenance of stocked coral fragments.
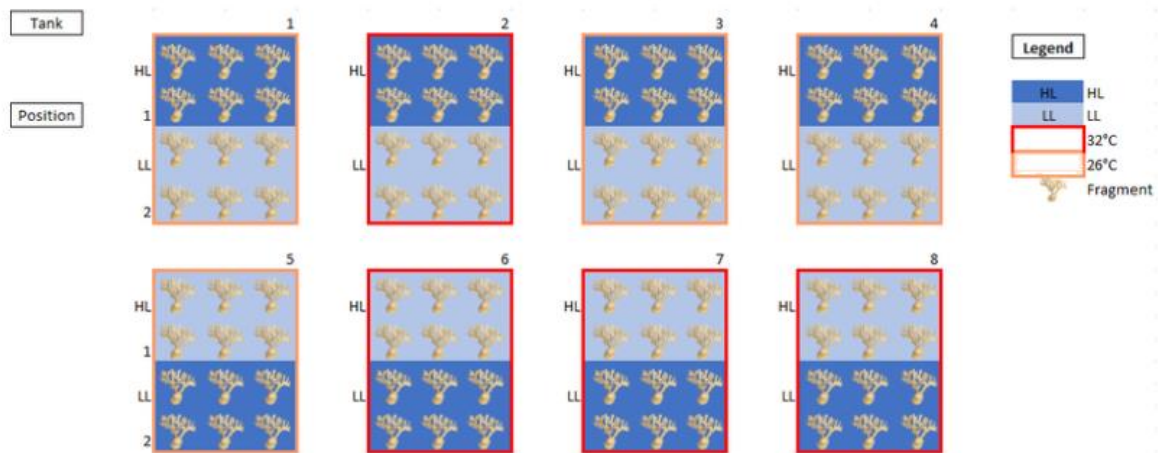
Figure 17 - Schematic representation of the experimental set up employed, with the random distribution of the 8 tanks across different temperature treatments and the placement of the 6 coral fragments on each light intensity zone (image by the author).

A total of 96 fragments were used in the experiment and were randomly distributed in the tanks. Twelve coral fragments were placed in each tank, 6 under each light intensity condition (Figure 17). Coral fragments were previously drip-acclimated to the water of the new tanks before being placed inside them. After this procedure, they were moved to the experimental tanks where they remained for a week to acclimate. According to McLachlan et al. (2020) the average number of days for coral acclimation prior to experimental procedures in heat-stress experiments is 5 days.

Coral fragments were placed in frag racks (two racks per tank, one in the LL zone and one in the HL zone) (Figure 17). Tanks were equipped with a trickle filter (EHEIM 2.5 W 380 l/h, Germany), a circulation pump (Turbelle nanostream 6015 3.5 W, Germany), a heater equipped with a thermostat (EHEIM 3616, 150 W, Germany) and a LED light (ViparSpectra, 165 W, China). All tanks were filled with filtrated (5 µm) and UV-irradiated natural seawater collected near the inlet of Ria de Aveiro coastal lagoon, with water quality parameters being kept at: 0 mg/l of ammonia, 0 mg/l of nitrites, 6.0 mg/l of

oxygen, salinity of 37, pH of 8.1 and temperature of 26 ˚C. Photoperiod was kept at 12L:12D.

Partial water changes (PWC) were performed using natural seawater previously heated to 26 ˚C, with salinity being corrected by tap water purified by a reverse osmosis system (Aqua-win RO-6080, Kaohsiung, Thailand). These changes were performed every day, so that about 1.5 L of water was used (approximately 2.5% PWC per day), giving a total of about 10.5 L per week (17% PWC weekly). This procedure allowed to replace a larger amount of water once a week, but in a more gradual way and, consequently, being less stressful for stocked organisms. Trickle filters and tanks were cleaned once a week.

Water temperature was monitored daily using a high precision thermometer (Tropic Marin, Germany), while salinity was also monitored daily using a seawater refractometer (HANNA, HI 96822). Additionally, pH and oxygen were also monitored using a WTW probe (ProfiLine pH, Cond 3320, Germany and Oxi 3310 SET1, Germany, respectively). Ammonia (NH_3), and nitrites (NO_2) were tested once a week using Profi Test (Salifert, Holland). The following values were recorded throughout the experiment: salinity (34.15 ± 1.05), pH (8.24 ± 0.14) ammonia (0.10 ± 0.06mg/l) and nitrites (0 mg/l).

The duration of the experiment was 72 days: 30 days for the "photoacclimation" phase, where fragments acclimated to the new conditions of light in the tanks, followed by a HW simulation with the duration of 10 days, in which temperature was increased from 26 to 32 ˚C at a rate of 1 ˚C h^{-1} (McLachlan et al., 2020). Following 10 days at 32 ˚C, temperature was reduced to match the control condition (26 ˚C) at a rate of 1 ˚C h^{-1}. A "recovery" phase lasting for 30 days at control temperature was then carried out to determine if corals that endure the HW would return to their initial state (see figure 18 for a schematic representation).
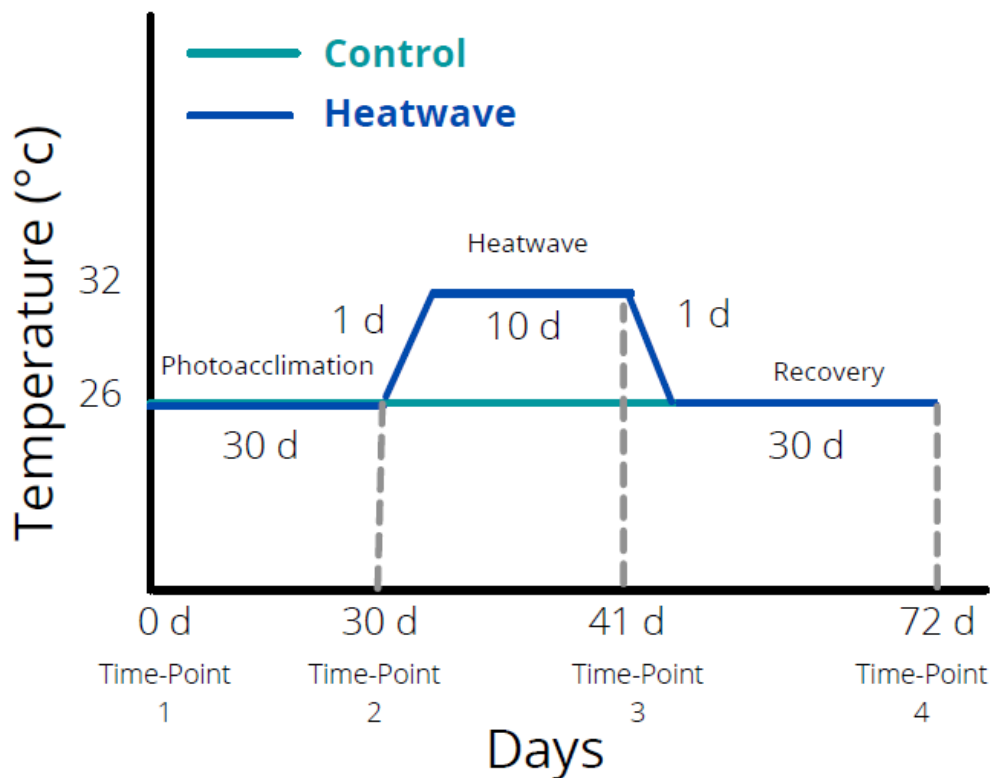
Figure 18 - Schematic representation of experimental Time-points/Sampling Times and duration (in days) of each experimental phase (Photoacclimation, HW and Recovery) concerning the two independent variables tested (Temperature and Light Intensity) for *Sarcophyton* cf. *glaucum* fragments (image by the author).

Coral fragments were not fed throughout the experiment, as an exogenous source of food may affect coral's physiological processes (Titlyanov et al., 2001), masking experimental results. Therefore, coral fragments subsisted on photosynthates provided by their photosynthetic endosymbionts.

2.2.1 Photoacclimation phase

This phase consisted of a period of 30 days when coral fragments acclimated to light conditions of each experimental tank. The duration of this period was similar to that described in previous studies employing corals. According to Anthony & Hoegh-Guldberg (2003), Kuguru et al. (2010) and Roth et al. (2010), photoacclimation of corals should not be lower than 30 days, although Lohr et al. (2019) states that 21 days are enough. As

referred above, light intensity was 663 µmol m^{-2} s^{-1} under HL intensity conditions and 253 µmol m^{-2} s^{-1} under the LL intensity conditions.

2.2.2 Heatwave phase

Heatwave simulation started after the photoacclimation phase, with temperature being raised from 26 °C, the average temperature for optimum growth in corals (optimum range between 23 °C to 29 °C) (NOAA, 2021), to 32 °C at a rate of about 1 °C per hour. This warming rate is commonly used in studies using shallow water/intertidal coral species of the Indo-Pacific (McLachlan et al., 2020). In fact, this heating rate was chosen for two reasons: (i) it has been the most used warming rate in heat-stress experiments in corals (mean ± SD: 1.2 ± 2.2 °C h^{-1}) (McLachlan et al., 2020); and (ii) temperature changes of 1 °C h^{-1} can be experienced by corals in the reef-flat zone (Willis & Berkelmans, 1999). Temperature was kept constant at 32 °C for 10 days, a typical HW period for tropical regions (HWs often span between 5 and 10 days) (Oliver et al., 2018). The temperature selected (32 °C) is known to occur in HW events in tropical seas (where temperatures can rise up to >34 °C) (MacKellar & McGowan, 2010). This temperature is also known to induce bleaching in *Sarcophyton* spp., as there are reports of bleached corals of this genera at water temperatures > 31 °C (Marshall & Baird, 2000).

2.2.3 Recovery phase

After the 10 days of the HW phase, a period of 30 days followed, with the temperature dropping back to values identical to those of control conditions (26 °C), also at a rate of 1 °C per hour (McLachlan et al., 2020). Although a period of 30 days may not be a precise indicative of recovery in corals, it has been previously used to test the recovery potential of western tropical Pacific reef building corals following warming events (Hueerkamp et al., 2001). Recovery times similar to the one used in the present experiment have also been applied to other coral species (38 days for *Acropora millepora* and 31 days for *Montipora tuberculosa*) (Fabricius et al., 2013).

2.3 Sampling

Samples from each treatment were collected at four Time-points of the experiment: in the first day of the photoacclimation phase, at the end of the photoacclimation phase (30 days after the beginning of the experiment), at the end of the HW period (41 days after the beginning of the experiment) and at the end of the recovery phase (72 days after the beginning of the experiment). All the sampled fragments were then used to measure physiological and molecular biomarkers.

In more detail, from each side of the different light intensities tested per tank, 1 fragment was randomly taken (one from the HL treatment and one from the LL treatment), so that at the end of each sampling time, a total of 16 fragments were collected. Each fragment was labeled by the number of the tank and its position on the coral rack (Figure 19) (TX_YZ, where X represents the number of the tank, Y the position of the fragment and Z the light treatment, so that T1_1HL means that the fragment belongs to tank 1, position 1 and the HL treatment).

Figure 19 - Scheme of the fragment's position in the coral racks placed on each tank (image by the author).

Sampled fragments were screened to assess photophysiological measures using PAM fluorometry and subsequently carefully placed in test cups, flash-frozen in liquid nitrogen and stored in an ultra-freezer at -80 °C until zooxanthellae quantification and molecular biomarker analyses.

Only living fragments at the time of sampling were considered for physiological and molecular analysis.

2.4 Photophysiological parameters

2.4.1 *in vivo* chlorophyll fluorescence

The photosynthetic activity of the endosymbionts of *S.* cf. *glaucum* was measured *in vivo* using PAM fluorometry (Figure 20) (Imaging PAM Fluorometer, Mini version, WALZ) (Schreiber et al., 1986), prior to the flash-freezing of the fragments (as refereed above). Yield of zooxanthellae's PSII given by $F_v/F_m = ((F_m - F_0)/F_m)$ (Serôdio et al., 2001), was calculated using the software ImagingWinGigE V2.56p and can be used as proxy to assess the photoinactivation of endosymbionts associated with thermally induced coral bleaching (as well as other stressors) (Warner et al., 1999).
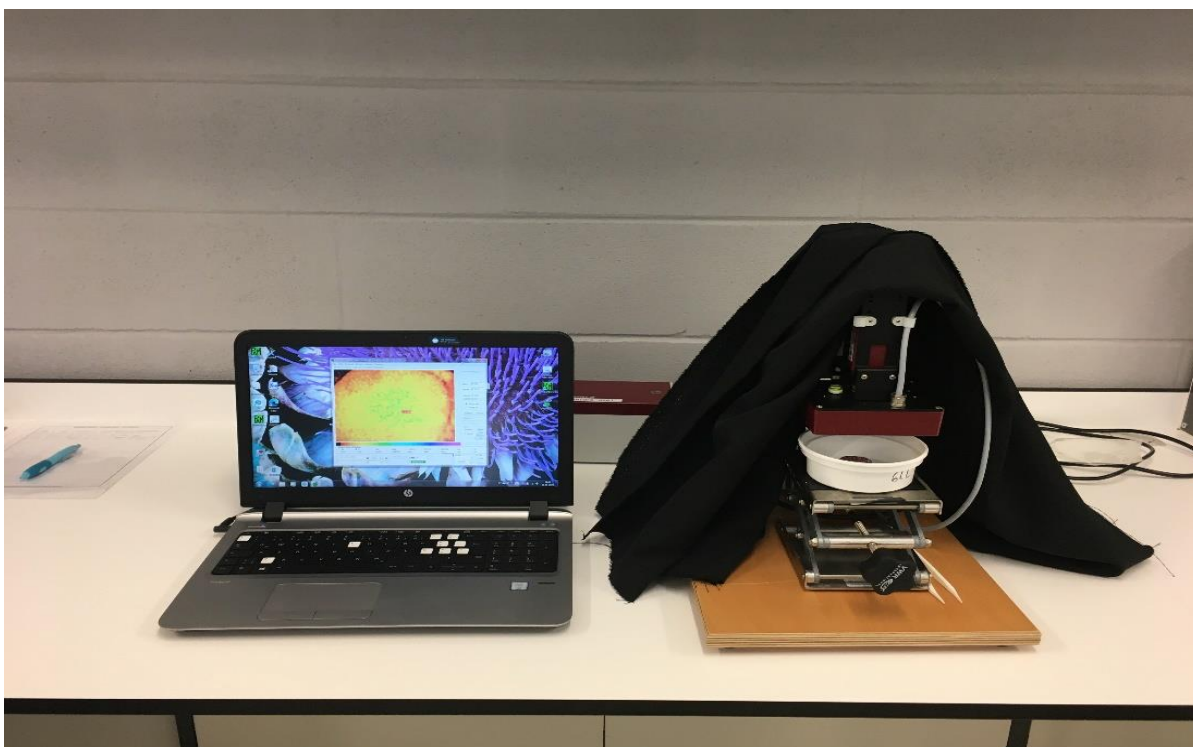
Figure 20 - Imaging PAM Fluorometer and software used to monitor photosynthetic parameters in fragments of *Sarcophyton* cf. *glaucum*.

Before PAM fluorometry analysis all coral samples were dark-adapted in a closed Styrofoam box (Figure 21). Coral fragments were held in plastic containers with water from their respective tank, being kept in a room with controlled temperature to avoid

shifts in water temperature. Dark adaptation was performed for a period of 15 min (in line with recommended periods described in the literature that range from 10-30 min (Warner et al., 2010)). It must be highlighted that dark adaptation for extended periods may give origin to artificially low F_v/F_m values. Dark adaptation prior to measurements ensures the full capacity of the photochemical apparatus to absorb light, which means that PSII reaction centers are fully open (Warner et al., 2010). Measurements always took place 2 h after the beginning of the daylight period to make sure that the photosynthetic apparatus was fully activated (Rocha et al., 2013b). One saturation pulse (0.8 s) was applied, to determine F_0 and F_m (Schreiber et al., 1986). Actinic and saturating lights were provided by a blue LED-lamp, with a measuring light of 1 μmol m^{-2} s^{-1}, while other parameters were kept as default. We measured three areas of interest (AOI) per coral fragment to obtain three F_0´s and F_m´s, which were then averaged to get a mean value per fragment. Subsequently, the maximum quantum yield of PSII was calculated in each coral fragment using the following equation:

$$(1)\ F_v/F_m = (F_m - F_0)/F_m$$

Figure 21 - Styrofoam box employed to perform dark-adaptation of fragments of *Sarcophyton* cf. *glaucum* before performing PAM fluorometry readings.

