Multilevel Assessment of Seagrass Response to Thermal Stress: Stress Memory and Epigenetic Changes

Thesis

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Hung Manh Nguyen

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Doctor of Philosophy

Biology

Open University UK

School of Life, Health and Chemical Sciences

Stazione Zoologica Anton Dohrn

Department of Integrative Marine Ecology

Director of studies

Dr. Gabriele Procaccini

Stazione Zoologica Anton Dohrn
Department of Integrative Marine Ecology
Villa Comunale 80121
Napoli
Italy

Supervisors

Dr. Lázaro Marín-Guirao

Spanish Institute of Oceanography
Oceanographic Center of Murcia
San Pedro del Pinatar 30740
Murcia
Spain

Dr. Mathieu Pernice

University of Technology Sydney
Climate Change Cluster
Ultimo 2007
Sydney
Australia

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Abstract

Seagrasses are being threatened globally due to human-induced environmental changes with ocean warming being one of the main players. A better understanding of the interaction between seagrasses and warming is, therefore, crucial to secure a sustainable future for these paramount foundation species.

Through a literature review and a series of *ad hoc* mesocosm and field experiments using four seagrass species from the northern (i.e. Mediterranean: *Posidonia oceanica, Cymodocea nodosa*) and southern (i.e. Australia: *Posidonia australis* and *Zostera muelleri*) hemisphere and by applying multi- and inter-disciplinary approaches [i.e. photo-physiology, growth, pigments, gene expression (RT-qPCR and RNA-seq), and genome screening (ddRADseq)], here I (***i***) identify potential commonalities in the effects of warming and the responses of seagrasses across different levels ranging from molecular to planetary [e.g. warming strongly affects seagrasses at all levels while seagrass responses diverge amongst species, populations and over depths]; (***ii***) demonstrate the existence of thermal stress memory for the first time in seagrasses [e.g. non-primed plants suffered significant reduction in photosynthetic capacity, leaf growth and pigments content, while heat-primed plants were able to cope better with recurrent stressful events]; (***iii***) reveal the molecular mechanisms that potentially govern the formation (priming phase) and activation (memory phase) of thermal stress memory in seagrasses [e.g. response to warming of non-primed plants required the involvement of several cellular compartments and processes while in heat-primed plants the response focused on a more limited group of processes]; (***iv***) explore the involvement of epigenetic modifications (DNA methylation and histone modifications in particular) in thermal stress response and thermal stress memory in seagrasses [e.g. results from gene expression analyses demonstrated a high activation of genes related to epigenetic modifications and thermal stress memory during the triggering event in both heat-primed and non-primed plants]; (***v***) broaden our knowledge in interspecific divergences in response to warming among seagrass species (northern versus southern hemisphere seagrasses and climax versus pioneer species) [e.g. results showed that northern hemisphere *Posidonia* better dealt with warming than its southern hemisphere counterpart and, in both hemispheres, pioneer seagrasses were more thermal tolerant than climax ones]; (***vi***) investigate the molecular basis of local adaptation to high temperature condition in seagrasses [e.g. ddRADseq data analysis identified several outlier loci potentially responsible for thermal stress response and epigenetics]; and finally (***vii***) suggest future directions for seagrass research

[e.g. studies involving additional species and populations, investigation of the seagrass holobiont, seagrasses as a solution to mitigate climate change among others].

This thesis provides novel insights into the field of seagrass ecology and yields potential implications for future seagrass conservation and restoration activities in an era of ocean warming.

Keywords: Seagrass, *Posidonia oceanica*, *Cymodocea nodosa*, *Posidonia australis*, *Zostera muelleri*, Marine heatwave, Heat stress, Stress memory, Epigenetics.

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For one of the biggest milestones in my life,

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This achievement would not be possible without you, ***THANK YOU SO VERY MUCH!***

‘Seagrasses are fascinating creatures that have been around for millions of years.
However, the ocean has been changing fast making their future to be uncertain.
I truly hope that our children's children will still be able to see them
not in documentaries or in the form of specimens in museums
but continue to thrive in the ocean.’

Hung Manh Nguyen

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List of figures and tables

List of original papers included in this thesis and author contribution

Chapter I + VI: The work presented in these chapters has been published previously:

Hung Manh Nguyen, Peter J. Ralph, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Seagrasses in an era of ocean warming: a review (2021) *Biological Reviews* **96**(5): 2009−2030. https://doi.org/10.1111/brv.12736

Author contributions: HMN, LMG, MP, GP conceptualized the review and selected literature. HMN wrote the first draft of the manuscript. PR, LMG, MP, GP revised the manuscript.

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Hung Manh Nguyen, Mikael Kim, Peter J. Ralph, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini (2020) Stress memory in seagrasses: first insight into the effects of thermal priming and the role of epigenetic modifications. *Frontiers in Plant Science* **11**:494. https://doi.org/10.3389/fpls.2020.00494

Author contributions: HMN, PR, LMG, MP and GP conceived and designed the experiment. HMN, MK performed the experiment. HMN analyzed the results. HMN wrote the first draft of the manuscript. MK, PR, LMG, MP and GP revised the manuscript.

Chapter III: The work presented in this chapter has been included in the following manuscript:

Hung Manh Nguyen, Uyen V. T. Hong, Miriam Ruocco, Emanuela Dattolo, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Beneath the memory: new insights on molecular mechanisms governing thermal stress memory in seagrasses. *In preparation* targeting to Journal of Ecology.

Author contributions: HMN, LMG, MP and GP conceived and designed the experiment. HMN performed the experiment. HMN analyzed the results with the help of UVTH, MR, ED, LMG and GP. HMN wrote the first draft of the manuscript. UVTH, MR, ED, LMG, MP and GP revised the manuscript.

Chapter IV: The work presented in this chapter has been published previously:

Hung Manh Nguyen, Fabio Bulleri, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Photo-physiology and morphology reveal divergent warming responses in northern and southern hemisphere seagrasses (2021). *Marine Biology* **168**, 129. https://doi.org/10.1007/s00227-021-03940-w

Author contributions: HMN, LMG, MP and GP conceived and designed the experiment. HMN performed the experiment. HMN analyzed the results with the help of FB, LMG and GP. HMN wrote the first draft of the manuscript. FB, LMG, MP and GP revised the manuscript.

Chapter V: The work presented in this chapter has been included in the following manuscript:

Hung Manh Nguyen, Miriam Ruocco, Emanuela Dattolo, AgostinoTomasello, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Signature of local adaptation to extreme environmental conditions in the Mediterranean seagrass *Posidonia oceanica*. *In preparation* targeting to Frontiers in Ecology and Evolution.

Author contributions: HMN, LMG, MP and GP conceived and designed the experiment. AT performed sample collection, morphological measurement and sea surface temperature analysis. MR and ED isolated DNA samples. HMN, MR and ED analyzed data. HMN wrote the first draft of the manuscript. MR, ED, AT, LMG, MP and GP revised the manuscript.

List of other publications during the candidature

Jessica Pazzaglia, **Hung Manh Nguyen***, Alex Santillán-Sarmiento, Miriam Ruocco, Emanuela Dattolo, Lázaro Marín-Guirao and Gabriele Procaccini (2021) The genetic component of seagrass restoration: what we know and the way forwards. *Water* 13(6): 829. https://doi.org/10.3390/w13060829

Author contributions: The paper was conceptualized by GP. JP and HMN led the bibliographic search, synthesis of information, and draft writing. GP and LMG led the paper writing. MR, ASS and ED equally contributed during the whole writing process.