2.4.2 Quantification of zooxanthellae

A sample of coral tissue of approximately 150 mg (weighted in a high precision scale, (A&D Company Limited, FX-5000i, Japan)) was removed with a scalpel from each fragment and weighted to the nearest 0.001 g before determining the density of zooxanthellae. Coral tissue was homogenized using 2 mL of a phosphate buffered saline solution (140 mM NaCl, 3 mMKCL, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH= 7.4) and an OMNI Tissue Homogenizer (OMNI TH, United States). Zooxanthellae counting was performed manually under an optical microscope (Leica, DM2500, Germany) at 10x magnification, using a haemocytometer - Neubauer improved counting chamber (Figure 22) (HEINZ HERENZ HAMBURG, Germany) (2 replicates per coral fragment were analyzed, each with 4 cell counts). Endosymbiont concentration was normalized to wet weight and expressed as the number of zooxanthellae per gram of wet weight of coral tissue. From this point ahead, results regarding zooxanthellae quantification will be referred to as "Number of Endosymbionts", considered here as photosynthetic endosymbionts commonly termed as zooxanthellae.

a)

b)
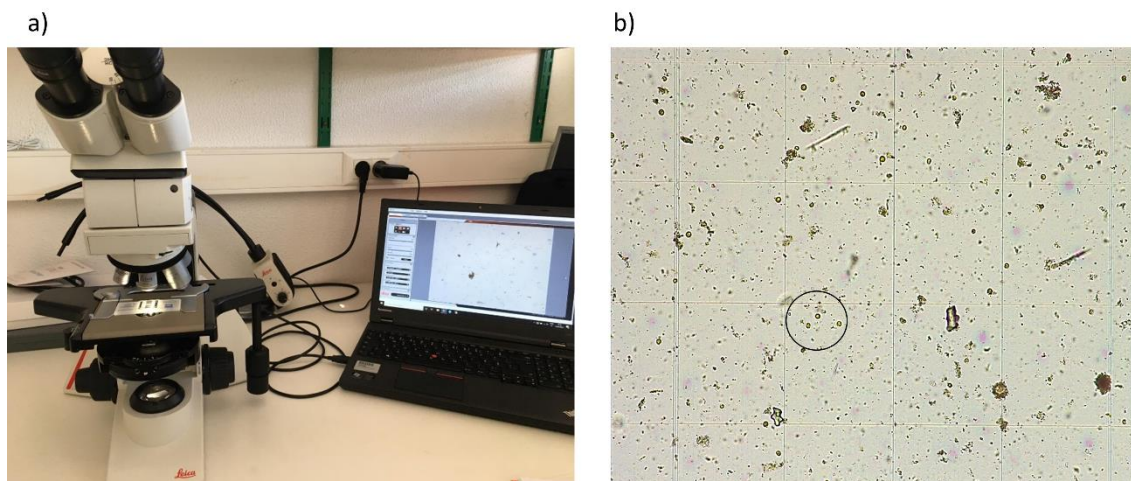
Figure 22 - a) Zooxanthellae quantification using a microscope; b) zooxanthellae in a Neubauer chamber (endosymbionts identified with a black circle).

2.5 Biomarkers

2.5.1 Protein extraction

The 64 coral samples previously collected and flash-frozen, were thawed and placed on ice. Then, with the help of a scalpel, 100 to 150 mg of tissue from each fragment were removed (weighted in a high precision scale (A&D Company Limited, FX-5000i, Japan)) crushed and then placed inside an Eppendorf tube (5.0 mL). After this procedure, samples were homogenized in 2 mL of a phosphate buffered saline solution (PBS) (140 mM NaCl, 3 mMKCL, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH= 7.4) and using an OMNI Tissue Homogenizer (OMNI TH, United States) for about 15 s at 5000 rpm.

2.5.2 Total protein quantification

Total protein quantification was carried out according to the Bradford method (Bradford, 1976) for 96-well microplates (Figure 23). A total of 20 µl of each coral sample (in duplicates) were added to each well of the microplate. Then, 180 µl of Bradford reagent (Supelco®, #B6916, Sigma-Aldrich, USA) were also added to each well. Absorbance was read at 595 nm using a Synergy™ HTX Multi-Mode Microplate BioTek® reader. Bovine Serum Albumin (BSA) (Sigma-Aldrich, USA) was used as standard (0 to 1 mg ml^{-1}) to build a seven-point calibration curve.

The total protein quantification was used to normalize biomarkers levels (Hsp70 kDa, Ubi, CAT, GST, SOD and TAC). Moreover, total protein content was also used as a biomarker of coral condition in the present study. For that, total protein content was normalized by the wet weight of each coral fragment.
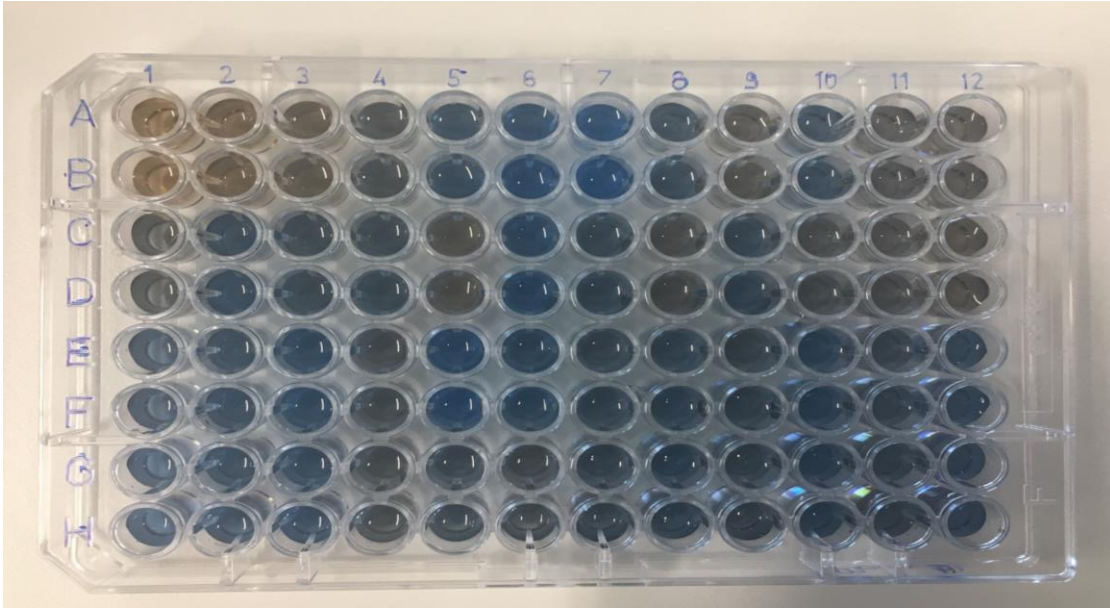
Figure 23 - Bradford Assay (Bradford, 1976) for a 96-well microplate.

2.5.3 Glutathione-s-transferase activity

The GST assay was performed according to the protocol described by Madeira et al. (2019), where duplicates of 40 µL were retrieved from each coral sample and placed in the microplate's wells. To each well it was added 160 µL of a reagent mix with 200 mM of reduced L-glutathione, and 100 mM CNDB and buffer Dulbecco (Sigma Aldrich, USA®). The absorbance was read at 340 nm every minute for 6 minutes, in the same microplate reader referred above. GST activity was calculated using a molar extinction coefficient of 0.00503 µM following the equations:

(2) GST Abs_{340}/min = (Abs_{340}final read – Abs_{340} initial read)/ reaction time (min)

(3) GST specific activity = (GST Abs_{340}/min / 0.00503 μM^{-1}) * (0.2 mL / 0.04 mL)

2.5.4 Catalase activity

The CAT assay was adapted from Madeira et al. (2019), in which duplicates of 40 µL were retrieved from coral samples and placed into the microplate's wells. Then, the

following reagents were added in the following order: 100 μL of assay buffer (100 mM potassium phosphate), 30 μL of methanol and 20 μL of 0.035 M H_2O_2. The microplates were incubated in a shaker at 80 rpm for 20 minutes. After this period, 30 μl of potassium hydroxide (10 M) and 30 μL of Purpald (34.2 mM in 0.5 M HCL) were added to each microplate well, with the microplate being incubated once again in the shaker at 80 rpm for 10 minutes. Then, 10 μL of Potassium periodate (65.2 mM in 0.5 M KOH) were added to each well and absorbance was read at 540 nm in the microplate reader mentioned above. A calibration curve was constructed using formaldehyde standards, ranging from 0 to 75 μM. Catalase activity was calculated considering that one unit of CAT is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C.

2.5.5 Superoxide dismutase inhibition

The SOD assay was performed following the protocol described by Madeira et al. (2019). Duplicates of 20 μL were retrieved from coral samples and placed in each microplate well, with 230 μL of a reagent mix containing EDTA (ethylenediaminetetraacetic acid) (3mM), Xanthine (3mM) and NBT (nitroblue tetrazolium chloride) (0.75 mM) being then added to each one of them. At the end of this procedure, 10 μL of Xanthine Oxidase (XOD) were added to the microplate wells to start the reaction. A negative control was also performed, only containing 20 μL of PBS and 230 μL of the reagent mix. The absorbance was read at 560 nm every 5 minutes for 20 minutes (including time zero) using the microplate reader mentioned above and SOD activity was calculated using the following equations for the % of inhibition:

(4) SOD Abs_{560}/min = Abs_{560} final read - Abs_{560} initial read) / reaction time (min)

(5) SOD % inhibition = ((Abs_{560}/min negative control - Abs_{560}/min sample) / (Abs_{560}/min negative control)) x 100

2.5.6 Lipid peroxidation

LPO assay was conducted following the thiobarbituric acid reactive substances method (TBARS) (Uchiyama and Mihara, 1978). Fifty µL of each coral sample were placed in a microtube, together with 12.5 µL of sodium dodecyl sulfate (SDS) 8.1%, 93.5 µL of trichloroacetic acid (TCA) 20% and 93.5 µL of thiobarbituric acid (TBA) 1%. Additionally, 50.5 µL of Milli-Q grade ultrapure water were also added to the mixture. Microtubes lids were punctured with a needle (to impair them from bursting) and were incubated in a block heater (PHMT - PSC24 (24 x 0.2 mL), UK) at 100 ˚C for 10 minutes. Subsequently, they were placed on ice for 5 minutes to cool and 62.5 µL of Milli-Q grade ultrapure water were added. Then, two portions of the microtube liquid (150 µL each) were placed into a 96-well microplate. Absorbance was read at 532 nm using the microplate reader mentioned above. To quantify lipid peroxides, an eight-point calibration curve, ranging from 0 to 0.3 µM, was constructed using malondialdehyde bis (dimethylacetal) (1uM) standards (Merk, Germany).

2.5.7 Hsp70 and total ubiquitin quantification

To quantify Hsp70 and Ubi, an enzyme linked immunosorbent assay (ELISA) was used (Madeira et al., 2014c). No dilution was performed for the quantification of Hsp 70, while for the quantification of Ubi a dilution of 1:2 was employed. First, 50 µL of each coral sample were placed in microplates wells (in duplicates) and incubated overnight at 4 °C. After this period, the microplates were washed three times in PBS 0.05% Tween-20 and blocked through the addition of 200 µL of 1% BSA in PBS. Another incubation period followed, this time at 37 °C for 90 minutes.

After washing, the primary antibodies (mouse monoclonal Hsp70/Hsc70, # TA326357, OriGene, USA for Hsp70 quantification and mouse monoclonal Ubi-1, #ab7254, Abcam, UK for Ubi quantification) were diluted to 2 µg mL^{-1} and 1.5 µg mL^{-1}, respectively, in 1% BSA in PBS. They were then added to the microplate's wells (50 µL in each well), which were subsequently incubated for 90 minutes at 37 °C. After this procedure, another washing process was performed for both microplates and a

secondary antibody (50 µL) was added to each well: anti-mouse IgG conjugated to alkaline phosphatase, Fc specific (#A1418 Sigma-Aldrich, USA) diluted to 2 µg mL^{-1} (Hsp70) and anti-mouse IgG conjugated to alkaline phosphatase, Fab specific (#A1293 Sigma-Aldrich, USA) diluted to 2 µg mL^{-1} (Ubi) both in 1% BSA in PBS. Another incubation period was performed at 37 °C for 90 minutes and another washing process followed. Then, 100 µL of substrate (made using SIGMA FAST^{TM} p-Nitrophenyl Phosphate Tablets for the total volume of 20 mL) was added to each microplate well and incubated for 20 minutes at 37 °C. After this step, absorbance was read in the 96-well microplate reader mentioned above at 405 nm.

Calibration curves were constructed, within the 0 to 1 µg mL^{-1} range, using serial dilutions of purified Hsp70 active protein (#AR03018PU-N, OriGene, USA) and purified Ubi (UbpBio, E-1100, USA).

2.5.8 Total antioxidant capacity

TAC was determined using duplicates of 20 µl from each coral sample and placing them in the microplates' wells. A total of 10 µl of myoglobin 90µM and 150 µl of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) 600µM were added to each well. To start the reaction, 40 µl of H_2O_2 500 µM (0.0017%) were added to each well. After a 5-minute incubation at room temperature, absorbance was read in the 96-well microplate reader mentioned above at 410 nm. Calibration curves were carried out using Trolox standards (0 to 0.330 mM) (Kambayashi et al., 2009).

2.6 Data analyses
2.6.1 Photophysiological and molecular parameters

Summary statistics (mean, standard deviation, coefficients of variation) were calculated for all response variables in each treatment at each time point sampled using Excel (Microsoft 365).

To unravel data structure and detect which photophysiological and molecular biomarkers contributed to explain the variance recorded in the dataset, a Principal Component Analysis (PCA) was performed at each time point sampled using PRIMER-E v6, following a data normalization to rescale different response variables into a common measurement scale. Then, a resemblance matrix among samples was assembled using Euclidean distances. Following the PCAs, a permutational analysis of variance (PERMANOVA) was carried out at each Time-point to test the overall effect of light intensity (Time-point 2: photoacclimation phase), temperature and their interaction (Time-point 3 and 4: HW and recovery phases) on overall photophysiological response variables and CSR and oxidative damage (i.e., molecular biomarkers). No PERMANOVA was performed for Time-Point 1, as this time-point was the initial control. To perform the PERMANOVAs, the Euclidean distance matrix of both photophysiological and molecular biomarkers, at each time point, was analyzed through PERMANOVA + add-on using PRIMER-E v.6, following a unrestricted permutation of raw data (9999 permutations) when there was only one factor involved (Time-point 2: photoacclimation, involving light intensity) and a permutation of residuals under a reduced model (9999 permutations) when there was more than one factor (Time-point 3 and 4, HW and recovery phases, involving temperature and light intensity); this last procedure is more indicated for multi-factorial designs (Legendre & Andersson, 1999; Braak & Anderson, 2003) . Additionally, pair-wise tests were also performed to detect differences between treatment groups. To identify which specific response variables were significantly affected by the tested stress factors, a PERMANOVA was performed on each response variable separately. According to Anderson (2017b), a PERMANOVA carried out on one response variable using Euclidean distance yields the classical univariate F statistic, avoiding the assumption of data normality. Again, pair-wise tests were performed for the response variables significantly affected by the stress factors being tested in the present study.

Moreover, a clustered heatmap was assembled using MetaboAnalyst (v5.0) to facilitate the visualization of response patterns of each photophysiological and molecular parameter in each Time-point along the different experimental treatments. Prior to this analysis, the data matrix was normalized by auto-scaling. The metrics used in the cluster

analysis were as follows: (i) distance measure: Euclidean distance, and (ii) clustering algorithm: Ward's linkage. The significance level considered for all the analyses was 0.05.

Furthermore, percentages of increase/decrease were calculated in Excel (Microsoft 365) for photophysiological and molecular   parameters to better understand the level of change occurring between experimental treatments.

In the original data set, there were 6 values which were considered Missing data (negative). These values were replaced by an average value of the respective treatment.

3. Results

3.1 Photophysiological and molecular parameters analyses

Overall, *Sarcophyton* cf. *glaucum* showed physiological and molecular changes when exposed to the different combinations of temperature and light intensity tested in the present experiment.

Summary statistics concerning all Time-points sampled and different experimental treatments are detailed in supplementary tables S1, S2, S3 and S4, describing means, standard deviations, and coefficients of variation.

3.1.1 Principal component analyses (PCA)

PCA was performed independently for all Time-points sampled. The highest cumulative explained variance for the first two components was found in Time-point 4 (recovery phase, 68.3%: 47.5% for PC1 and 20.9% for PC2), followed by Time-point 3 (HW phase, 66.6%: 51.7% for PC1 and 14.9% for PC2). The lowest value was found for Time-point 2 (photoacclimation phase, 56.4%: 37.5% for PC1 and 14.9% for PC2).

Overall, a differentiation between temperature and light intensity groups was detected, especially at Time-point 3 (HW phase) and 4 (recovery phase), although physiological and molecular profile differences are more visible for temperature groups (Figure 25 b and d). At Time-point 2 (photoacclimation phase) there is a slight separation of light intensities and at Time-point 1 no groups can be detected, as this was the control Time-point (beginning of the experiment, no stress factors applied) (Figure 24 a and b). A few outliers within the dataset were also evidenced by the PCAs.

Overall, there is a change in the type of correlation (positive or negative) between response variables and PCs between Time-point 1 and the rest: the parameters that correlate to PC1 positively, become negatively correlated to PC1 in Time-point 2, 3 and 4. The same happens for PC2. No parameter was highly negative or positive correlated to either of the axes in all time points sampled (Pearson r > $|0,7|$). In fact, the highest correlation found was a negative one in PC2 of Time-point 4 belonging to the biomarker SOD (Pearson r = - 0.555), followed by CAT at Time-point 1 in PC2 (Pearson r = - 0.0552), Ubi at Time-point 4 in PC2 (Pearson r = - 0.537), F_0 at Time-point 3 in PC2 (Pearson r = -

0.535) and Ubi at Time-point 2 also in PC2 (Pearson r = - 0.520). There is a group that seems to be formed by one of the biomarkers and two parameters related to the organisms' photophysiological performance: Total Protein, F_v/F_m and F_0, respectively. This group of response variables is always negatively correlated to PC1 after Time-point 1, indicating a decrease in these parameters in corals exposed to elevated temperature, as temperature groups separate along PC1 axis (Figure 25 b and d). Another set of response variables, this time always positively correlated to PC1 especially in Time-points 3 and 4, is composed by the molecular parameters GST, LPO, SOD, Ubi, HSP70 and TAC, indicating that HW-exposed corals have higher abundance of these biomarkers (Figure 25 b and d).
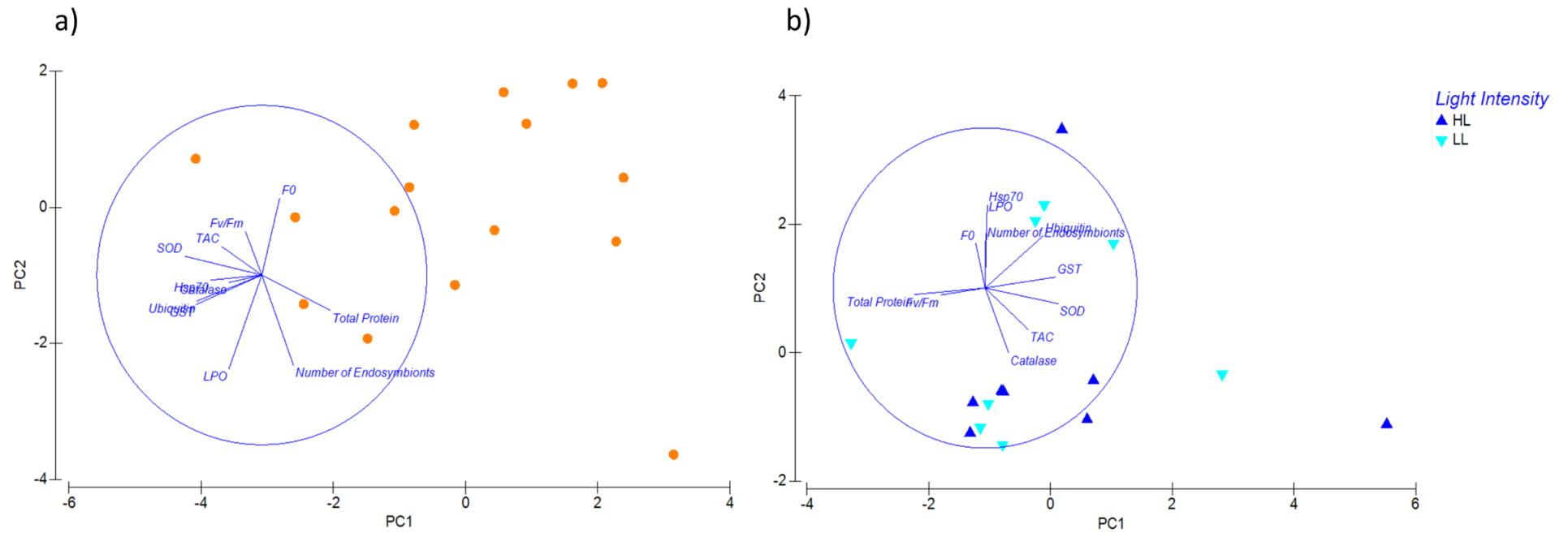
Figure 24 - Principal Components Analysis performed for all markers (including photophysiological and molecular) monitored to survey the response to stress of the soft coral *Sarcophyton* cf. *glaucum*. (a) Time-point 1 (control at the beginning of the experiment) before stress was imposed and (b) Time-point 2 (photoacclimation phase) when corals were subjected to two levels of Light Intensity (HL: 663 µmol m^{-2} s^{-1} and LL: 253 µmol m^{-2} s^{-1}) for 30 days. Markers: Hsp70 – Heat Shock Protein 70, Ubi – Total Ubiquitin, SOD – Superoxide dismutase, GST – Glutathione-s-transferase, CAT – Catalase, TAC – Total antioxidant capacity, LPO – Lipid Peroxidation, F_v/F_m – maximum quantum yield of photosystem II, F_0 - minimum fluorescence, used as a proxy of Cl *a* content.
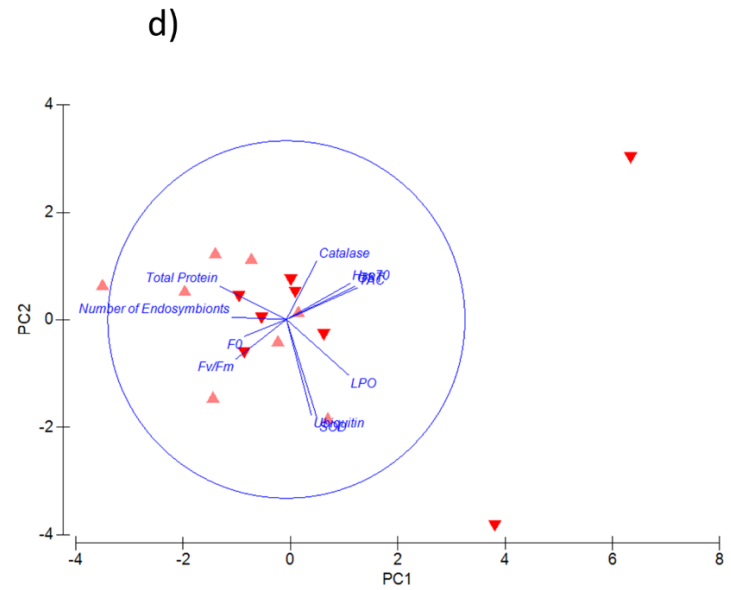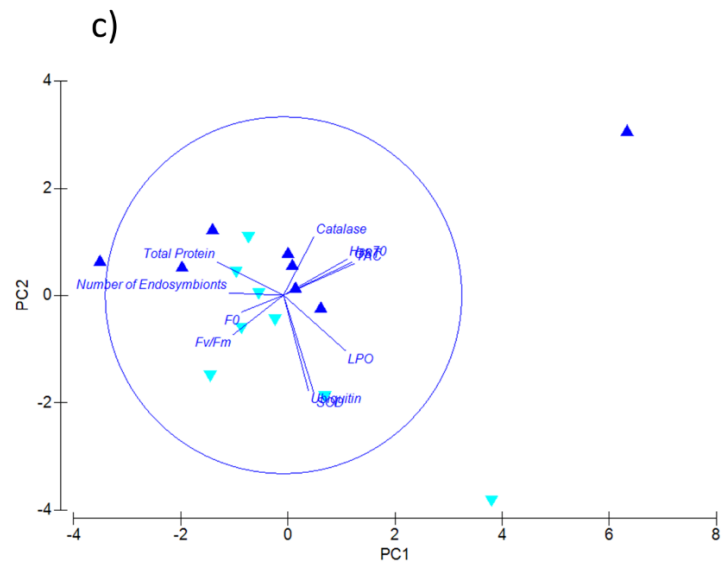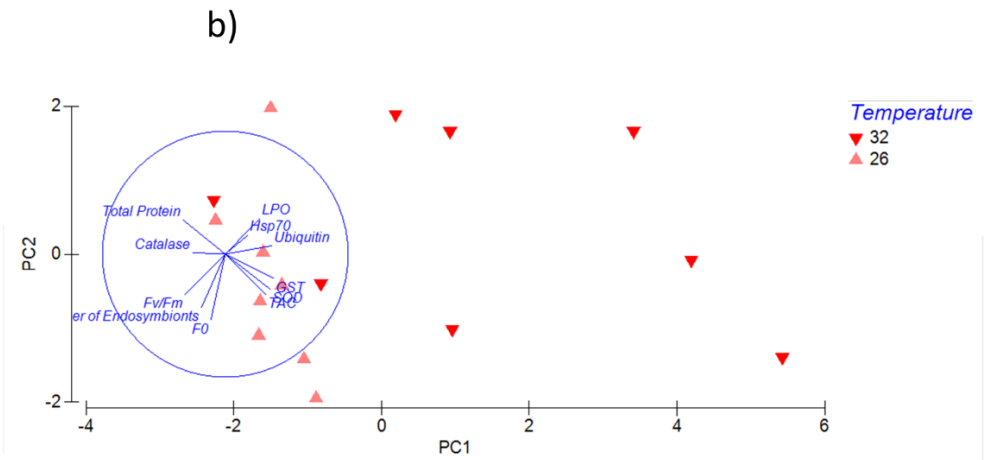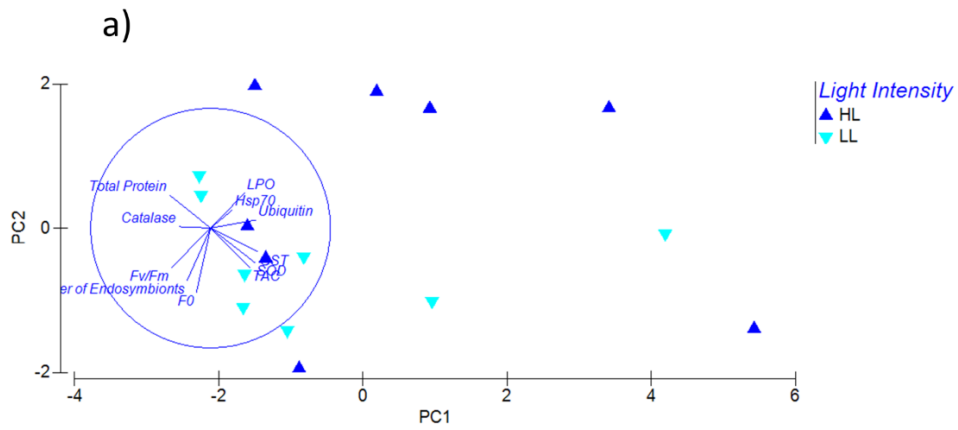
Figure 25 - Principal Components Analysis performed for all markers (including photophysiological and molecular) monitored to survey the response to stress (temperature and light) of the soft coral *Sarcophyton* cf. *glaucum*. (a, b) Time-point 3 (HW phase), when corals where subjected to 32 ˚C for 10 days under different light intensities (HL: 663 µmol m^{-2} s^{-1} and LL: 253 µmol m^{-2} s^{-1}), with (a) representing group separation according to light intensity and (b) representing group separation according to temperature; (c, d) Time-point 4 (recovery phase) in which corals were returned to control temperature for 30 days, under the different light intensities, with (c) representing group separation according to light intensity and (d) representing group separation according to temperature (i.e. previous exposure to HW conditions). Markers: Hsp70 – Heat Shock Protein 70, Ubi – Total Ubiquitin, SOD – Superoxide dismutase, GST – Glutathione-s-transferase, CAT – Catalase, TAC – Total antioxidant capacity, LPO – Lipid Peroxidation, F_v/F_m – maximum quantum yield of photosystem II, F_0 - minimum fluorescence, used as a proxy of Cl *a* content.

3.1.2 Photophysiological parameters: permutational analysis of variance (PERMANOVA)

Main results from PERMANOVA performed at the photoacclimation phase (Time-point 2), HW phase (Time-point 3) and recovery phase (Time-point 4) for the photophysiological parameters studied (F_v/F_m, F_0 and number of endosymbionts) showed a significant effect of the factor light intensity in Time-point 2 over these parameters (p = 0.0002, Table 1). At Time-point 3, temperature and light intensity had a significant effect over the same parameters (p = 0.0009 and p = 0.0007, respectively, Table 1). Photophysiological parameters at Time-point 4 only seemed to be affected by temperature (p = 0.0054, Table 1). Additionally, no significant interaction was found between both factors (Light Intensity x Temperature) at any of the Time-points sampled.

Still, at Time-point 3, pairwise tests (Table 2) revealed significant differences between light intensities within temperature 32 ˚C (p = 0.028, Table 2) but not at control temperature (26 ˚C, p=0.054). However, corals exposed to different temperatures (26 vs 32 ˚C) showed differences in their photophysiology, regardless of light intensity (26 vs 32 ˚C under HL: p = 0.0285 and 26 vs 32 ˚C under LL: p = 0.0255, Table 2). At Time-point 4, the differences between temperature 26 and 32 ˚C were only detected in HL intensity (p = 0.030).

Table 1 - Main results of PERMANOVA performed to analyse the effect of stress factors imposed at each Time-point (2 – photoacclimation, 3 – HW and 4 – recovery phases) on photophysiological parameters (F_v/F_m, F_0 and number of endosymbionts) regarding *Sarcophyton* cf. *glaucum*. Significant effects over the markers are represented in bold. Factors: L – Light Intensity, T – Temperature, L x T – Interaction between the two factors.