* Co-first author

Chapter I – Introduction

The work presented in this chapter (1.3) has been published previously:

Hung Manh Nguyen, Peter J. Ralph, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Seagrasses in an era of ocean warming: a review (2021) *Biological Reviews* **96**(5): 2009−2030. https://doi.org/10.1111/brv.12736

1.1 Stress, stress response and stress memory in plants

1.1.1 Definitions of stress in plants

Historically, the 'stress' term in biology was initially originated from physics and mechanics where it was introduced into the theory of elasticity as the amount of force applied in a given unit area (Wardlaw, 1972). It is derived from the fact that if we apply a certain amount of force (i.e. stress) on an elastic material the effect will be reversible, however, if the material is plastic then the effect can be irreversible and the material can be broken. Similarly, in biology, when organisms are exposed to unfavorable conditions within a certain limit, they can return to their normal stage after the condition comes back to normal. If this certain limit is exceeded, it can result in permanent damage or even death (Mosa, Ismail and Helmy, 2017).

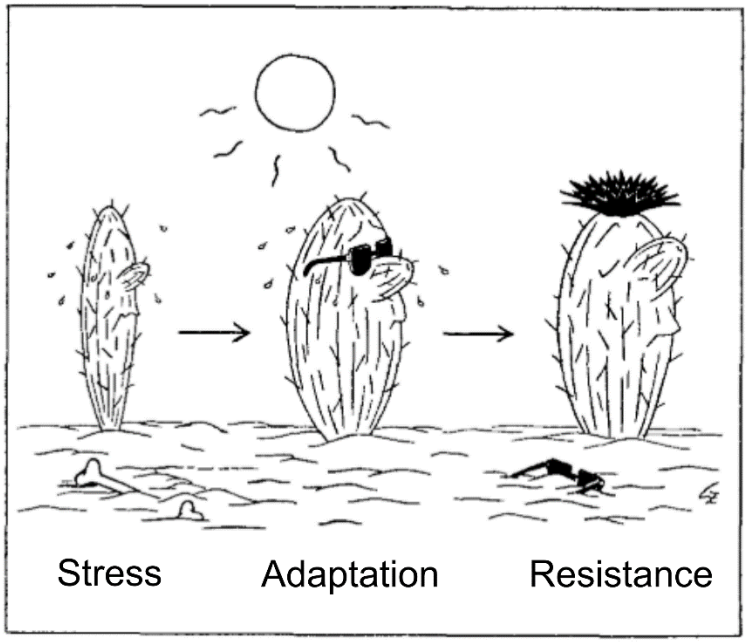

Figure 1.1 *A drawing illustrates stress in plants (Figure was taken and translated from Larcher 1987).*

To date, several definitions of plant stress have been formulated. For instance, plant stress was first defined by the Austrian botanist Walter Larcher in 1987 as "*a situation in which increasing conditions made upon a plant induce a disruption of functions, followed by normalization and improved resistance. If the limits of tolerance are exceeded and the adaptive capacity is overloaded, permanent damage or even death may result*" (see **Fig. 1.1**) (Larcher, 1987). It was also described as "*any unfavorable condition or substance that impacts or inhibits a plant's metabolism, growth or development*" (Lichtenthaler, 1996), "*changes in physiology*

that happen when species are exposed to extreme conditions that not necessarily result a threat to life but will produce an alarm response" (Gaspar *et al.*, 2002) or more recently as "*a condition caused by factors that tend to alter an equilibrium*" (Kranner *et al.*, 2010).

In this thesis, and adopting the concept of plant stress from Larcher (1987), I have used the term 'stress' for describing a plant response to changing conditions that impact the normal functions of plants, improve plant resistance, but that can cause permanent damage or even death to the plants if the limits of tolerance of the plants are exceeded.

Plant stress can be divided into different types following several categories as summarized in **Table 1.1**. As the main focus of this thesis is about thermal stress, I used the term 'stressor' to indicate an environmental factor (abiotic or biotic) that induces stress in plants.

Table 1.1 *Types of plant stress according to different categories (see related references for more explanations and examples).*

Category	**Plant stress**	**Description**	**Reference**
Type of factor	Abiotic stress	Caused by non-living factors (e.g. temperature, light, etc.)	(Mosa, Ismail and Helmy, 2017)
	Biotic stress	Caused by living factors (e.g. bacteria, insects, etc.)	
Effect of stress	Eustress	With positive effects on the plant	(Jansen and Potters, 2017)
	Distress	With negative effects on the plant	
Persistence of stress	Short-term stress	Endurable through persistent, acclimation, and adaptation	(Lichtenthaler, 1996)
	Long-term stress	Result in substantial and irreversible damages	
Origin of stress	Internal stress	From within the plant	(Kranner *et al.*, 2010)
	External stress	From outside the plant	

1.1.2 Plant stress responses

Being sessile organisms, plants cannot escape from potentially stressful conditions (except for the means of dispersals of fragments, gametes or seeds) that impact their performance, growth, development, productivity, and survival. As a consequence, plants have evolved sophisticated mechanisms to perceive these environmental stressors and to activate the appropriate responses

in order to survive and reproduce (Gaspar *et al.*, 2002; Kotak *et al.*, 2007; Kranner *et al.*, 2010). Plant stress responses can be influenced by several factors including the characteristics of the stressor and of the plant itself **(Fig. 1.2)**.

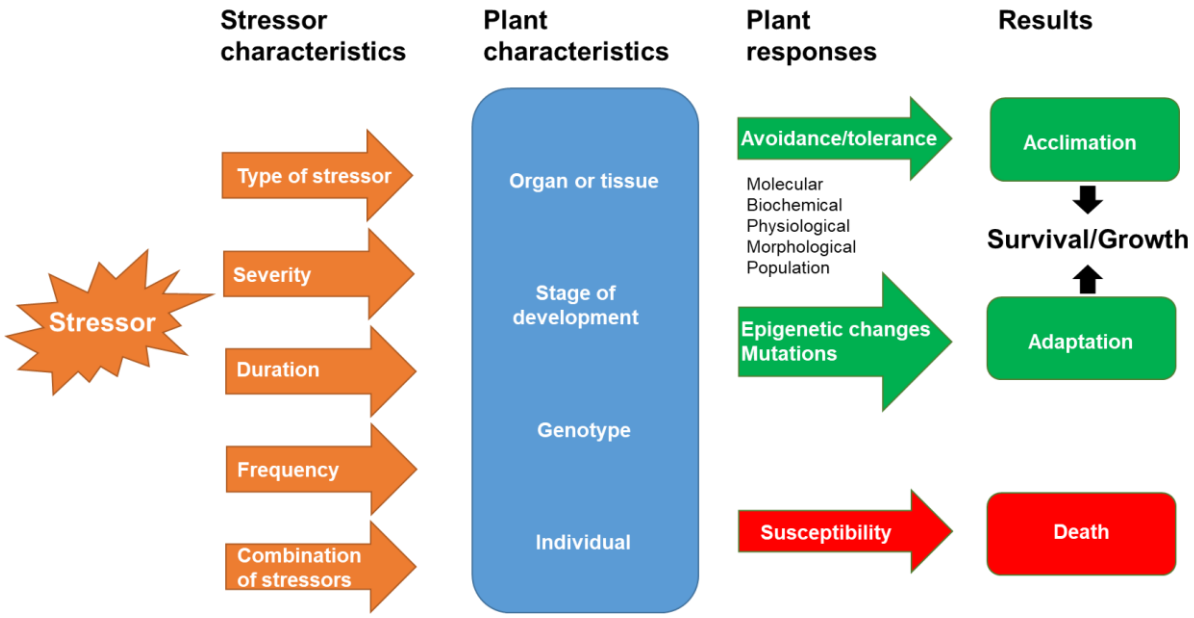

Figure 1.2 *Factors affect plant stress responses. Figure was created by adopting Figure 3 in Gaspar et al., (2002).*

Stressor characteristics determine how it impacts a plant and how the plant responds (**Fig. 1.2**). Different types of stressors can target different organs or can affect the same organ differently. For instance, heavy metals affect the root system while light stress occurs in the above-ground part of plants. In terrestrial plants, salinity, heavy metals, and drought are 'below-ground' stressors, however, their impacts on plants as well as the corresponding plant stress responses can vary (see a review by Kul *et al.*, 2020). On the other hand, the severity and duration of a stressful condition also strongly influence plant responses. Stress experienced by a plant can be extreme if the stressor is applied for a short duration but with high intensity or for a long duration with low intensity (Gaspar *et al.*, 2002). The frequency or number of times a plant is exposed to a stressor can also induce different responses (see section 1.1.3). Finally, a combination of different kinds of stressors also determines how plants respond and can result in 'additive', 'antagonistic', or 'synergistic' effects on a plant (see a review by Gunderson, Armstrong and Stillman, 2016).