	Time Point	Factors	Markers	df	SS	MS	Pseudo-F	P (perm)	UP
						Values			
Physiological Parameters	2	L	Fv/Fm	1	5.4177	5.4177	7.9153	0.0123	5056
			F0	1	8.32	8.32	17.437	0.002	2355
	3	L	Fv/Fm	1	4.4512	4.4512	44.022	0.0003	9817
			F0	1	6.0171	6.0171	9.4767	0.0101	9832
		T	Fv/Fm	1	8.6854	8.6854	85.899	0.0004	9780
			Symbionts	1	6.2823	6.2823	9.2239	0.0088	9839
		L xT	Fv/Fm	1	0.65013	0.65013	6.4299	0.0285	9818
	4	L	Fv/Fm	1	4.0289	4.0289	11.875	0.0049	9829
		T	Fv/Fm	1	6.3683	6.3683	18.769	0.0019	9844
			Symbionts	1	4.6763	4.6763	5.6421	0.0314	9838

Table 2 - Results of the Pair-wise tests performed for the stressors (Temperature and Light Intensity) that significantly affected photophysiological parameters of *Sarcophyton* cf. *glaucum* during the present study at Time-points 3 (HW) and 4 (recovery). Significant effects are represented in bold

	Time-Point	Levels of Factor	Within levels of factor	Groups	t	P (perm)	UP
Physiological Parametrs	A3	Light	**Temperature 32**	**HL vs LL**	**2.9803**	**0.028**	**35**
			Temperature 26	HL vs LL	1.4946	0.0549	35
		Temperature	**Light Intensity HL**	**32 vs 26**	**2.6248**	**0.0285**	**35**
			Light Intensity LL	**32 vs 26**	**2.202**	**0.0255**	**35**
	A4	Temperature	**Light Intensity HL**	**32 vs 26**	**2.6376**	**0.0301**	**35**
			Light Intensity LL	32 vs 26	1.3052	0.205	35

To detect which specific photophysiological parameters varied according to treatment, individual PERMANOVAs were performed separately for each photophysiological response variable, helping to discern which ones contributed to the effects recorded in the main PERMANOVA (Table 3).

At Time-point 2, light intensity had a significant effect over F_v/F_m (p = 0.0123) and F_0 (p = 0.002) (Table 3). In more detail, both parameters decreased when corals were exposed to HL, with F_v/F_m decreasing 17% and F_0 declining 39% from LL to HL (see Figure 26). At Time-point 3, F_v/F_m was affected by light intensity (p = 0.0003), temperature (p = 0.0004) and the combination of both factors (p = 0.0285) (Table 3). F_v/F_m decreased an average of 43% from LL to HL-exposed corals and an average of 55% from 26 to 32 ˚C-exposed corals. According to the interaction detected between light and temperature, the percentage decrease of F_v/F_m was 36% from 26 to 32° C when corals were exposed to LL conditions and 80% from 26 to 32° C when corals were exposed to HL conditions (see heatmap Figure 27 and pairwise tests Table 4). At this same Time-point (3), F_0 was only affected by light intensity (p = 0.0101) and the number of endosymbionts affected by temperature (p = 0.0088). In more detail, F_0 declined 40% from LL to HL and the number of endosymbionts decreased 58% from 26 to 32° C. At Time-point 4, F_v/F_m was still the only parameter that responded to both stressors (p = 0.0019 for temperature and p = 0.0049 for light intensity). The number of endosymbionts was also affected by temperature (p = 0.0314) (Table 3). At this Time-point, F_v/F_m declined 26% from LL to HL and 32% from 26 to 32° C. In the case of number of endosymbionts, a decrease of 64% was observed from 26 to 32° C-exposed corals (see heatmap Figure 27).

Table 3 - Main results of PERMANOVA performed to analyze the effect of stress factors imposed at each Time-point (2 – photoacclimation, 3 – HW and 4 – recovery phases) on photophysiological parameters (F_v/F_m, F_0 and number of endosymbionts) regarding *Sarcophyton* cf. *glaucum*, to detect which ones had the highest contribution to the results found in the main PERMANOVA. Only significant results are presented in the table. Factors: L – Light Intensity, T – Temperature, L x T – Interaction between the two factors.

	Time Point	Factors	Markers	Values					
				df	SS	MS	Pseudo-F	P (perm)	UP
Physiological Parameters	2	L	Fv/Fm	1	5.4177	5.4177	7.9153	0.0123	5056
			F0	1	8.32	8.32	17.437	0.002	2355
	3	L	Fv/Fm	1	4.4512	4.4512	44.022	0.0003	9817
			F0	1	6.0171	6.0171	9.4767	0.0101	9832
		T	Fv/Fm	1	8.6854	8.6854	85.899	0.0004	9780
			Symbionts	1	6.2823	6.2823	9.2239	0.0088	9839
		L x T	Fv/Fm	1	0.65013	0.65013	6.4299	0.0285	9818
	4	L	Fv/Fm	1	4.0289	4.0289	11.875	0.0049	9829
		T	Fv/Fm	1	6.3683	6.3683	18.769	0.0019	9844
			Symbionts	1	4.6763	4.6763	5.6421	0.0314	9838

Table 4 - Results of the Pair-wise tests performed for the physiological parameter F_v/F_m of *Sarcophyton* cf. *glaucum*, to detect at which level of the interaction L x T at Time-point 3 (HW), a significant result can be found. Significant effects are represented in bold. Factors: L – Light Intensity, T – Temperature, L x T – Interaction between the two factors.

	Time-Point	Marker	Levels of Factor	Within levels of factor	Groups	t	P (perm)	UP
Physiological Parameters	A3	Fv/Fm	T x L	26	HL x LL	4.3577	0.0271	35
				32	HL x LL	5.1959	0.0288	35
				HL	26 x 32	8.4386	0.0312	35
				LL	26 x 32	4.7098	0.0313	35

3.1.3 Molecular parameters: permutational analysis of variance (PERMANOVA)

Concerning the analysis of molecular parameters, the effect of stress factors was less pronounced than photophysiological ones. Temperature was the only stressor to significantly affect the biomarkers surveyed during the present study (p = 0.0412), solely at Time-point 3 (HW phase) (see PERMANOVA Table 5).

Pairwise tests, however, did not show significant differences between temperature groups (Table 6), although the comparison between 26 vs 32 ˚C within HL conditions was borderline non-significant (p = 0.057).

Table 5 - Main results of PERMANOVA performed to analyse the effect of stress factors imposed at each Time-point (2 – photoacclimation, 3 – HW and 4 – recovery phases) on molecular parameters (Total Protein, Hsp70, Ubi, SOD, GST, CAT, TAC and LPO) regarding *Sarcophyton* cf. *glaucum*. Significant effects over the markers are represented in bold. Factors: L – Light Intensity, T – Temperature, L x T – Interaction between the two factors.

	Time Point	Factors	Values					
			df	SS	MS	Pseudo-F	P (perm)	UP
Molecular Parameters	2	L	1	3.5274	3.5274	0.424	0.8544	5080
	3	L	1	4.4085	4.4085	0.61671	0.6399	9938
		T	**1**	**24.167**	**24.167**	**3.3807**	**0.0412**	**9935**
		L x T	1	5.6437	5.6437	0.7895	0.5295	9936
	4	L	1	10.279	10.279	1.2989	0.2651	9932
		T	1	8.5122	8.5122	1.0756	0.3951	9943
		L x T	1	6.2453	6.2453	0.78918	0.5344	9923

Table 6 - Results of the Pair-wise tests performed for the stressor Temperature that significantly affected molecular parameters of *Sarcophyton* cf. *glaucum* during the present study at Time-points 3 (HW). No significant results were detected.

	Time-Point	Levels of Factor	Within levels of factor	Groups	t	P (perm)	UP
Molecular Parameters	A3	Temperature	Light Intensity HL	32 vs 26	1.8244	0.0575	35
			Light Intensity LL	32 vs 26	1.0737	0.4019	35

As PERMANOVA detected a main effect for temperature over the multivariate dataset of molecular parameters, individual PERMANOVAs were also performed for each specific biomarker, to identify which ones were indeed affected by environmental changes. Molecular parameters were less affected by the stressors tested in this study than photophysiological parameters. Catalase was the only variable affected by light intensity (p = 0.0302, average decrease of 42% between LL and HL) and temperature x light intensity (p = 0.0196) at Time-point 3 (Table 7). Regarding this interaction, the response of CAT to temperature was dependent on light intensity, with an opposite pattern being observed between light intensities. In particular, a 41% increase in CAT was observed between 26 and 32 °C in LL and a decrease of 68% was detected between 26 and 32 °C in HL (see heatmap Figure 27 and pairwise tests Table 8). Moreover, the response of CAT to light intensity was also dependent on temperature, as significant differences between light intensities were only found at temperature 32 °C, with CAT showing a decrease of 76% between LL and HL (see heatmap Figure 27 and pairwise tests Table 8). In addition, at Time-point 3, Ubi (p = 0.0392), SOD (p = 0.0469) and GST (p = 0.0255) were significantly affected by temperature (Table 7). In more detail, SOD increased 45% from 26 to 32°C, GST increased 216% between the same treatments and Ubi 135% in the same conditions. At Time-point 4, only SOD suffered a change when corals were exposed to the stressors being studied, in this case light intensity (p = 0.0406) (Table 7), with this biomarker showing a decline of 48% between LL and HL conditions (Figure 27).

Table 7 - Main results of PERMANOVA performed to analyze the effect of stress factors imposed at each Time-point (2 – photoacclimation, 3 – HW and 4 – recovery phases) on molecular parameters (Total Protein, Hsp70, Ubi, SOD, GST, CAT, TAC and LPO) regarding *Sarcophyton* cf. *glaucum*, to detect which ones had the highest contribution to the results found in the main PERMANOVA. Only significant results are presented in the table. Factors: L – Light Intensity, T – Temperature, L x T – Interaction between the two factors.

	Time Point	Factors	Markers	Values					
				df	SS	MS	Pseudo-F	P (perm)	UP
Molecular Parameters	3	L	Catalase	1	3.4313	5.4313	5.8224	0.0302	9836
		T	Ubiquitin	1	4.1044	4.1044	4.7835	0.0392	9826
			SOD	1	4.1539	4.1539	4.6934	0.0469	9815
			GST	1	4.7292	4.7292	5.8272	0.0255	9862
		L x T	Catalase	1	4.1927	4.1927	7.1144	0.0196	9836
	4	L	SOD	1	4.119	4.119	4.74	0.0406	9846

Table 8 - Results of the Pair-wise tests performed on the molecular parameter CAT of *Sarcophyton* cf. *glaucum*, to detect at which level of the interaction L x T at Time-point 3 (HW), a significant result can be found. Significant effects are represented in bold. Factors: L – Light Intensity, T – Temperature, L x T – Interaction between the two factors.

	Time-Point	Marker	Levels of Factor	Within levels of factor	Groups	t	P (perm)	UP
Molecular Parameters	A3	Catalase	T x L	26	HL x LL	0.21773	0.8589	35
				32	HL x LL	3.1291	0.031	35
				HL	26 x 32	4. 1122	0.0309	35
				LL	26 x 32	1.0692	0.333	35

3.1.4 Heatmaps

To better understand all photophysiological and molecular changes that occurred in *Sarcophyton* cf. *glaucum* under different treatments and along the Time-points surveyed, clustered heatmaps were performed for each Time-point (photoacclimation, HW and recovery phase) (Figure 26 and 27). By analyzing the heatmap regarding Time-point 2 (Figure 26), there seems to be some variability in responses across coral fragments. Still, photophysiological parameters such as F_0 and F_v/F_m tend to be higher in LL conditions (red color). In the heatmap performed for Time-point 3 (Figure 27 a) a pattern was detected. Overall, the lowest scores for the photophysiological parameters occurred at 32 °C in HL and the highest scores for the molecular parameters occurred in the same conditions. At Time-point 4 (Figure 27 b), most biomarkers seemed to have returned to their control values, even though photophysiological parameters values remain very low compared to control conditions.
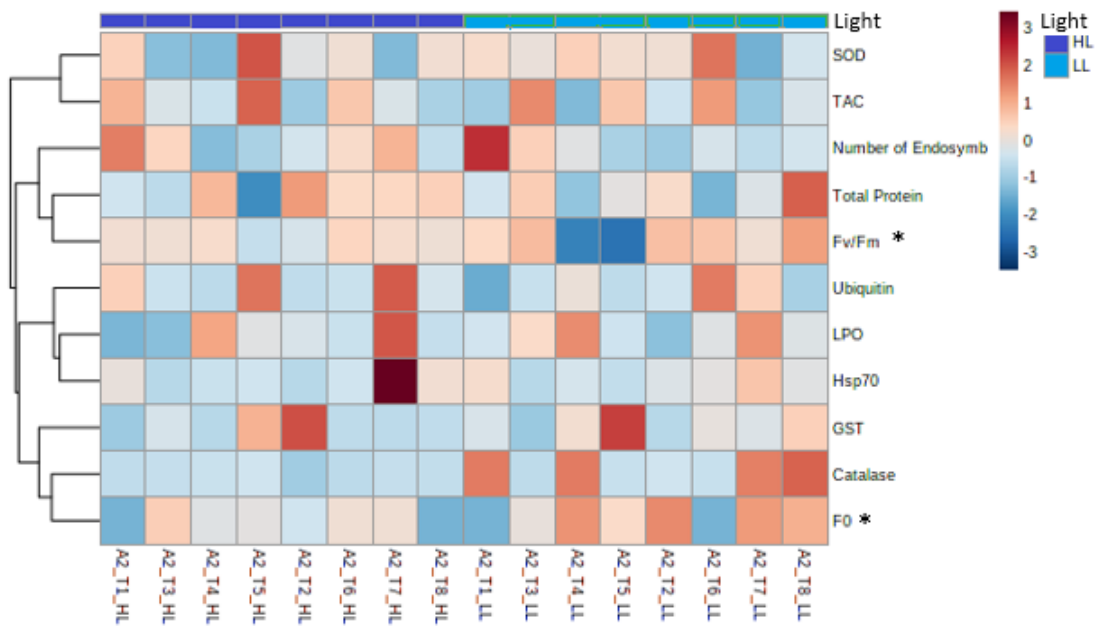
Figure 26 - Clustered heatmap representing the photophysiological and molecular profiles of the soft coral *Sarcophyton* cf. *glaucum* at Time-point 2, after 30 days of photoacclimation to different light intensity treatments (HL: 663 µmol m^{-2} s^{-1} and LL: 253 µmol m^{-2} s^{-1}). Red colors correspond to the highest scores and blue ones represent the lowest. Markers for which significant effects were detected in PERMANOVAs are marked with an asterisk. Markers: Hsp70 – Heat Shock Protein 70, Ubi – Total Ubiquitin, SOD – Superoxide dismutase, GST – Glutathione-s-transferase, CAT – Catalase, TAC – Total antioxidant capacity, LPO – Lipid Peroxidation, F_v/F_m – maximum quantum yield of photosystem II, F_0 - minimum fluorescence, used as a proxy of Cl *a* content.
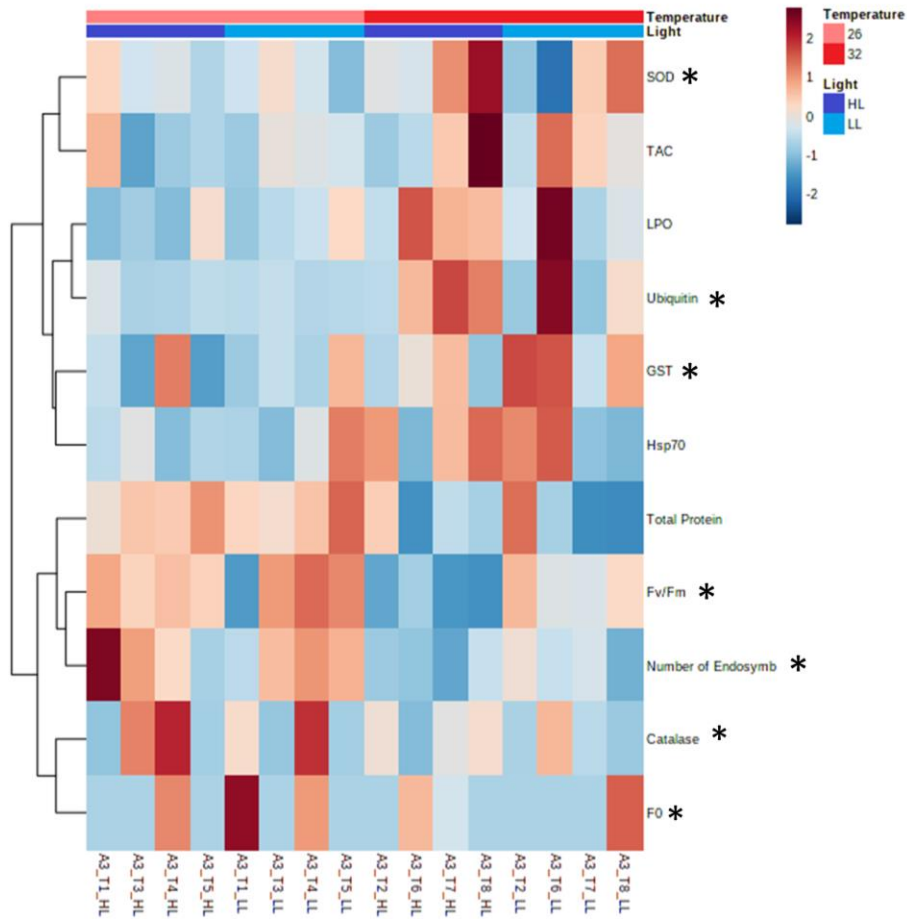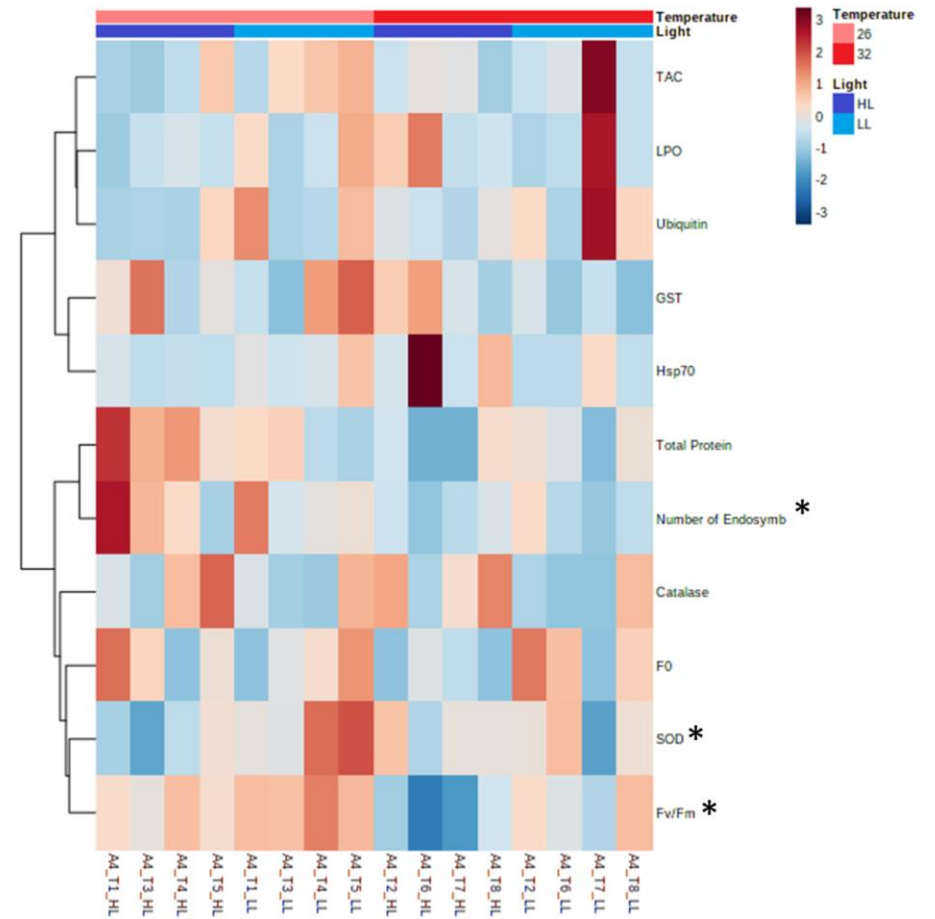
Figure 27 - Clustered heatmaps representing the photophysiological and molecular profiles of the soft coral *Sarcophyton* cf. *glaucum* at (a) Time-point 3, when corals were subjected to a 10 day-HW simulation (32 ˚C vs control 26 ˚C) under different light intensities (HL: 663 µmol m^{-2} s^{-1} and LL: 253 µmol m^{-2} s^{-1}) and (b) Time-point 4, when corals previously exposed to 32 ˚C (labelled here as 32) were allowed to recover for 30 days at control temperature, under the different light intensities tested. Red colors correspond to the highest scores and blue ones represent the lowest. Markers for which significant effects were detected in PERMANOVAs are marked with an asterisk. Markers: Hsp70 – Heat Shock Protein 70, Ubi – Total Ubiquitin, SOD – Superoxide dismutase, GST – Glutathione-S-transferase, CAT – Catalase, TAC – Total antioxidant capacity, LPO – Lipid Peroxidation, F_v/F_m – maximum quantum yield of photosystem II, F_0 - minimum fluorescence, used as a proxy of Cl *a* content.