Not only the characteristics of the stressor but also the intrinsic features of a plant, such as organ or tissue types, stages of development and genotype features are strong determinants of

plant stress responses (**Fig. 1.2**). Different organs or tissue types can be affected by a stressor in different ways and at different levels, therefore exhibiting divergences in response to the same stress condition. For example, high salinity levels caused different oxidative stress and antioxidant responses on different organs (roots, mature leaves and young leaves) of maize (*Zea mays L.*) seedlings (AbdElgawad *et al.*, 2016). Plants are either more or less sensitive to a particular stressor at specific developmental stages. Due to this developmental-stage sensitivity, a stress response may be induced directly by stress or indirectly by a stress-induced injury (Gaspar *et al.*, 2002). Developmental stages can also be defined as 'ages' and ages determine how stress signals are perceived and processed within the plant, which ultimately define the way the plant responds (see a review by Rankenberg *et al.*, 2021). Genotype is another important component for determining plant stress responses (Bray, Baily-Serres and Weretilnyk, 2000). In most cases, different genotypes exhibit different capabilities to cope with stressors (Bray, Baily-Serres and Weretilnyk, 2000). However, it also happens that different individuals of the same genotype (i.e. clones) can respond in different ways to the same stressor (Buchanan, Gruissem and Jones, 2015).

Plant stress responses range from mechanisms ensuring survival (avoidance and tolerance) to mechanisms that can lead to adaptation but also cause negative responses (epigenetic changes and clonal/somatic mutations). Avoidance prevents plants from exposure to the stressor while tolerance allows plants to deal with the stressor through adjustments of physiological and structural attributes. Both avoidance and tolerance are typically genetically dependent and ultimately result in acclimation (Bray, Baily-Serres and Weretilnyk, 2000; Gaspar *et al.*, 2002; Buchanan, Gruissem and Jones, 2015). On the other hand, stressors can induce epigenetic changes and/or mutations that enhance the stress tolerance level of plants and potentially result in adaptation (Gaspar *et al.*, 2002). Nevertheless, mutations (both clonal and somatic mutations) are not always beneficial as they can also introduce negative responses such as neoplastic progression leading to true cancer cells or true generalized cancers at the shoot level, with death as an ultimate consequence (Gaspar *et al.*, 2002). It is important to distinguish between acclimation (proximal) and adaptation (ultimate). Acclimation occurs within the life span of a single individual while adaptation occurs at the population level over many generations. In addition, plant stress responses can also be categorized by biological organizations (e.g. molecular, biochemical, physiological, etc. see also section 1.3.3). When the stress exceeds the limit of tolerance of the plant, the stressor can ultimately lead to death (**Fig. 1.2**). Plant stress responses also depend on the ability of the plant to exhibit different

phenotypic states in terms of chemistry, physiology, morphology, and gene expression when it is exposed to different environmental conditions (Pazzaglia, Reusch, *et al.*, 2021). This phenomenon is known as phenotypic plasticity and can contribute to plant stress responses at the individual level but also at the population level, especially in the case of rapid environmental changes (see Pazzaglia, Reusch, *et al.*, 2021 for a comprehensive review on seagrass phenotypic plasticity under rapid global changes).

1.1.3 Stress memory in plants

Repeated exposure to stressors can alter subsequent plant stress responses. A stressful condition can erode plant fitness, increasing its sensitivity when the stress recurs, or it can train the plant, increasing its tolerance to a subsequence stress exposure. The later phenomenon can be related to the so-called 'plant stress memory' which is defined as the capacity of plants experiencing recurrent stress to 'remember' past stressful events and better respond when stressful conditions occur again (Bruce *et al.*, 2007). Previous studies in terrestrial plants demonstrated that, in some cases, plants exposed to cyclic or episodic perturbations have shown increased tolerances when the stress recurs and these modified stress responses are commonly known as hardening, priming, conditioning, or acclimation (Zwiazek, 1991; Goh, Gil Nam and Shin Park, 2003; Biber, Kenworthy and Paerl, 2009; Kinoshita and Seki, 2014). Stress memory includes stress-induced structural, genetic and biochemical modifications (Baldwin and Schmelz, 1996; Bruce *et al.*, 2007; Jaskiewicz, Conrath and Peterhälnsel, 2011; Yakovlev *et al.*, 2011). It has been suggested that stress memory can last from several days to months and even years, and in some cases, it can be transmitted to the next generation (Baldwin and Schmelz, 1996; Iqbal and Ashraf, 2007; Rendina González *et al.*, 2018).

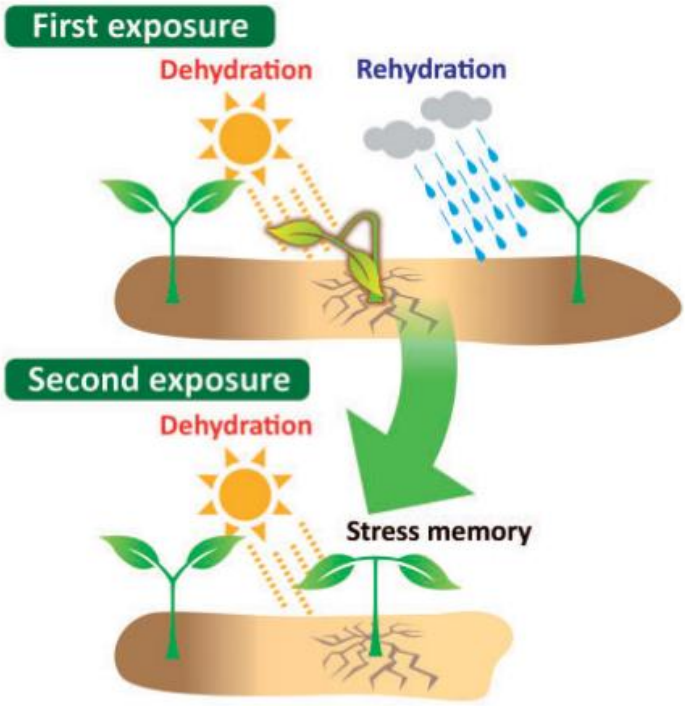

Figure 1.3 *An example of stress memory in plants. For example, a plant that experiences a period of drought wilts under the dehydration stress and then recovers after rehydration (upper panel); during second drought stress, the plant 'remembers' the past drought experience, allowing it to achieve better resistance to dehydration and improve its survival prospects (lower panel) (Ding, Fromm and Avramova, 2012). Figure was taken from Kinoshita and Seki (2014).*

1.1.4 Epigenetic modifications in plant stress response and plant stress memory

Epigenetic modifications are molecular modifications that alter gene expression in response to internal (e.g. ontogenetic processes) or external (e.g. environmental changes) triggers, without changes in the underlying DNA sequence (Bossdorf, Richards and Pigliucci, 2008). Epigenetic modifications occur in the form of DNA methylation, histone modifications and noncoding micro RNAs (Bossdorf, Richards and Pigliucci, 2008; Bonasio, Tu and Reinberg, 2010). To date, DNA methylation is the most frequently studied and best understood epigenetic mechanism in plants (Chinnusamy and Zhu, 2009; Kinoshita and Seki, 2014; Liu *et al.*, 2015).