4. Discussion

4.1 Photophysiological parameters: combined influence of temperature and light intensity

The photophysiological plasticity of the soft coral *S.* cf. *glaucum* was evaluated through the estimation of photobiology-related parameters, namely maximum quantum yield of PSII (F_v/F_m), a measure of photosynthetic activity, dark-level fluorescence (F_0), as a proxy of Cl *a* content and the number of endosymbionts on each coral fragment surveyed at different Time-points.

As expected, these photophysiological parameters varied with temperature and light intensity, as previously observed for *S.* cf. *glaucum* (e.g. Farag et al., 2021; Rocha et al., 2013a) and other soft coral species (Khalesi et al., 2009; Rocha et al., 2013b), as well as several stony corals (Gardner et al., 2017a; Hoadley et al., 2019; Jones et al., 1998; Wicks et al., 2010). Specifically, F_v/F_m decreased when coral fragments were exposed to high temperature and high light intensity in all Time-points, comparing to control conditions. Also, this was the only photophysiological variable for which an interaction between temperature and light was detected at Time-point 3 (HW phase under different light conditions). The number of endosymbionts, however, was not affected by light in any of the treatments, only by temperature, decreasing in heat-stressed corals. The contrary occurred to F_0, which was not affected by temperature, only by light intensity, decreasing under HL intensity. These results suggest that photosynthetic efficiency of soft corals especially decreases under a combination of heat and HL stress.

The impairment of photosynthesis by Simbiodiniaceae is the most likely reason for the occurrence of the first steps of bleaching (Karim et al., 2015a). In fact, bleaching can result from two concomitant processes, namely the loss of zooxanthellae from the coral host and the loss of pigments from the remaining zooxanthellae (Brown, 1997a; Kleppel et al., 1989). Several mechanisms have been proposed to explain the occurrence of bleaching when corals are exposed to stress factors, such as elevated temperature and variations in light intensity. For example, 1) excessive photon absorption by light harvesting antennae causes a disruption of the PSII reaction centers, in particular the D1 protein (Warner et al., 1999); 2) stress may limit photosynthesis by destabilizing the thylakoid membranes (Tchernov et al., 2004); 3) damaged PSII reaction centers

mentioned above cannot be replaced by newly re-synthesized D1 protein because stress usually inhibits protein synthesis and may lead to an increase in D1 turnover rate (Takahashi et al., 2009, Warner et al. 1999); 4) Rubisco's activity can become limited in the face of stress events (Lilley et al., 2010); and 5) suppression of the synthesis of light harvesting antennae proteins, resulting in the loss of the major light harvesting proteins in the photochemical pathway (Takahashi et al., 2008).

However, bleaching susceptibility of corals may be dependent on the photochemical efficiencies and photosensitivity of different clades of Simbiodiniaceae to environmental stress (see Berkelmans & Van Oppen, 2006; Grégoire et al., 2017; Karim et al., 2015a). For example, in a study comparing photochemical efficiencies of 6 different Simbiodiniaceae under control (25 ˚C) and heat-stress (33 ˚C) temperatures, Karim et al. (2015a) found that F_v/F_m decreased significantly between both temperatures in only three out of six clades surveyed. This finding possibly means that the effects of temperature vary between strains of Symbiodiniaceae. The same occurs when facing different light intensities (Karim et al., 2015b). Accordingly, Berkelmans and van Oppen (2006) report a direct causal link between the type of zooxanthellae and corals' thermal tolerance, which has been confirmed by other studies as well (e.g. Jones & Berkelmans, 2012; Qin et al., 2019; Rowan, 2004; Sampayo et al., 2008). Moreover, corals are known to shift their symbiont community in response to stress, possibly associating with more tolerant Symbiodiniaceae clades (Kemp et al., 2014), despite energetic trade-offs (Jones & Berkelmans, 2010). However, most of this knowledge comes from studies addressing hard corals. Flexibility in algal symbiosis has been less studied in soft corals, although some studies have already reported the existence of such variability (Lewis & Coffroth, 2004). Still, it seems that soft corals' capacity to establish multi-clade symbiosis is rather reduced when compared to hard corals (Baker & Romanski, 2007; Goulet et al., 2008; Goulet & Coffroth, 2003). A recent study also highlights the complexity of non-random coral-zooxanthellae interactions, which are shaped by symbiont transmission mode (horizontal vs vertical transmition), evolutionary history and biogeography, affecting the overall thermal resilience of coral species (Swain et al., 2021). Although endosymbiont clades were not evaluated in this experiment, future studies should address the plasticity of this

symbiosis, as well as its constraints, in response to stress in different species of soft corals.

Concerning the main effects of light on *Sarcophyton* cf. *glaucum*, a significant decrease of photosynthetic activity (indicated by a reduction in F_v/F_m) was observed under HL conditions, suggesting that HL intensity leads to photoinhibition and/or photodamage of the photosynthetic apparatus. In two previous studies by Rocha et al. (2013a, b), corals stocked under three different PAR values (50, 80 and 120 µmol m^{-2}.s^{-1}), presented an inverse relationship between F_v/F_m and light intensity, with corals exposed to 50 µmol m^{-2} s^{-1} having a higher F_v/F_m, when compared to corals exposed to 120 µmol m^{-2} s^{-1}. This same pattern was also observed in the present study, despite the different PAR values employed. Also, according to the same authors, although some differences were found among light treatments, F_v/F_m values were always high and relatively close to the maximum values reported in literature for corals (approx. 0.6) (Rocha et al., 2013a). Somewhat similar results were obtained in this study as, under optimal conditions (26 ˚C, LL), *S.* cf. *glaucum*'s F_v/F_m values were around 0.5. Lower values (approx. 0.3) were found in sub-optimal conditions (heat and/or HL stress), similarly to what has been previously reported in other coral species under temperature stress (Jones et al., 2000; Nielsen et al., 2018; Rowan et al., 1997; Warner et al., 1999) and light stress (Gardner et al., 2017a; Kuguru et al., 2010; Wicks et al., 2010) .

The negative effects of HL intensity on the photosynthesis of corals seemed to be exacerbated by elevated temperature (and vice-versa) (Berg et al., 2020), as observed in the present study (in particular for the parameter F_v/F_m). Previous research has shown that synergistic effects of these stressors on corals play a key role in coral bleaching responses, usually leading to photo-oxidative stress, persistent photosystem damage and longer recovery periods (Berg et al., 2020; Lesser, 2011). For example, according to Karim et al. (2015a), when corals are exposed to HL, decreases in F_v/F_m are greater at 33 °C when compared to 25 °C, as in the present study. Indeed, in our experiment a decrease in F_v/F_m of 80% was found between 26 °C and 32 °C under HL conditions vs 36% between the same temperatures at LL. Thus, coral health may be especially disrupted if heat stress is combined with HL conditions, as shown by the pronounced declines in F_v/F_m in the

present study. This finding is also in agreement with a study by Wicks et al. (2010), on which the combined effect of both temperature and light intensity was tested in the stony coral *Pocillopora damicornis*. These authors report that the lowest mean values of F_v/F_m were recorded for the treatment with HL intensity and the highest temperature tested (29 °C). Wicks et al. (2010) also state that an exacerbated effect of light and temperature was found in coral endosymbionts, with HL and high temperature having the most pronounced effect on the photosynthetic efficiency of PSII.

If algal endosymbionts of *S.* cf. *glaucum* were photodamaged by this interactive combination of temperature and light intensity, F_v/F_m values may not recover so easily, as the effects of these stressors may become additive over successive days (Jones et al., 2000). This could be the case of HW occurring under high irradiance, which can be one of the reasons for why F_v/F_m values did not attain basal levels in *S.* cf. *glaucum* after 30 days of recovery in the present experiment. This result is in line with the findings by Jones et al. (2000) referred above. Interestingly, Berg et al. (2020) states that the recovery of the photochemical quantum yield may take three times as much as the duration of the treatment (i.e., stress imposed). Laboratory studies with the hard coral *Plesiastrea versipora* show that F_v/F_m can became reduced for a great amount of time following a heat stress event, providing a measure for this type of stress (Warner et al., 1999), agreeing with our findings. The reduction of F_v/F_m could be due to an overexcitation of the photosynthetic apparatus when corals are exposed to thermal stress (and/or high irradiances), resulting in excess energy and electrons that need to be diverted from the photosynthetic apparatus by means of heat dissipation and via alternative electron flows (photoprotection mechanisms) (e.g. Jones et al., 1998; Roberty et al., 2014). Under these conditions, the electron flow is increased via the alternative Mehler reaction, which reduces oxygen (O_2) to the ROS superoxide anion ($O_2^{\bullet-}$) by donation of an electron in PSI, being a major source of ROS under stress (see Lesser, 2010 and Robertry, 2014). This ROS is rapidly converted into water by antioxidants but under stress, the amount of ROS produced may easily surpass antioxidant capacities, leading to cellular damages, including in PSII (Robertry 2014). The overwhelming of this system is thus related to reductions in F_v/F_m, although some authors refer that the primary effect of heat stress is the

impairment of the carboxylation step in the Calvin cycle, by lowering the activity of Rubisco (e.g. Jones et al. 2002). Still, heat stress is also known to affect the ability of symbionts to resynthesize or substitute D1 protein, the main protein that suffers the damages of stress. However, an incapacity to repair thylakoid proteins could be another explanation (Karim et al., 2015a), ultimately leading to a reduced F_v/F_m and even apoptosis, when cellular damage is severe (Zhang et al., 2018).

When it comes to HL intensity, reductions in F_v/F_m are most likely explained by the excess energy overwhelming photoprotection mechanisms, resulting in photodamage in the face of stress, representing the physiological compromise of symbionts which were not able to dissipate the energy created by excess light (Smith et al., 2005). Moreover, it could also mean that zooxanthellae were unable to repair their PSII or compensate the electron flow through remaining functional reaction centers (Behrenfeld et al., 1998). It is still unclear whether this damage is representative of a primary or secondary response to stress (Jones et al., 1998).

F_0 and the number of endosymbionts were the two other photophysiological parameters monitored on this study that were affected by light and temperature, respectively, with no interactions being detected. Results concerning F_0 were similar to those reported in other studies (Rocha et al., 2013a, b) where HL intensity leads to a lower F_0 and hence a putatively lower Cl a concentration. According to Berg et al. (2020), a reduction in F_0 can be interpreted as an activation of photoprotection mechanisms, on which pigments of the antenna dissipate excess light, while increases in F_0 may signal photodamage. In the present study, F_0 was only significantly affected by light intensity, displaying a reduction of about 40% when PAR values increase from an average of 253 µmol m^{-2} s^{-1} to an average of 663 µmol m^{-2} s^{-1}. According to Hoegh-Guldbergl & Smith (1989), after exposure to full sunlight for 8 h, Cl a content of the stony coral *Stylophora pistillata* did not reveal any significant differences between stress and control conditions. Although this contradicts the findings of the present study, one of the obvious reasons could be related to the different exposure times, as *S.* cf. *glaucum* fragments were exposed for at least 30 days (in Time-point 2, photoacclimation phase, where the only factor intervening was light). In another study, where corals of the species *S. pistillata*

were exposed to different light levels for 10 days, both values of Cl a per area^{-1} and per zooxanthellae^{-1} decreased as PAR increased (Hoegh-Guldbergl & Smith, 1989). The same occurred in two studies by Rocha et al. (2013a, b), one of them addressing *S.* cf. *glaucum*, with Cl a content being higher in the fragments reared under the lowest PAR value. Acclimation to a LL intensity is known to promote an increase in Cl a concentration in photosynthetic endosymbionts, leading to a higher capacity to absorb light than corals acclimated at a HL intensity, overall explaining the decrease in Cl a from LL to HL conditions (Berg et al., 2020; Cohen & Dubinsky, 2015; Dubinsky et al., 1990). With regards to temperature, it showed no effect on *S.* cf. *glaucum*'s F_0. This finding is supported by other studies in various species of hard corals (for example *S. pistillata*, *Sertatopora hystrix* (Hoegh-Guldbergl & Smith, 1989)), on which Cl a content per zooxanthellae was not affected by temperature. However, in a study by Gardner et al. (2017a), it is possible to detect a significant decrease regarding Cl a concentration in response to a high temperature (32 °C). One explanation could be the fact that this only occurred after a 70% loss of endosymbionts, which may somehow justify such result (a reduction in the number of endosymbionts can be responsible by a reduction of Cl a concentration) (Gardner et al., 2017a). Nonetheless, as mentioned before, symbiont clade differences is one of the most explored theory for this variation of response (some clades may be more resilient to stress conditions, remaining with the coral host, thus avoiding a decrease in pigment concentration) (Abrego et al., 2008; Loya et al., 2010).

As stated before, pigment concentration can also be influenced by zooxanthellae density and/or pigment concentration per zooxanthellae (Rocha et al., 2013b). In fact, a decrease in F_0 is often an indicative of the loss of zooxanthellae (Fitt et al., 1993; Hoegh-Guldbergl & Smith, 1989). There was an overall significant effect of light intensity on the concentration of zooxanthellae in both studies by Rocha et al. (2013a, b), contradicting the findings of the present study, in which no significant effects of light were observed on zooxanthellae density over the whole experiment. One of the hypothesis could be that there was not enough time to detect changes, since they commonly occur within 40 days (Titlyanov., et al 2001). However, even though this might have happened in Time-point 2 (it only lasts 30 days), it does not justify the fact that this variable was not affected by

light within the other Time-points surveyed. Also, since there was a decrease in Cl a content (F_0) between light intensities, it was expected that this could be due to a significant loss of symbionts (Hoegh-Guldbergl & Smith, 1989), which was not the case. This could indicate that the reduction of F_0 happened due to a reduction of Cl a per symbiont rather than due to the loss of endosymbiont. Other authors also state that the effect of light may only be visible at near lethal temperatures (Coles & Jokiel 1978). This could explain the lack of effect of light over zooxanthellae abundance, likely because the 32 °C temperature imposed during the HW phase of the experiment were not a near death temperature for *S.* cf. *glaucum*, hence light effect did not promote a pronounced effect on the density of zooxanthellae.

Although light intensity did not significantly impact the density of zooxanthellae in the present study, significant changes in this parameter occurred between corals exposed to different temperatures. In particular, decreases of around 60% were detected in Symbiodiniaceae numbers of heat-exposed corals at Time-points 3 (HW phase) and 4 (recovery phase). The decline in the number of endosymbionts is well-known to be indicative of stress under elevated temperatures (Brown & Howard, 1985), as reported by several authors (Hueerkamp et al., 2001; Jones et al., 2000; Nielsen et al., 2018; Tang et al., 2020; Yakovleva et al., 2004). In these studies, a reduction in zooxanthellae number was observed in the hard coral species *Plesiastrea versipora*, *Platygyra ryukyuensis*, *Stylophora pistillata*, *Pocillopora damicornis*, *Pocillopora elegans*, *Porites lobata*, *Pavona clavus* and *Pavona gigantea* exposed to heat stress. The expulsion of endosymbionts from the coral host can be considered a defensive mechanism that prevents cellular damage from oxidative stress induced by water temperature or HL intensity, thus allowing the coral to adjust its photosynthetic supply to nutritional demands (Lesser & Shick, 1989). Since in the present study symbionts concentration did not change significantly in the face of high irradiance, this could mean that coral fragments were not stressed enough to activate this defense mechanism and instead, only lowered cl a (F_0) content. However, previous studies have shown that other anthozoans, such as the corallimorpharians *Rhodactis rhodostoma* and *Discosoma unguja*, decrease both Cl a concentrations and zooxanthellae abundance with increasing levels of irradiance (Kuguru et al., 2010).

After a recovery period, represented by Time-point 4, photophysiological parameters were expected to return to their basal (i.e. control) values, based on the assumption that a recovery process, such as PSII repair cycle within the photosynthetic endosymbionts, could be underway (e.g. Aro et al., 1993). However, from the photophysiological parameters tested, only F_0 values returned to control levels.

Curiously, Hoegh-Guldbergl & Smith (1989) suggested that bleaching may involve an initial phase in which a decline in Cl a content may be visible. After that, a period of recovery follows, where values increase. This is in line with the findings of the present study. The same may also occur to zooxanthellae concentration, but this was not visible for *S.* cf. *glaucum*, as the number of endosymbionts was still lower in corals that had been exposed to heat stress, even after 30 days of recovery (Time-point 4). Although Hoegh-Guldbergl & Smith (1989) did not find a recovery on the number of endosymbionts after heat-stressed corals were returned to control temperature for 4 days, they did find a recovery of endosymbionts after 23 days in the species *Stylophora pistillata*. While our data show an opposite trend, several factors could contribute to a different recovery ability among coral species, such as strain-specific endosymbionts infectivity and species-specific responses. For example, photosynthetic endosymbionts can be recovered by the coral host by recruiting them from the remaining population or through an external media (Baird & Marshall, 2002; Lewis & Coffroth, 2004). However, heat-stress has been shown to reduce the ability of some strains of zooxanthellae to infect host cells, limiting recovery potential (Kishimoto et al., 2020; Schreiber et al., 1986). Moreover, differences in recovery times have also been recorded between species: while the hard coral *Porites lobata* took only 25 days to recover from a heat stress treatment (30.7 °C), the hard coral *Pavona clavus* required 75 days to do so (Hueerkamp et al., 2001). As such, it is possible that *S.* cf. *glaucum* may need more time to recover its number of endosymbionts when compared to other coral species that endure an exposure to heat stress. Moreover, as already mentioned, the duration of such recovery period can vary from 3 months up to 2 or more years in the case of zooxanthellate corals (Brown & Suharsono, 1990; Glynn & D'Croz, 1990). The same can be said for F_v/F_m (for temperature and light). In fact, according to Thomas & Palumbi (2017), a great part of the transcriptome of the host can

remain affected for several months after bleaching, even after pigmentation returns to the coral colony previously bleached. The impacts of such a traumatic event can last up to 12 months, thus reflecting possible longer recovery times for some species (Thomas & Palumbi, 2017).

Overall, it is important to understand that the effects of environmental stress in any study is dependent on the rates and intensity of environmental change, as well as the duration of experiments. Thus, if an experiment is extended for a longer period, such as weeks or months instead of days (as in several studies referred above), different responses in terms of bleaching and tolerance to stressors can be observed (e.g. Aichelman et al., 2021; Cumbo et al., 2013; Wicks et al., 2010). This is particularly interesting considering the different possible durations of a HW, which can last from 5 (minimum number of days that a thermal anomaly must span to be considered a HW) to 95 days, one of the longest HWs recorded to date that occurred in Western Australia (Hobday et al., 2016). However, it is possible to find records of longer MHWs on other regions of the planet. Interestingly, Kuanui et al. (2020) also highlighted that those coral responses to changes in environmental factors can be age-dependent. Also, the temperature threshold at which deleterious effects occur for temperate corals, such of *P. versipora* (28 °C) is different and lower than the temperature required to negatively impact tropical corals in the Caribbean or the Indo-Pacific (32-34 °C) (Fitt & Warner, 1995; Jones et al., 1998, 2000). Bleaching usually occurs when zooxanthellae lose photosynthetic pigments, or when the coral host loses its zooxanthellae (Hoegh-Guldbergl & Smith, 1989). In these terms, we can consider that *S.* cf. *glaucum* bleached in the present study, since a significant loss of Cl *a* (i.e. F_0) and zooxanthellae, along with a decrease in F_v/F_m, was observed. However, a discoloration of tissue is most commonly associated with bleaching (Jones et al., 2000). In the present species however, this feature is not so easily visualized. Nevertheless, a visual indicative of bleaching could be noticed, particularly before sampling at Time-point 3, which occurred mainly in the fragments that were exposed to 32 °C. Some tissue breakdowns in these fragments seemed to be occurring, and partial colonization by algae was observed, indicating that partial necrosis could be occurring in these fragments. In fact, 32_HL was the treatment with the lowest

levels of F_v/F_m, followed by 32_LL at Time-point 3 and 4. The same occurred for F_0 and the number of endosymbionts, supporting that, in fact, these corals endured a bleaching event caused by the HW simulated over 10 days under HL intensity.

One thing that cannot be left unsaid is the fact that, when it comes to the interaction between temperature and light , there is a pattern for corals to pale their upper sunlight exposed surfaces; this is often termed a "shade effect" (Glynn, 1983). In fact, Jones et al. (1998) showed that the effect of temperature tends to be more severe on the upper surface of corals, the areas which also experience a higher irradiance. It has been proposed that this effect occurs due to the presence of a light/temperature-sensitive clade of endosymbionts on that area (Rowan et al., 1997), something that may also occur in *S.* cf. *glaucum*. Therefore, since parameters such as F_0 and F_v/F_m were only measured on this type of surfaces, it may help to explain the pronounced effect on these photophysiological parameters recorded in the present study. If these variables were monitored on the sides of the coral fragment, one cannot exclude the possibility of higher values having been recorded.

4.2 Molecular parameters: stress biomarkers under the combined influence of temperature and light intensity

A high number of invertebrates, including corals, can induce a set of similar metabolic pathways to cope with environmental stressors (Jin et al., 2016). These pathways are associated with the universal mechanism of CSR and include the up-regulation of defenses against ROS, commonly enhanced under stressful conditions, as well as the induction of apoptosis, protein quality control, cytoskeleton reorganization and an innate immune response (Kültz, 2005, 2020; Palumbi et al., 2014). Reactive Oxygen Species (i.e., superoxide anion (O_2^-), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO·) (Richier et al., 2006)), can be created due to an exposure to an abiotic stressor, such as temperature and light intensity. This exposure can be of two types: chronic stress (i.e. long-term stress), also known as routine, meaning that it represents conditions that corals experience in the long-term (for example long-term rate of sea surface warming) and to which they could acclimatize or even adapt to, and

acute stress (occurring over shorter time-scales and that can result in sporadic bleaching), which is the stress that occurs for example during a HW and that can develop for weeks to months (Magris et al., 2015; Ulstrup et al., 2006). Both types of stress can lead to cellular damage, including cellular membrane peroxidation (LPO), DNA degradation and protein denaturation (Richier et al., 2005; Weis, 2008). To prevent this from happening, both the coral host and its endosymbionts activate defense mechanisms, like antioxidant enzymes (enzymatic and non-enzymatic), to maintain cellular functions intact (Lesser, 1997; Lesser, 1996).

Reactive Oxygen Species accumulation can be associated with heat and light stress in the coral holobiont (Brown et al., 2002; Cziesielski et al., 2019; Lesser et al., 1990) and it can be responsible for coral bleaching (Lesser, 2011), a theory termed "Oxidative Theory of Bleaching". This theory states that heat and light stress trigger the coral bleaching process by destabilizing symbiont photosynthesis, which leads to a high production of ROS in the symbiont that saturate corals' antioxidant defenses and culminates in even more ROS accumulation. Subsequently, these ROS diffuse into the coral host resulting in a disruption of symbiosis (Weis, 2008).

In the present study, there was an overall increase in biomarkers levels when corals were exposed to the environmental stressors addressed. However, this increase was only visible in four out of the eight biomarkers tested. CAT was affected by light (reduction of 42% on its value when corals were exposed to HL, in comparison to LL) and the combined effect of both factors (an increase of 41% from 26 to 32 °C under LL and a reduction of 68% from 26 to 32 °C under HL) at Time-point 3. SOD suffered a significant increase in *S.* cf. *glaucum* exposed to elevated temperatures at Time-point 3 and a significant decrease when exposed to HL at Time-point 4. Ubi and GST were only affected by temperature at Time-point 3, being significantly increased in heat-stressed corals.

Yakovleva et al. (2004) studied the effect of thermal stress (33 °C) on the antioxidant enzymes SOD and CAT of two stony coral species, *Platygyra ryukyuensis* and *Stylophora pistillata*, with elevated temperature having a significant effect on these species' enzymatic activity, although species-specific responses were observed. In the coral tissue of *P. ryukyuensis*, SOD activity was not affected by temperature throughout

the experiment, while in *S. pistillata* it increased. When it comes to the same activity, but this time in their zooxanthellae, *P. ryukyuensis* SOD only increased slightly after 12 h of exposure, while in *S. pistillata* it increased significantly after 6 h and decreased by 50% after 12 h. These results agree with the findings of the present study, in which very similar temperature treatments were used. In fact, this increase in SOD under high water temperatures could be explained by the fact that this antioxidant enzyme is the first one involved in ROS elimination, acting very close to the site of ROS production (Lesser, 2011). Catalase activity in *P. ryukyuensis* was unaffected by temperature during the first 6 h, but later it increased (Yakovleva et al., 2004). In the stony corals *Acropora millepora* and *Montipora digitata*, high levels of CAT were also detected upon exposure to an elevated temperature (33 ˚C), although no antioxidant response was detected in endosymbionts (Krueger et al., 2015). Interestingly several studies mention that the up-regulation of the coral host antioxidant defenses is independent of symbiont antioxidant production and precedes bleaching (Hawkins et al., 2015; Krueger et al., 2015). This suggests that host redox regulation may in fact not be dictated by photophysiological responses of its endosymbionts (Krueger et al. 2015). In the experimental study by Hawkins et al. (2015), the hard coral *Stylophora pistillata* increased CAT activity under heat and HL stress, but this was only observed in colonies that had been collected from deeper waters, whereas conspecifics from shallow-waters exhibited no changes in their antioxidant system. This suggests that previous habitat conditions influence the ability of corals to display an antioxidant response in the face of stress (Hawkins et al. 2015). In the present experiment, temperature did not display a main effect on CAT activity in *S.* cf. *glaucum*, but an interaction between temperature and light was found. This result suggests that the effect of temperature on this enzyme is likely dependent on light levels experienced by corals.

Another similar study evaluated the effect of elevated temperature (30 °C) and low salinity on oxidative stress biomarkers (LPO, SOD, CAT and GST) along 60 days on nine hard coral species (one massive species (*Galaxea fascicularis*), one encrusting species (*Montipora capricornis* green morphotype), three planting species (*Montipora capricornis* brown morphotype, *Turbinaria reniformis* and *Echinopora lamellosa*) and four branching

species (*Acropora tenuis, Pocillopora damicornis, Stylophora pistillata* and *Psammocora contigua*)) that occur in the Indo-Pacific region (just like *S.* cf. *glaucum*) (Dias et al., 2019a). The authors reported that, unlike in the present study, LPO and CAT levels were significantly affected by temperature (Dias et al., 2019a). The same was recorded for GST (Dias et al., 2019a), which was also affected by temperature in our study on *S.* cf. *glaucum*. Same results regarding LPO, CAT and GST were obtained in another study by the same author (Dias et al., 2019b). According to Dias et al. (2019a), SOD was affected by the interaction between temperature and salinity, while for *S.* cf. *glaucum* was affected both by temperature and light intensity. In most biological systems, CAT is the main antioxidant responsible for the elimination of H_2O_2, a product of oxidative stress (Munoz-Munoz et al., 2009). However, coral fluorescent proteins (FPs) may also be responsible for this scavenging (Palmer et al., 2009). Therefore, this may explain why a main effect of temperature was not observed for CAT, as these FPs could have been sufficient to cope with the elimination of H_2O_2, as already proposed by Dias et al. (2019a, b) . However, as an interaction between temperature and light intensity was detected in our study, with CAT activity being induced by heat stress under LL but a reduction being recorded under HL, it seems that the combination of heat stress and HL inhibits the scavenging activity of CAT. However, in the present study, no significant increase was detected in the level of products resulting from oxidative damage, here measured as LPO, with increasing temperature and light. One explanation for this finding may be the fact that an increase in antioxidant enzymes activities (like SOD and GST for *S.* cf. *glaucum*), was enough to counterbalance the deleterious effects of ROS, reducing the buildup of oxidative damage products, such as lipid peroxides, by avoiding cellular membrane damage (Dias et al., 2019a, b). Still, future studies may include markers of DNA damage (8-hydroxy-2'-deoxyguanosine or 8-oxo-7,8-dihydro-2' -deoxyguanosine, known as 8-OHdG and 8-oxodG, respectively) and protein damage (e.g. protein carbonylation), in order to confirm these findings.