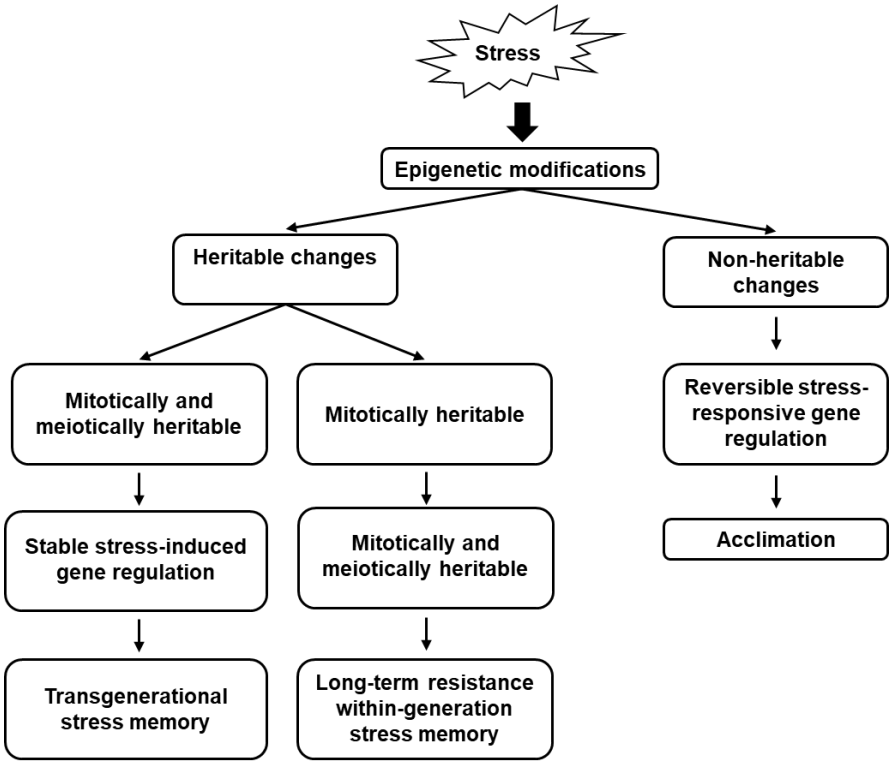

Figure 1.4 *Epigenetic modifications with plant stress response and plant stress memory. Figure was created by adopting Figure 1 in Chinnusamy and Zhu (2009).*

Epigenetic modifications induce changes in gene expression to cope with stresses (Wada *et al.*, 2004; Yaish, Colasanti and Rothstein, 2011; Dowen *et al.*, 2012; Greco *et al.*, 2013; Secco *et al.*, 2015). For instance, a study on tobacco plants (*Nicotiana tabacum*) subjected to pathogen stress demonstrated that levels of DNA methylation change upon exposure to stress, and that these changes highly correspond with the expression of stress-responsive genes (Wada *et al.*, 2004). Another study provided shreds of evidence of DNA methylation changes in regulating gene expression in response to salicylic acid (Dowen *et al.*, 2012).

Epigenetic modifications can be either not heritable and result in plant acclimation or heritable and formulate stress memory (**Fig. 1.4**) via activating, enhancing or speeding up responses to coping with recurrent stressors (Crisp *et al.*, 2016). Epigenetic modifications can also increase phenotypic plasticity and accelerate adaptation in plants (Verhoeven, vonHoldt and Sork, 2016; Richards *et al.*, 2017). Bruce *et al.*, (2007) suggested two possible mechanisms for the formation of plant stress memory including (*i*) accumulation of signaling proteins or transcription factors and (*ii*) epigenetic modifications. While the first mechanism mediates more transient or short-term effects, epigenetic modifications can enable longer stress memory (Bruce *et al.*, 2007). Indeed, it has been demonstrated in several studies in terrestrial plants that

DNA methylation can transmit the effect of stressors on gene expression (i.e. stress memory) to the next generations. For instance, when Molinier *et al.*, (2006) treated *Arabidopsis thaliana* with short-wavelength radiation (ultraviolet-C), they observed epigenetic changes (in this case, enhanced homologous recombination) that lead to increased genomic flexibility of untreated generations and that may increase the potential for adaptation for the subsequent generations (see also reviews by Angers, Castonguay and Massicotte, 2010; Iwasaki and Paszkowski, 2014; Rendina González *et al.*, 2016).

1.2 Seagrass biology, ecology and evolution

1.2.1 Seagrass biology, ecology and evolution

Seagrasses are a unique group of angiosperms that have recolonized the marine realm 60–90 million years ago on at least three separate events (Les, Cleland and Waycott, 1997; Waycott *et al.*, 2004) (**Fig. 1.5**). To overcome the numerous challenges of a submerged lifestyle in the marine environment, seagrasses have developed a range of specialized adaptive characteristics (Invers, Perez and Romero, 1999; Borum *et al.*, 2007; Wissler *et al.*, 2011; Hogarth, 2015; Olsen *et al.*, 2016). Leaves of most seagrass species are narrow and strap-shaped (linear) and young leaves are protected by leaf sheaths, a design that would tend to limit damage from wave and ocean currents (Hogarth, 2015). Seagrass leaves lack stomata and have a very thin cuticle because there is no need to protect blades from drying (Olsen *et al.*, 2016). Moreover, thin cuticle layers also facilitate gas and nutrient exchange, which is crucial for sustaining an aerobic condition in the belowground parts to withstand toxic sedimentary environments (Borum *et al.*, 2007). Different from typical land plants, photosynthesis in seagrasses takes place almost exclusively within the epidermis (i.e. the outermost layer of leaf cells) (Hogarth, 2015) and uses mostly carbonic acid and bicarbonate instead of CO_2 (Invers, Perez and Romero, 1999). Other special mechanisms have also been evolved in seagrasses to deal with high salinity levels of seawater (Walker and McComb, 1990).

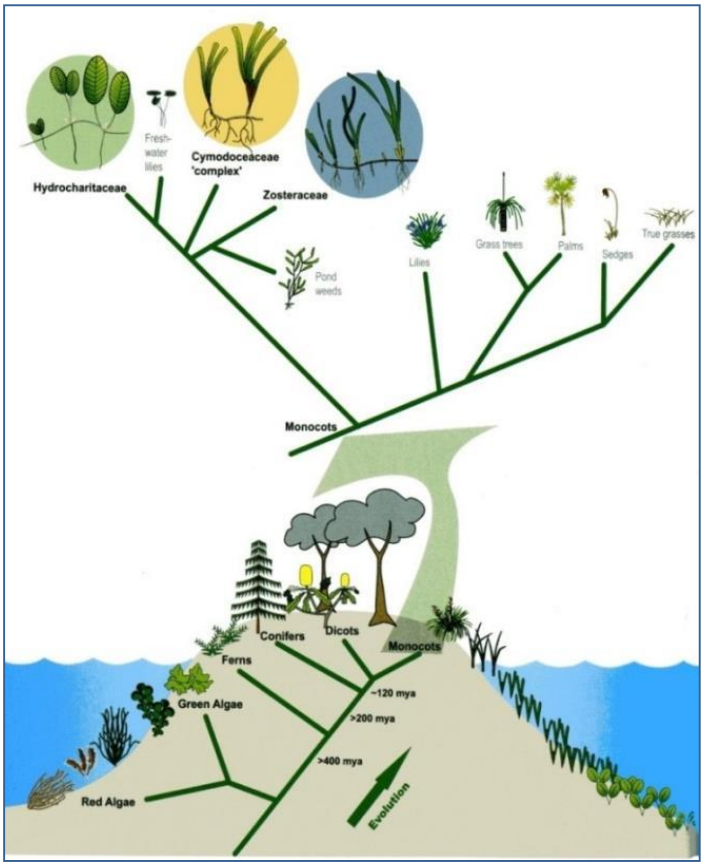

Figure 1.5 *The evolution of seagrasses (Waycott et al., 2004).*

Even with a surprisingly small number of species (~60–70 species), seagrasses are found along thousands of kilometers of the sedimentary shorelines from sub-Artic to tropical regions with the regions of insular Southeast Asia and north tropical Australia being the hotspots of seagrass biodiversity with an average of 12-15 species (Short *et al.*, 2007) (**Fig. 1.6**).