Another biomarker study performed by Madeira et al. (2015) evaluated the effect of different seasons (Spring and Summer) on the octocoral *Veretillum cynomorium*, considering Summer as the warmest period. It was possible to detect a significant effect

of the factor season over all the stress biomarkers tested (Hsp70, Ubi, CAT, SOD and LPO), although biomarker levels were reduced during summer (Madeira et al. 2015). However, as this was a field study, many other factors other than temperature could have contributed to these results, namely food availability, reproductive status, or previous exposure to stressful events (Madeira et al. 2015).

According to the literature, it is possible that some thermal tolerant coral species (e.g., *Porites cylindrica*) present higher levels of SOD and Hsps than more susceptible ones (*Stylophora pistillata*) (Fitt et al., 2009). In fact, there are some marine invertebrate species considered very resistant to heat stress whose Hsp70 levels are constitutively elevated. This is a protective mechanism known to be helpful in environments with constant and extreme temperature fluctuations (Dong et al., 2008; Madeira et al., 2014b). A similar strategy has already been reported in corals, termed constitutive frontloading (Barshis et al., 2013). These previous authors compared the molecular profiles (transcriptome) of conspecific corals that differed in physiological resilience to environmental stress, revealing that Hsps, antioxidants, and genes involved in apoptosis regulation, innate immune responses and cell adhesion were frontloaded (high constitutive expression) in resilient stony coral *Acropora hyacinthus* (Barshis et al., 2013). These results were also corroborated by Jin et al. (2016), which found that Hsps and antioxidant genes were frontloaded in the stony coral *Acropora millepora*. In classical thermotolerance studies in *Drosophila*, small to moderate increases in Hsp70 are responsible for an increase in thermotolerance. However, a large increase in this protein reduces this ability (Rowan et al., 1997). Therefore, frontloaded genes may have optimized expression levels, resulting from evolutionary processes or local adaptation in organisms consistently exposed to variable environmental conditions. The lack of a significant increase in Hsp70 in the face of stress that occurred in the present study, may indicate that *S.* cf. *glaucum* is a species that is well-adapted to warm and variable environments. This assumption makes sense considering that this species inhabits intertidal reef flats (Fabricius & Alderslade, 2001), where large temperature (1 °C h^{-1}, (Willis & Berkelmans, 1999)) and irradiance fluctuations occur, following an emersion/immersion period associated to tidal cycles. This indicates that Hsp70 protein,

despite being effective in heat shock response, may not be the best thermal stress biomarker for all coral species (Kenkel et al., 2011). In fact, some studies state that Hsp16 may be a better marker, as it increased dramatically (about 800-fold) when the hard corals *Porites astreoides* and *P. lobata* were exposed to heat stress (Kenkel et al., 2011). Future studies should address the suitability of Hsp16 as a biomarker for thermal stress in different species of stony and soft corals.

Despite the lack of change in HSP70 in this study, previous authors have reported its induction in coral species subjected to thermal stress (Coles & Brown, 2003; Desalvo et al., 2010; Hernández-Elizárraga et al., 2019; Petrou et al., 2021), along with the induction of other Hsps (for example 60 kDa and 90 kDa, as reported in Coles & Brown, 2003; Desalvo et al., 2010; Petrou et al., 2021; Seveso et al., 2014). Heat shock proteins are often used as indicators of the level of protein damage/unfolding of a cell, representing an indirect measure of stress (Ciechanover, 1998). On the other hand, Ubi is considered a direct measurement of protein damage since it truly indicates protein loss via the ubiquitin-proteasome pathway (Ciechanover, 1998). Nonetheless, these two parameters are used to indicate how environmental stress impacts organismal function, as protein damage is known to occur at the limits of physiological tolerance (e.g. Hofmann & Somero, 1995; Madeira et al., 2014c). Taking this into account, in the present study, Ubi levels significantly increased (135%) in heat-stressed corals. This can indicate that the cellular protein pool suffered a substantial amount of misfolding, thus being translocated to the proteasome for degradation (Hershko, 1996). However, if this occurred, Hsp70 was expected to change as well, but this did not happen. Still, it is possible that other Hsps could be at play in this process, a hypothesis that should be further investigated in *S.* cf. *glaucum*. Interestingly, Ubi levels have been shown to correlate with environmental temperature in the hard coral *Acropora millepora* sampled from the Great Barrier Reef (Lundgren et al., 2013). Moreover, Barshis et al. (2010) states that Ubi levels in the hard coral *Porites lobata* are consistently higher in colonies sampled from variable reef sites, even when they are transplanted to other sites. These findings suggest that genetically-based differences may explain Ubi levels, and these may contribute to differences in coral thermal tolerance (Lundgren et al., 2013).

Regarding TAC levels, no changes were detected in *S.* cf. *glaucum* according to temperature or light intensity-exposure. However, a study by Marangoni et al., (2019) presents opposite results in other species of corals, namely the scleratinian coral *Mussismilia harttii* and the hydrocoral *Millepora alcicornis*. In this study, an overall significant effect of temperature was found for TAC, as it increased in corals facing thermal stress. Again, such results suggest that changes in antioxidants may be species-specific and dependent on stress intensity and duration, as Marangoni et al. (2019) studied corals *in situ* for several months and the present study is based on an *ex situ* experimental simulation of a 10-days HW. In another study, the hard coral species *Acropora eurystoma* and *Pocillopora damicornis* were exposed to a simulated light pollution stress during night-time with significant differences in TAC being recorded; these were likely dependent on exposure time and the monochromatic wavelengths employed to simulate different types of light pollution (Ayalon et al., 2019). Nevertheless, the fact that other antioxidant mechanisms, such as specific enzymes (e.g., SOD and GST), were contributing to restore *S. glaucum*'s redox status in the present study, could explain why TAC did not suffer a significant increase.

There is a lack of studies that address the influence of light intensity on biomarkers levels. However, according to the literature, light intensity modulates the oxidative status of the coral holobiont, as light stress is known to induce ROS production (Downs et al., 2002). This process occurs in chloroplasts through various mechanisms that are associated with PSI and II-catalyzed electron transfer, where the most notable ones are the Mehler reaction and the formation of H_2O_2 by the oxygen-evolving process (Downs et al., 2002). This H_2O_2 leaves the chloroplast of the symbionts and enters the host cytoplasm. In here, it can be eliminated through enzymatic or non-enzymatic pathways or even be catalyzed through the Fenton reaction to hydroxyl (OH) radical that, when in high levels, will cause the host to expel or destroy the symbiotic zooxanthellae as a manner of defense (Downs et al., 2002).

Interestingly, all the biomarkers that were somehow influenced by light (SOD and CAT) in *S.* cf. *glaucum* experienced a decrease when light intensity increased compared to control conditions. This was not expected since ROS levels should have a tendency to rise

under HL conditions. This response was more noticeable in CAT, which was significantly affected by the interaction between light and temperature. One hypothesis is that the HL intensity used (663 μmol m^{-2} s^{-1}) was so stressful for coral fragments that it led to an exhaustion of the antioxidant production system and consequently a decrease in antioxidant levels. This effect could explain why (i) CAT decreased 68% in corals exposed to heat and HL stress, but not in corals exposed to heat under LL (at Time-point 3) and (ii) SOD decreased in corals exposed to HL conditions at Time-point 4. The hypothesis of an exhaustion of the antioxidant system was also proposed by Dias et al. (2019b) to interpret the findings recorded on other coral species exposed to heat stress. Also, there is a chance that these lower values of CAT and SOD recorded in *S.* cf. *glaucum* when facing light stress could be due to a much lower production of oxygen radicals (see for example Brown et al., 2002). There are many types of host cnidarian species, including corals, that possess a photoprotective mechanism when under HL. When exposed to high irradiance, corals can engage in a tissue retraction that not only protects them from a high intensity light at a photosynthetic level, but also limits the production of ROS (Shick & Dykens, 2014).

The SOD-CAT antioxidant pathway is essential to regulate the oxidative status of cells (Halliwell, 2006). These enzymes constitute the first line of defense against ROS, limiting the damage to intracellular macromolecules, even though not being completely efficient at this process (Hayes & McLellan, 1999). The chemicals produced from the interaction of ROS with these intracellular molecules, become very reactive and also have to be detoxified before they further damage lipids, proteins and DNA, culminating in cell death (Dixon et al., 2010). So there must be a second line of defense preventing these occurrences, and GST is responsible for it, detoxifying lipid peroxides and repairing cells structure (Hayes & McLellan, 1999). Considering the results of the present study, if corals are exposed to severe stress due to the combined effect of temperature and high irradiance, this SOD-CAT pathway can be compromised, impairing the first line of defense against ROS. However, other antioxidants (like GST) could potentially cover the detoxification process underway and avoid significant cell damage.

At Time-point 4, biomarkers, in general, returned to their control levels. This suggests that a recovery process was taking place at the molecular level for most coral fragments of *S.* cf. *glaucum*. However, SOD levels were lower in HL-exposed corals when compared to LL at this Time-point, suggesting that this specific biomarker was inhibited, or its production system reached exhaustion after a prolonged exposure to HL conditions. Also, this general decrease could be explained by a damage to ROS-sensitive enzymes upon exposure to stressful conditions (Martindale & Holbrook, 2002).

Total protein concentration apparently maintained its values throughout the whole experimental period. This may indicate that *S.* cf. *glaucum* did not have the need to engage in an heterotrophic feeding behavior, since its energetic reserves remained unaltered (Rodrigues & Grottoli, 2007). However, since bleaching appeared to have occurred, an alteration on this species energy reserves was somehow expected (loss of symbionts usually leads to a loss of energy reserves) in order to fulfill energetic needs (see Fitt et al., 1993; Knochel, 2017; Rodrigues & Grottoli, 2007). Nonetheless, it is important to notice that an organism pool of energetic reserves is not limited to proteins, as lipids and carbohydrates also play a relevant role (Grottoli et al., 2004; Rodrigues & Grottoli, 2007). In this way, *S.* cf. *glaucum* may have relied on these compounds to fulfil its energetic needs, without having to degrade proteins. This assumption may be confirmed in future studies by measuring lipid and carbohydrate levels in corals.

Just like for the photophysiological parameters determined during the present work, treatment 32_HL was the one with overall higher biomarker values when compared to other treatments, followed by 32_LL. This finding can indicate that these treatments were the most stressful ones at molecular level to fragments of *S.* cf. *glaucum*. This fits the general observation that coral fragments undergoing a more pronounced bleaching were the ones in these two experimental treatments.

4.3 Integrated response of photophysiological and molecular parameters

As already referred, a decrease in F_v/F_m during environmental stressing highlights the occurrence of damage to the photosynthetic apparatus of zooxanthellae (Jones et al.,

2000; Warner et al., 1999). This is commonly caused by a disruption between the degradation and resynthesis of protein D1 reaction center, which promotes the accumulation of symbionts with nonfunctional PSIIs (Vasilikiotis & Melis, 1994). The damage caused to this protein involves active oxygen, whose toxicity can be a mechanism responsible for the decrease of photosynthetic efficiency of zooxanthellae exposed to thermal stress and high irradiance, as high temperatures and HL intensity increase the flux of these oxygen products (ROS) (Lesser, 1996). In fact, the production of singlet oxygen ($^1O_2$), considered the most harmful ROS, seems to be responsible for light- and heat-stress induced loss of PSII activity, in symbiont chloroplasts (Krieger-Liszkay, 2005; Nielsen et al., 2018). In this sense, the induction of protein quality control (to counteract protein damage) and antioxidant defense systems (to regulate the redox status) by corals is an important line of defense against these stress-generated ROS (Somero, 2020). If these defense systems are not enough to prevent cellular damage, then bleaching is triggered, resulting from an inhibition of PSII, reduced photosynthetic efficiency and a decrease in zooxanthellae density due to the expulsion of Symbiodiniaceae by the coral host (Warner et al., 1999).

Interestingly, bleaching susceptibility is known to increase with zooxanthellae density, meaning that a higher number of endosymbionts leads to a higher accumulation of ROS in the coral host, leading to an earlier bleaching event (Cunning & Baker, 2013; Gardner et al., 2017b). However, differences in host regulation of symbionts may also contribute to different bleaching susceptibilities (Nitschke et al., 2015). For example, according to Gardner et al. (2017a), *S. pistillata* is a vertical transmitter, meaning that has tight specific host-symbiont associations. In this way, this coral species presents an earlier regulation of antioxidant enzymes in their symbionts: it prefers to activate antioxidant activity than to regulate symbiont density through their expulsion (Gardner et al., 2017a).

Other authors present other correlation theories between the collapse of PSII in symbionts and the upregulation of antioxidant enzymes. The "Oxidative theory of coral bleaching", mentioned above, states that an increase in ROS production by zooxanthellae, due to a decline in photosynthetic activity, results in the diffusion of H_2O_2 into the coral host, which leads to a rise of antioxidant defenses as a way to remove it, proving a

connection between symbiont stress (that could be reflected in their loss or the decrease in F_v/F_m or F_0) and host antioxidant regulation (Gardner et al., 2017a). If this verifies, then a decrease in photosynthetic function would be initially detected before an increase in the antioxidant defense mechanism and the expulsion of photosynthetic endosymbionts. However, since the enzymatic upregulation is considered a secondary and late response to bleaching, it may indicate that using the health of symbiont photosystem as a proxy of the beginning of bleaching could be wrong. In fact, studies that only focus on this type of markers may hold incorrect information (Gardner et al., 2017a).

According to the same authors, there is also the possibility that the loss of symbionts and the activation of antioxidant defenses occur before the collapse of photophysiological parameters, like F_v/F_m and F_0 (Gardner et al., 2017a). If this is true, then we can attribute the initial cause of the bleaching event of *S.* cf. *glaucum* in the present study to the upregulation of the molecular biomarkers and some loss of symbionts. The secondary boost occurs with the loss of Cl a (F_0) and photosynthetic activity (F_v/F_m) by the symbionts that remain.

However, regarding the effect of light in the present study, *S.* cf. *glaucum* presented an initial photosynthetic decline at Time-point 2, associated with HL, but no biomarkers were up-regulated then. Regarding the effect of temperature, to identify the mechanism underway in *S.* cf. *glaucum*, more sampling time-points should have been included during the HW-phase of the experiment. This would have allowed us to discern which changes were triggered first.

Curiously, according to Downs et al. (2013), temperature and light stress cause different effects on symbionts during the first steps of bleaching. In fact, during the first 48 h of heat stress at 32 °C, a disorganization of thylakoids allows the occurrence of photo-oxidative stress (Downs et al., 2013). In the case of HL stress (2007 µmoles m^{-2} s^{-1}), condensation or fusion of multiple thylakoid lamellae happened at the same time as the levels of oxidative damage increased, which indicates that this oxidative stress was the reason for the membrane damage recorded on the chloroplasts of endosymbionts. Furthermore, the combination of both factors of stress (high temperature and high light) induces both pathomorphologies (Downs et al., 2013).