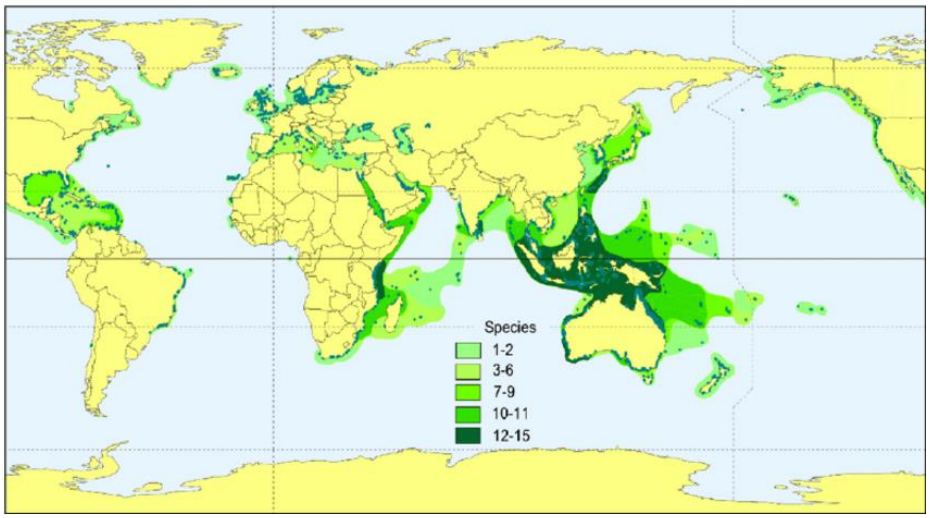

Figure 1.6 *The distribution of seagrasses in the world (Short et al., 2007).*

Seagrasses belong to four families including *Posidoniaceae*, *Zosteraceae*, *Cymodoceae*, *Hydrocharitaceae* (Green and Short, 2003) thriving in both tropical and temperate regions (Short *et al.*, 2007)**.** Most seagrass species grow in large meadows and exhibit a mixture of sexual and clonal reproduction (Short *et al.*, 2007). In terms of plant structure, a seagrass plant consists of horizontal or vertical shoots which are composed of leaf bundles, orthotropic or plagiotropic rhizomes and roots (**Fig. 1.7**). Seagrasses range from small species with thin leaves (e.g. *Halophila*, *Halodule*) to large species with thick leaves (e.g. *Thalassia*, *Enhalus*, and *Posidonia*) (Papenbrock, 2012).

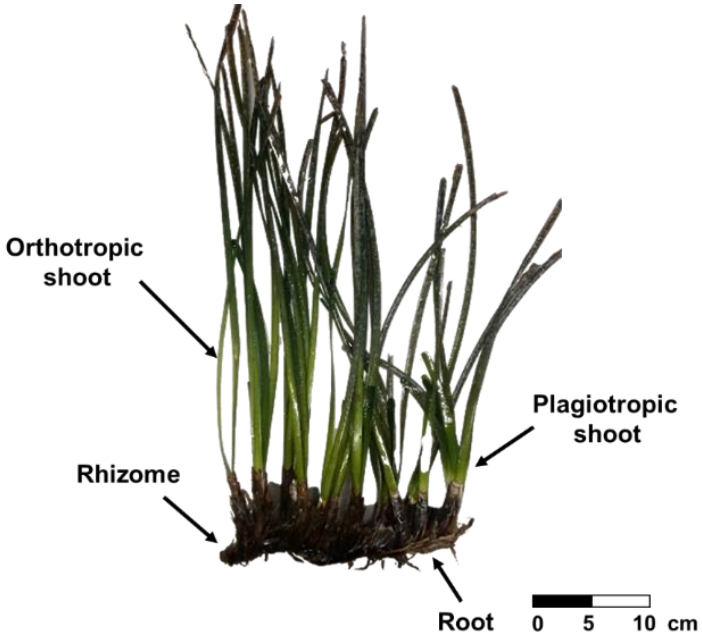

Figure 1.7 *Plant structure of Posidonia oceanica.*

1.2.2 The importance of seagrasses

As foundation species, seagrasses form a paramount coastal ecosystem, the underwater seagrass meadows supply a wide range of essential ecosystem services. Seagrass meadows are highly productive, recycle nutrients, enrich the water with oxygen, provide the habitat for economically important fish and crustaceans, and are crucial in the production and burial of organic carbon, acting as efficient carbon traps (Orth *et al.*, 2006; Fourqurean *et al.*, 2012). They also help to stabilize coastal sediments and to prevent coastal erosion (Orth *et al.*, 2006) and reduce pathogenic threats for humans, fish, and invertebrates (Lamb *et al.*, 2017). Economically, seagrasses are ranked as one of the most valuable marine ecosystems on Earth with an estimated value of $1.9 trillion per annum only for their function in the global carbon cycle (Costanza *et al.*, 2014). Given that a vast majority of the human population inhabits

coastal areas, seagrasses directly or indirectly influence the livelihoods of billions of people worldwide (Bertelli and Unsworth, 2014). Due to their importance, seagrass meadows have been legally recognized in the European Union Water Framework Directive as a key coastal ecosystem and they have been identified as bio-indicators of ecosystem quality (Marbà *et al.*, 2013). Moreover, seagrasses represent one of the most significant natural carbon sinks on Earth (Fourqurean *et al.*, 2012; Macreadie and Hardy, 2018) playing hence a crucial role to mitigate climate change (Gattuso *et al.*, 2018; Bertram *et al.*, 2021).

1.2.3 Threats to seagrass ecosystems

Despite their critical values, seagrass meadows are suffering a global decline driven mainly by the growing number of pressures linked directly or indirectly to human activities [e.g. ocean warming, coastal modification, water quality degradation, aquaculture, boat damage, agricultural runoff, dredging, etc. (Orth *et al.*, 2006; Waycott *et al.*, 2009; Grech *et al.*, 2012)]. Across the globe, human activities are wiping out over 100 km^2 of seagrass meadows per year. As a result, nearly 20% of their areal extent has been lost since 1884 (Dunic *et al.*, 2021). Indeed, ten seagrass species (~14% of the total seagrass species) have already been listed at risk of extinction while the other three species have been listed as endangered species (Short *et al.*, 2011). For instance, the Mediterranean endemic species (*Posidonia oceanica*) already lost approximately 13-50% of its total areal extent since the mid-19th century (Telesca *et al.*, 2015). Recently, the rate of seagrass loss, at least at a regional scale, seems to have been lessened as a result of the implementation of management plans, such as the European environmental protection measures (de los Santos *et al.*, 2019). However, the decline of *P. oceanica* meadows is likely to continue as this species' ecological functions have even been predicted to go extinct by the end of this century (Marbà and Duarte, 2010; Chefaoui, Duarte and Serrão, 2018).

1.3 Seagrasses in an era of ocean warming

1.3.1 Ocean warming is happening at an alarming rate

Covering over 70% of the Earth's surface, the ocean plays a fundamental role in the Earth's climate, and it is a habitat for an estimated 50–80% of all life on Earth. Nevertheless, the ocean is warming at an alarming rate, especially in coastal areas where the temperature increase has been reported as much as 0.17 ± 0.11 ºC/decade (Liao *et al.*, 2015). In semi-closed seas (e.g. the Red Sea and the Mediterranean Sea), the rate is even faster (Nguyen, Yadav, *et al.*, 2020).

Ocean warming is not only reflected by a pronounced upward trend in the average seawater temperature but also by an increased frequency of extreme climatic events, known as marine heatwaves (MHWs) (Oliver *et al.*, 2018). MHWs are extreme warm periods that last for at least five days with a temperature level exceeding the 90th percentile, based on three-decade historical baseline temperature values (Hobday *et al.*, 2016). Ocean warming, and especially MHWs, is already causing catastrophic consequences in coastal benthic communities worldwide (Coma *et al.*, 2009; Harley *et al.*, 2012; Wernberg *et al.*, 2016). Indeed, the impact of MHWs is of more concern than the increase in average seawater temperature because organisms are generally more vulnerable to sudden temperature changes than to progressive changes (Smale *et al.*, 2019). Therefore, MHWs may trigger destructive chronic impacts on marine creatures that can result in shifts in species distributions and even local extinctions (Easterling *et al.*, 2000).

1.3.2 Seagrasses are being strongly impacted by ocean warming

A recent study listed seagrasses as one of the habitat-forming species that are likely to disappear as a consequence of climate change, or more specifically ocean warming (Trisos, Merow and Pigot, 2020). Seagrass die-offs, as a consequence of MHWs, have been reported for different species worldwide including *P. oceanica* (Marbà and Duarte, 2010), *Zostera marina* (Reusch *et al.*, 2005; Jarvis, Brush and Moore, 2014), and *Amphibolis antarctica* (Seddon, Connolly and Edyvane, 2000; Arias-Ortiz *et al.*, 2018; Strydom *et al.*, 2020). The observed mass mortality of several seagrass populations after extreme MHWs (Arias-Ortiz *et al.*, 2018; Strydom *et al.*, 2020) and the projected warming trend for the next decades have motivated the predictions of a functional extinction of seagrass meadows in the near future (Marbà and Duarte, 2010; Jordà, Marbà and Duarte, 2012; Chefaoui, Duarte and Serrão, 2018). It is worth mentioning that while MHWs have already caused extensive local extinctions of seaweed species across hundreds of kilometers (Smale, 2020), the comparative effects on seagrasses might appear to be lesser. This sheds light on the need to further investigate the mechanisms driving potential differences in the resilience between seagrasses and other marine macrophytes.

1.3.3 Effects of warming and seagrass responses

Here, I reviewed previous studies on the effect of temperature on seagrasses to identify potential commonalities in the effects of warming as well as the responses of seagrasses across

different levels of biological organization: molecular, biochemical/physiological, morphological/population, and ecosystem/planetary (**Fig. 1.8** & **Fig. 1.9**).

The molecular basis of seagrass responses to a warming ocean can uncover seagrass traits that can be correlated to their persistence under changing climatic conditions (Procaccini, Olsen and Reusch, 2007; Reusch and Wood, 2007). Innovative molecular experiments in parallel with routine physiological and phenological/morphological measurements can provide early warning measures to detect changes in the ecological status of seagrass meadows well before any signs of mortality appear (Procaccini, Olsen and Reusch, 2007; Pernice *et al.*, 2015; Schliep *et al.*, 2015; Ceccherelli *et al.*, 2018). The extensive application of gene expression studies (transcriptomics) over the last decade (Davey *et al.*, 2016) and the availability of two seagrass genomes, *Z. marina* (Olsen *et al.*, 2016) and *Z. muelleri* (Lee *et al.*, 2016), have greatly fostered our understanding of seagrass responses to environmental changes at the molecular level. We are now much closer to integrating the fields of seagrass ecophysiology and ecological genomics, as anticipated almost a decade ago (Procaccini *et al.*, 2012). To date, gene expression studies [quantitative reverse transcription polymerase chain reaction (qRT-PCR) and RNA sequencing (RNA-seq)] have been conducted for only a handful of seagrass species. Large transcriptomic differences observed in seagrasses that had recovered from long-term acute temperature stress (3 weeks at 26℃) identified transcriptomic resilience as a predictor of thermal adaptation (Franssen *et al.*, 2011; Jueterbock *et al.*, 2016). Other studies found different transcriptomic responses to short-term acute temperature stress (5 days at 32℃) leading to the identification of molecular mechanisms involved in maintaining photosynthetic stability and respiratory acclimation of seagrasses under heat stress (Marín-Guirao *et al.*, 2017). Investigations of the responses of seagrasses to thermal stress have also revealed some interspecific similarities (see **Fig. 1.7**), comparable to those observed in the heat response of terrestrial plants, including refolding of proteins, activation of oxidative-stress defense, and cell wall fortification (Franssen *et al.*, 2011; Gu *et al.*, 2012; Marín-Guirao *et al.*, 2016; Marín-Guirao *et al.*, 2017).

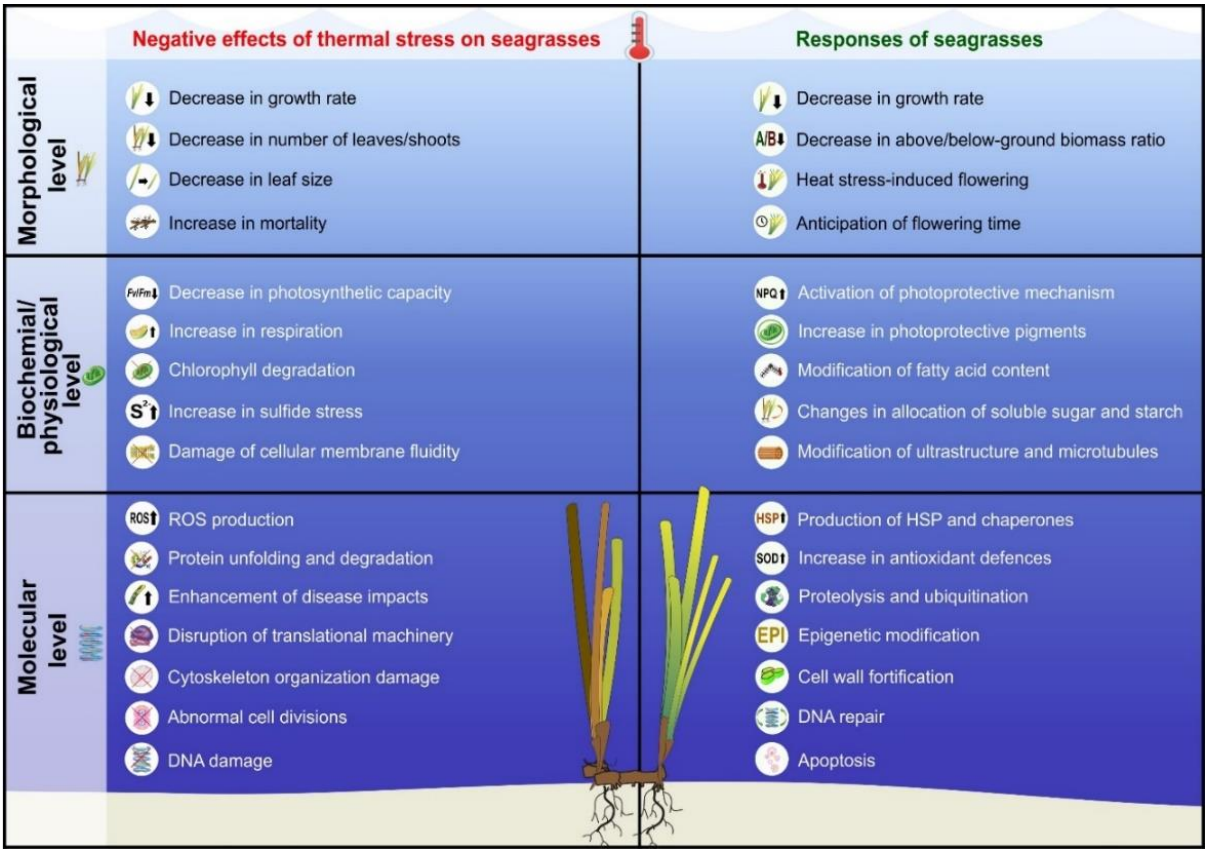

Figure 1.8 *The common effects of thermal stress and responses of seagrasses at molecular, biochemical/physiological and morphological level.*