To conclude, biochemical biomarkers, like SOD, CAT, GST and LPO, are known to be more repeatable and predictable as stress biomarkers , although being limited in their capacity to predict biological effects (Bartell, 2006). Markers at organismic level, like the photophysiological ones applied in the present study, are more ecologically relevant and represent a more integrated measure of the state of an organism; nonetheless, they are slower to respond to environmental stressors and, consequently, harder to detect. It therefore becomes important to integrate both types of markers in future studies on this topic, as they will certainly provide a more in-depth understanding on how different coral species deal cope with environmental stressors (Dias et al., 2020).

5. Conclusion and Future Perspectives

The main purpose of the present study was to evaluate the photophysiological and molecular performance of the soft coral *S.* cf. *glaucum* under different global change scenarios (temperature increase, 26 vs 32 ˚C), taking in account the depth distribution of the species across intertidal and subtidal habitats (by manipulating light intensity, exposing specimens to HL and LL intensity treatments, respectively).

Sarcophyton cf. *glaucum* was photophysiologically compromised under heat and HL stress, as F_v/F_m, F_0 and the number of photosynthetic endosymbionts were significantly affected by both stressors. F_v/F_m seemed to be the most negatively affected one, being the sole parameter monitored that was affected by the two stressors and their interaction. F_0 reacted most to light stress, while the number of endosymbionts responded to the increase in water temperature. Although F_0 and zooxanthellae density can be related (the decrease in F_0 can be a consequence of the reduction in the number of endosymbionts) this was not verified, suggesting that changes in F_0 rather reflected shifts of Cl *a* content per endosymbiont. After the recovery period, only F_0 returned to control levels, a finding that indicates that this coral was still photophysiologically affected and its photosynthetic efficiency was somehow still compromised, even after 1 month of recovery.

At molecular level, antioxidant defenses were the main component of the CSR mechanisms being activated, with increases in SOD, GST and CAT activity likely being due to high levels of ROS. Temperature was the factor that seemed to affect molecular biomarkers most strongly. Light intensity was only responsible for an effect over CAT and SOD but, instead of increasing the activity of these enzymes, it led to a decrease. This result was rather unexpected, as ROS levels should have increased under HL conditions. This finding may indicate that a prolonged exposure to HL results in an exhaustion of the antioxidant system of *S.* cf. *glaucum*. When it comes to Protein Quality Control, Ubi increased when corals were exposed to high temperatures indicating that heat induces protein damage, and these misfolded proteins were being tagged for degradation in the proteasome. At Time-point 4 (after 1-month of recovery), most molecular markers seemed to have returned to control conditions, which indicates a true recovery at the molecular level. This suggests that corals have some molecular plasticity to be able to

deal with increased temperatures, returning to a basal (control) state afterwards. On the other hand, it seems that *S.* cf. *glaucum* may need more time to recover at the photophysiological level. Corals did bleach during the experiment, as seen by the decrease in zooxanthellae density and F_0 concentration.

Global changes are a major threat to coral reefs around the world, leading to increased risk of bleaching and jeopardizing ecosystem services provided by these unique habitats (Smale et al., 2019). This study helps to better understand how climate change can impact one of the most iconic soft corals on Indo-Pacific coral reefs, the leather coral *S.* cf. *glaucum*. This study reveals some of its photophysiological tolerance limits in the face of stressful events, such as a MHW, whose effect is exacerbated by an increase in light intensity that can be experienced by specimens occurring in intertidal areas. However, this study also highlighted that this coral species is phenotypically plastic at a molecular level, being able to counteract ROS effects (at least to some extent) during heat stress events.

One point that needs to be considered is that the duration of a thermal stress event is one of the main factors affecting the results obtained. A large number of studies performs experiments using heat stress events with a duration of up to 60 days , as that by Dias et al. (2019b). Even though the total duration of the experiment matches the one performed in the present study, the duration of the heat-stress itself varies (26 days vs 10 days in the present work). Therefore, it is only logical to think that, if the duration of the HW simulation had been longer, the responses obtained could have been different, with deleterious effects on the corals exposed to heat stress being even more pronounced. Another crucial factor is the heating rate used, which can affect the way organisms respond to stress (Middlebrook et al., 2010). In this study, a heating rate of 1 °C h^{-1} was used, which could be considered a slower rate compared to many other studies (3 h to go from 30 °C to 39 °C and 1 h to decrease the temperature back to the 30 °C (Voolstra et al., 2021) and going from 29 to 34 °C in also in 3 h (Thomas et al., 2018)). Although, in the review article of McLachlan et al. (2020), a mean rate of 1.2 ± 2.2 °C h^{-1} was calculated taking into account a high number of studies regarding temperature stress on coral reefs, slower rates are able to somewhat delay the responses of the corals and their

photosynthetic endosymbionts (Middlebrook et al., 2010), potentially explaining why most of the coral fragments did not die in the present study. Further studies should therefore consider the standardization of heating rates for more comparable results.

Another point that should be taken into account is the lack of studies testing the effect of light intensity on soft corals at cellular and molecular level. If this type of experiments were more regularly performed, it would be easier to understand how high temperature and HL intensity can influence corals and how molecular and physiological parameters can correlate with each other. Moreover, future studies in corals should also consider the implementation of multi-omics techniques, which provide a broader overview of the molecular changes underpinning physiological tolerance (some examples are already available e.g. Cziesielski et al., 2018; Pathmanathan et al., 2021). This approach would improve knowledge in the climate change field concerning its impacts on coral reefs and contribute towards their conservation by identifying measures that are more likely to be effective. By understanding how different species of corals react in the face of climate change, it is possible to create more effective measures of conservation and recovery of corals around the world, contributing to the preservation of our oceans. Interesting examples of new tools in coral reef conservation that derived from physiological and molecular experiments are already available, namely assisted evolution tools (microbiome manipulation, hybridization, endosymbiont selection, pre-conditioning, transgenerational acclimation, CRISPR/Cas9-mediated genome editing) (Chakravarti & van Oppen, 2018; Chan et al., 2019; Cleves et al., 2018; Damjanovic et al., 2019; Van Oppen et al., 2015). Conservation of coral species is also relevant to the blue economy sector, since these organisms are responsible for several tourism attractions (Bruckner, 2002) and are also a target of biotechnological applications and medical investment, since they produce many bioactive compounds used for the medical industry, being of a high commercial value (McFadden et al., 2006).

6. References

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7. Supplementary Material

Supplementary Table S1 - Mean, Standard Deviation and Coeficient of Variation (% CV) of each marker (Photophysiological and Molecular) used to evaluate *Sarcophyton* cf. *glaucum* response to stress throughout Time-point 1. Markers: F_v/F_m Yield, F_0 Clorophyll a, Number of Endossymbionts (number of cells.g of wet weight^{-1}), Total Protein (mg.ml^{-1}), Hsp70 Heat shock protein 70 kDa (µg of Hsp70.mg^{-1} of total protein), Ubiquitin (µg of Ubi.mg^{-1} of total protein), SOD Superoxide Dismutase (% of inhibition.mg^{-1} of total protein), GST Gluthation-S-Transferase (nmol.min^{-1}.mg^{-1} of total protein) , Catalase (nmol.min^{-1}.mg^{-1} of total protein), TAC Total Antioxidant Capacity (mmol.mg^{-1} of total protein), LPO Lipid Peroxidation (MDA concentration nmol.mg^{-1} of total protein).

A1	Markers	Data	Initial Control
Physiological Parameters	F_v/F_m	Mean	0.297
		SD	0.087
		% CV	29%
	F_0	Mean	0.178
		SD	0.051
		% CV	28%
	Number of Endosymbionts	Mean	2.50E+06
		SD	1.81E+06
		% CV	73%
Molecular Parameters	Total Protein	Mean	3.523
		SD	1.431
		% CV	41%
	Hsp70	Mean	0.453
		SD	0.879
		% CV	194%
	Ubiquitin	Mean	0.446
		SD	0.242
		% CV	54%
	SOD	Mean	379.176
		SD	112.285
		% CV	30%
	GST	Mean	24009.02
		SD	14088.407
		% CV	59%
	Catalase	Mean	124.600
		SD	37.180
		% CV	30%
	TAC	Mean	0.321
		SD	0.107
		% CV	33%
	LPO	Mean	0.001
		SD	0.001
		% CV	58%

Supplementary Table S2 - Mean, Standard Deviation and Coeficient of Variation (% CV) of each marker (Photophysiological and Molecular) used to evaluate *Sarcophyton* cf. *glaucum* response to stress throughout Time-point 2. Markers: F_v/F_m Yield, F_0 Clorophyll a, Number of Endossymbionts (number of cells.g of wet weight^{-1}), Total Protein (mg.ml^{-1}), Hsp70 Heat shock protein 70 kDa (μg of Hsp70.mg^{-1} of total protein), Ubiquitin (μg of Ubi.mg^{-1} of total protein), SOD Superoxide Dismutase (% of inhibition.mg^{-1} of total protein), GST Gluthation-S-Transferase (nmol.min^{-1}.mg^{-1} of total protein) , Catalase (nmol.min^{-1}.mg^{-1} of total protein), TAC Total Antioxidant Capacity (mmol.mg^{-1} of total protein), LPO Lipid Peroxidation (MDA concentration nmol.mg^{-1} of total protein).

A2	Markers	Data	HL	LL
Physiological Parameters	F_v/F_m	Mean	0.414404	0.498
		SD	0.052164	0.066
		% CV	13%	13%
	F_0	Mean	0.125875	0.207
		SD	0.026926	0.048
		% CV	21%	23%
	Number of Endosymbionts	Mean	2.14E+06	2.09E+06
		SD	7.32E+05	8.47E+05
		% CV	34%	40%
Molecular Parameters	Total Protein	Mean	3.902014	3.786
		SD	1.197775	1.200
		% CV	31%	32%
	Hsp70	Mean	0.414267	0.313
		SD	0.563454	0.181
		% CV	136%	58%
	Ubiquitin	Mean	0.420295	0.383
		SD	0.226129	0.172
		% CV	54%	45%
	SOD	Mean	353.9845	337.702
		SD	132.63	108.807
		% CV	37%	32%
	GST	Mean	20077.71	19155.22
		SD	16345.92	11706.3
		% CV	81%	61%
	Catalase	Mean	145.8615	115.168
		SD	22.6087	42.534
		% CV	16%	37%
	TAC	Mean	0.35651	0.345
		SD	0.235336	0.230
		% CV	66%	67%
	LPO	Mean	0.001833	0.002
		SD	0.001128	0.001
		% CV	62%	43%

Supplementary Table S3 - Mean, Standard Deviation and Coeficient of Variation (% CV) of each marker (Photophysiological and Molecular) used to evaluate *Sarcophyton* cf. *glaucum* response to stress throughout Time-point 3. Markers: F_v/F_m Yield, F_0 Clorophyll a, Number of Endossymbionts (number of cells.g of wet weight^{-1}), Total Protein (mg.ml^{-1}), Hsp70 Heat shock protein 70 kDa (μg of Hsp70.mg^{-1} of total protein), Ubiquitin (μg of Ubi.mg^{-1} of total protein), SOD Superoxide Dismutase (% of inhibition.mg^{-1} of total protein), GST Gluthation-S-Transferase (nmol.min^{-1}.mg^{-1} of total protein) , Catalase (nmol.min^{-1}.mg^{-1} of total protein), TAC Total Antioxidant Capacity (mmol.mg^{-1} of total protein), LPO Lipid Peroxidation (MDA concentration nmol.mg^{-1} of total protein).

A3	Markers	Data	26_HL	26_LL	32_HL	32_LL
Physiological Parameters	F_v/F_m	Mean	0.431	0.551	0.086	0.354
		SD	0.044	0.033	0.069	0.077
		% CV	13%	6%	81%	22%
	F_0	Mean	0.116	0.189	0.090	0.152
		SD	0.050	0.051	0.046	0.023
		% CV	43%	27%	51%	15%
	Number of Endosymbionts	Mean	2.60E+06	2.27E+06	7.78E+05	1.27E+06
		SD	1.53E+06	7.57E+05	4.06E+05	6.06E+05
		% CV	59%	33%	52%	48%
Molecular Parameters	Total Protein	Mean	3.628	3.776	2.597	2.772
		SD	0.633	0.906	0.899	1.516
		% CV	17%	24%	35%	55%
	Hsp70	Mean	0.284	0.449	0.665	0.519
		SD	0.093	0.334	0.379	0.530
		% CV	33%	74%	57%	102%
	Ubiquitin	Mean	0.335	0.324	0.895	0.655
		SD	0.096	0.030	0.418	0.691
		% CV	29%	9%	47%	106%
	SOD	Mean	349.392	320.942	505.378	464.962
		SD	65.971	82.040	195.694	165.312
		% CV	19%	26%	39%	36%
	GST	Mean	14898.55	14829.41	54640.51	39390.48
		SD	7752.608	7527.029	43217	29220.92
		% CV	52%	51%	79%	74%
	Catalase	Mean	93.177	88.434	30.039	124.778
		SD	24.911	35.735	17.955	57.829
		% CV	27%	40%	60%	46%
	TAC	Mean	0.586	0.559	0.946	0.871
		SD	0.335	0.173	0.799	0.393
		% CV	57%	31%	84%	45%
	LPO	Mean	0.001	0.002	0.004	0.003
		SD	0.001	0.001	0.002	0.003
		% CV	85%	67%	49%	103%

Supplementary Table S4 - Mean, Standard Deviation and Coeficient of Variation (% CV) of each marker (Photophysiological and Molecular) used to evaluate *Sarcophyton* cf. *glaucum* response to stress throughout Time-point 4. Markers: F_v/F_m Yield, F_0 Clorophyll a, Number of Endossymbionts (number of cells.g of wet weight^{-1}), Total Protein (mg.ml^{-1}), Hsp70 Heat shock protein 70 kDa (µg of Hsp70.mg^{-1} of total protein), Ubiquitin (µg of Ubi.mg^{-1} of total protein), SOD Superoxide Dismutase (% of inhibition.mg^{-1} of total protein), GST Gluthation-S-Transferase (nmol.min^{-1}.mg^{-1} of total protein) , Catalase (nmol.min^{-1}.mg^{-1} of total protein), TAC Total Antioxidant Capacity (mmol.mg^{-1} of total protein), LPO Lipid Peroxidation (MDA concentration nmol.mg^{-1} of total protein).

A4	Markers	Data	26_HL	26_LL	32_HL	32_LL
Physiological Parameters	F_v/F_m	Mean	0.468	0.549	0.261	0.435
		SD	0.041	0.047	0.107	0.082
		% CV	9%	9%	41%	19%
	F_0	Mean	0.182	0.167	0.074	0.169
		SD	0.063	0.066	0.0180	0.064
		% CV	35%	40%	24%	38%
	Number of Endosymbionts	Mean	3.01E+06	2.34E+06	8.83E+05	1.05E+06
		SD	2.31E+06	1.32E+06	6.03E+05	9.22E+05
		% CV	77%	56%	68%	88%
Molecular Parameters	Total Protein	Mean	4.549	2.366	2.096	2.113
		SD	1.450	1.130	1.315	1.071
		% CV	32%	48%	63%	51%
	Hsp70	Mean	0.084	0.131	0.308	0.088
		SD	0.093	0.099	0.357	0.075
		% CV	110%	75%	116%	86%
	Ubiquitin	Mean	0.335	0.742	0.369	1.098
		SD	0.399	0.761	0.209	1.049
		% CV	119%	103%	57%	96%
	SOD	Mean	282.309	581.645	378.486	686.838
		SD	98.928	251.479	132.083	470.27
		% CV	35%	43%	35%	68%
	GST	Mean	12057.81	40259.29	149949	54260.96
		SD	10463.92	24753.94	253402.6	78579.48
		% CV	87%	61%	169%	145%
	Catalase	Mean	78.491	82.027	89.682	92.951
		SD	39.317	40.711	47.578	49.914
		% CV	50%	50%	53%	54%
	TAC	Mean	0.597	1.023	2.413	1.136
		SD	0.468	0.525	3.563	1.298
		% CV	78%	51%	148%	114%
	LPO	Mean	0.005	0.009	0.010	0.010
		SD	0.002	0.005	0.006	0.010
		% CV	33%	57%	57%	103%