Biochemical and physiological responses to thermal stress in seagrasses have been studied extensively since the 1990s, with earlier studies summarized in previous reviews (Bulthuis, 1987; Lee, Park and Kim, 2007; Koch *et al.*, 2013). Thermal stress tends to inhibit photosynthetic activity while simultaneously enhancing respiration. Recent findings suggest that extreme temperature changes could cause the degradation of chlorophylls as well as affect the fluidity of the cellular membrane, among other impacts (e.g. see Marín-Guirao *et al.*, 2016, 2017, 2018; Yaping *et al.*, 2019; Nguyen, Kim, *et al.*, 2020; Nguyen, Yadav, *et al.*, 2020). In return, seagrasses tend to activate protective mechanisms such as the accumulation of photo-protective pigments and modification of fatty acid contents, among others (**Fig. 1.8;** see also Koch *et al.*, 2007; Beca-Carretero, Guihéneuf, *et al.*, 2018; Marín-Guirao *et al.*, 2018).

Warming has a strong effect on seagrass growth rates (Collier, Uthicke and Waycott, 2011; Olsen *et al.*, 2012; Collier and Waycott, 2014; Hammer *et al.*, 2018; Marín-Guirao *et al.*, 2018; Nguyen, Kim, *et al.*, 2020; Nguyen, Yadav, *et al.*, 2020), leaf traits (York *et al.*, 2013), and leaf/shoot number (Mayot, Boudouresque and Leriche, 2005; Nejrup and Pedersen, 2008; Beca-Carretero, Olesen, *et al.*, 2018). While modifications of the above-ground part can result

in a reduction of the above- to below-ground biomass ratio (York *et al.*, 2013; Collier *et al.*, 2017; Marín-Guirao *et al.*, 2018), warming can also increase the above- to below-ground biomass ratio in rapid-growing seagrass species (Collier, Uthicke and Waycott, 2011; Marín-Guirao *et al.*, 2018), reducing the biomass of non-photosynthetic (below-ground) tissues and increasing photosynthetic biomass to offset the negative impacts of heat stress-enhanced respiration (**Fig. 1.8**).

Population responses to warming of seagrasses are summarized in **Fig. 1.9**. **Acclimatization/adaptation (1→2):** seagrass meadows can acclimatize or adapt to environmental changes. Seagrass meadows that normally experience large fluctuations in environmental parameters (such as temperature, light, etc.) are more likely to survive ocean warming (Massa *et al.*, 2009; Collier, Uthicke and Waycott, 2011; Marín-Guirao *et al.*, 2018; Soissons *et al.*, 2018). In addition, the resilience of seagrass meadows depends on the genetic diversity of the populations (Williams, 2001; Hughes and Stachowicz, 2004; Ehlers, Worm and Reusch, 2008). **Escape in space and time (1→3):** Warming can alter flowering in seagrasses, thus providing an escape mechanism through sexual reproduction and seed dispersal. Warming induces flowering in some species (Diaz-Almela, Marbà and Duarte, 2007; Ruiz *et al.*, 2018) and advances the onset of flowering in other cases (Blok, Olesen and Krause-Jensen, 2018; Marín-Guirao *et al.*, 2019). Through sexual reproduction, warming induces an increase in genetic diversity of seagrass populations, thus potentially sustaining the resilience of seagrass meadows (Massa *et al.*, 2009; Soissons *et al.*, 2018). Not only in space, sexual reproduction provides seagrasses with an escape mechanism also in time. For instance, in some seagrass species, their seeds have a resting stage, which can last up to two years [e.g. *Zostera*, *Halodule* and *Syringodium* (Orth *et al.*, 2000)]. **Die-off: (1 → 4):** when the environmental temperatures are too extreme, they can be deleterious. Massive die-offs of seagrasses due to ocean warming, especially after MHWs, have been reported recently (Marbà and Duarte, 2010; Arias-Ortiz *et al.*, 2018; Strydom *et al.*, 2020). Increased mortality due to warming has been observed in adult plants and also in seedlings (Olsen *et al.*, 2012; Guerrero-Meseguer, Marín and Sanz-Lázaro, 2017; Hernán *et al.*, 2017; Pereda-Briones, Terrados and Tomas, 2019). After such massive mortality, seagrass meadows can recover naturally in some cases, although the recovery can take decades, especially for slow-growing species (O'Brien *et al.*, 2018). **Recolonization (4→2):** after a local extinction, the same seagrass population can potentially recolonize its former space by asexual reproduction (i.e. vegetative recruitment) of acclimatized/adapted plants, and/or by sexual reproduction through seed dispersal and seed dormancy (Diaz-Almela,

Marbà and Duarte, 2007; Blok, Olesen and Krause-Jensen, 2018; Ruiz *et al.*, 2018; Marín-Guirao *et al.*, 2019). This phenomenon has been documented following physical disturbance (Olesen *et al.*, 2004), warm-induced anoxia events (Plus, Deslous-Paoli and Dagault, 2003), or microalgal blooms (Lee *et al.*, 2007). **New colonization (4→5):** the disappearance of local populations of seagrasses due to ocean warming can create an empty niche for colonization by new thermally tolerant species. The rapid expansion of the tropical seagrass *H. stipulacea* in the Mediterranean is an example of this phenomenon (Lipkin, 1975; Gambi, Barbieri and Bianchi, 2009). Finally, in an era of rapid ocean change, the future of seagrasses is indeed difficult to forecast (**2→4** & **5→4**). Although some thermal-adapted/thermal-tolerant seagrasses could potentially survive and even benefit from ocean warming in the near future (Saha *et al.*, 2019; Nguyen, Yadav, *et al.*, 2020), the existence of these species/populations may be challenged due to the ongoing increased frequency of extreme climatic events and human-induced impacts on the marine environment (Ralph *et al.*, 2007; Oliver *et al.*, 2019). However, seagrass management and restoration could effectively contribute to sustaining seagrass meadows and their services into the future (Reynolds, McGlathery and Waycott, 2012; Ramesh *et al.*, 2019; Valdez *et al.*, 2020). Will warm-adapted/thermal-tolerant seagrasses survive future ocean change? To the best of my knowledge, an answer to this question remains open.

At the ecosystem/planetary level, warming can switch seagrass ecosystems from autotrophic to heterotrophic (Burkholz, Duarte and Garcias-Bonet, 2019) enhancing carbon dioxide and methane fluxes from seagrass meadows into the atmosphere (Burkholz, Garcias-Bonet and Duarte, 2020). Therefore, this phenomenon not only reduces the ability of seagrass ecosystems to buffer climate warming but also contributes to it (see **Fig. 1.9**). After the massive mortality of seagrasses in Shark Bay (Australia), substantial quantities of carbon dioxide were released into the atmosphere contributing to the greenhouse effect (Arias-Ortiz *et al.*, 2018). A trophic transformation is not always solely dependent on ocean warming, but it depends also on seagrass species, co-occurring stressors (Macreadie and Hardy, 2018), and sometimes the diversity of seagrass meadows (Burkholz, Duarte and Garcias-Bonet, 2019). Warming threatens the distribution of large and long-lived species of seagrass [e.g. *P. oceanica* (Marbà and Duarte, 2010; Jordà, Marbà and Duarte, 2012)] while favours the expansion of some small rapid-growing species [e.g. *H. stipulacea* (Georgiou *et al.*, 2016; Nguyen, Yadav, *et al.*, 2020)]. Thus, warming is accelerating the tropicalization of temperate meadows (Hyndes *et al.*, 2016). When the ecosystem functions of seagrasses rely strongly on their primary production (i.e. their biomass), a switch from large species to small species due to warming could significantly

reduce their values in terms of ecosystem services as well as blue carbon storage (blue carbon is the carbon stored in coastal and marine ecosystems).

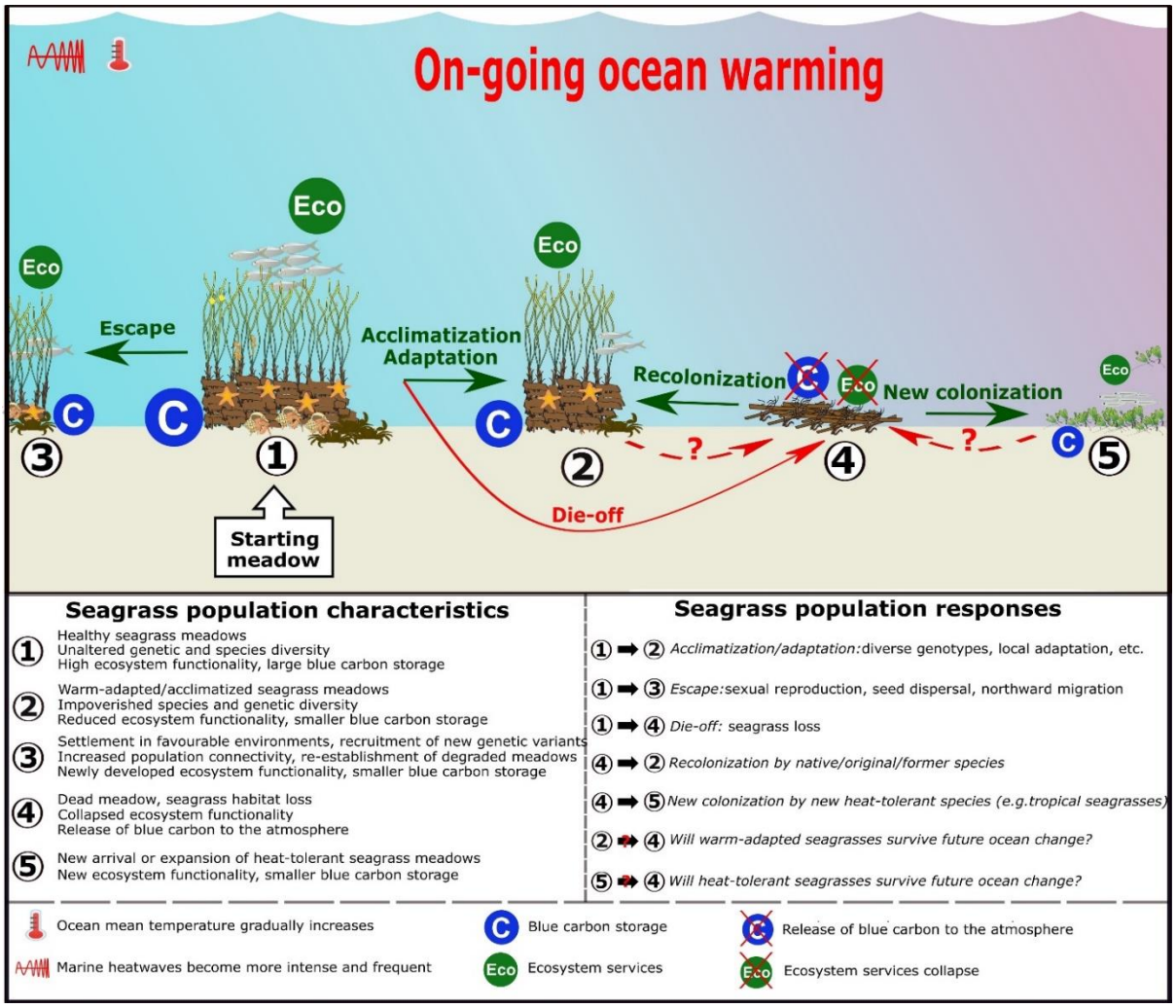

Figure 1.9 *Conceptual diagram summarizing the fate of seagrasses in the face of ocean warming as illustrated by the case of P. oceanica in the Mediterranean Sea.*

Under natural conditions, environmental stressors do not occur individually, but concurrently and synergistically (Sandifer and Sutton-Grier, 2014). Hence, studying the interaction of ocean warming with other stressors is essential for the comprehensive and precise understanding of seagrass responses to the changing environment (Gunderson, Armstrong and Stillman, 2016). See Nguyen *et al.*, (2021) for a comprehensive review about combined effects of warming and other stressors on seagrasses.

1.4 The thesis

1.4.1 Main aims of the thesis

As ocean warming continues to rise, seagrasses are facing a critical time in their evolutionary history in which their existence and their related ecological services will depend on our actions including research, restoration, and management activities. In addition, while the role of epigenetic modifications and thermal stress memory has been widely studied in terrestrial plants, these studies are lagging far behind in seagrasses. To the best of my knowledge, by the time I started this thesis, studies supporting the effect of thermal stress memory in seagrasses were not available and only one transcriptomic study suggested the involvement of epigenetic modification in the responses of seagrasses to thermal stress (Marín-Guirao *et al.*, 2017). A better understanding of the effect of thermal stress memory and thermal-induced epigenetic modifications will not only fill important gaps in our knowledge on seagrass biology but will also hold great potential implications for the management and restoration of seagrass meadows. According to that, the goal of my thesis is to improve the knowledge related to the relationship between seagrasses and warming, and to give insights into thermal stress memory that can foster the restoration of degraded meadows and the reinforcement of natural populations. More specifically, with this thesis, I aim to ***(i)*** demonstrate the existence of the effect of thermal stress memory in seagrasses, ***(ii)*** reveal the molecular mechanisms that govern thermal stress memory in seagrasses, ***(iii)*** explore the involvements of epigenetic modifications in thermal stress response and thermal stress memory in seagrasses, ***(iv)*** broaden our knowledge in inter-specific divergences in response to warming among seagrass species, ***(v)*** study local adaptation to high-temperature condition in seagrasses, and finally ***(vi)*** suggest directions for future seagrass research.

To this end, a series of *ad hoc* mesocosm and a field experiment were conducted using four seagrass species from both the northern (Mediterranean Sea: *Posidonia oceanica* and *Cymodocea nodosa*) and southern (Australian Sea: *Posidonia australis* and *Zostera muelleri*) hemisphere (see section 1.4.2 for more details on model species). To fulfill my goal, I used several approaches including photosynthesis, growth, pigment, and molecular tools (i.e. RT-qPCR, RNA-seq and ddRAD-seq) to assess the effects of warming on seagrasses as well as seagrasses' response (see section 1.4.3 for more details on experiments and measurements).

1.4.2 Targeted species

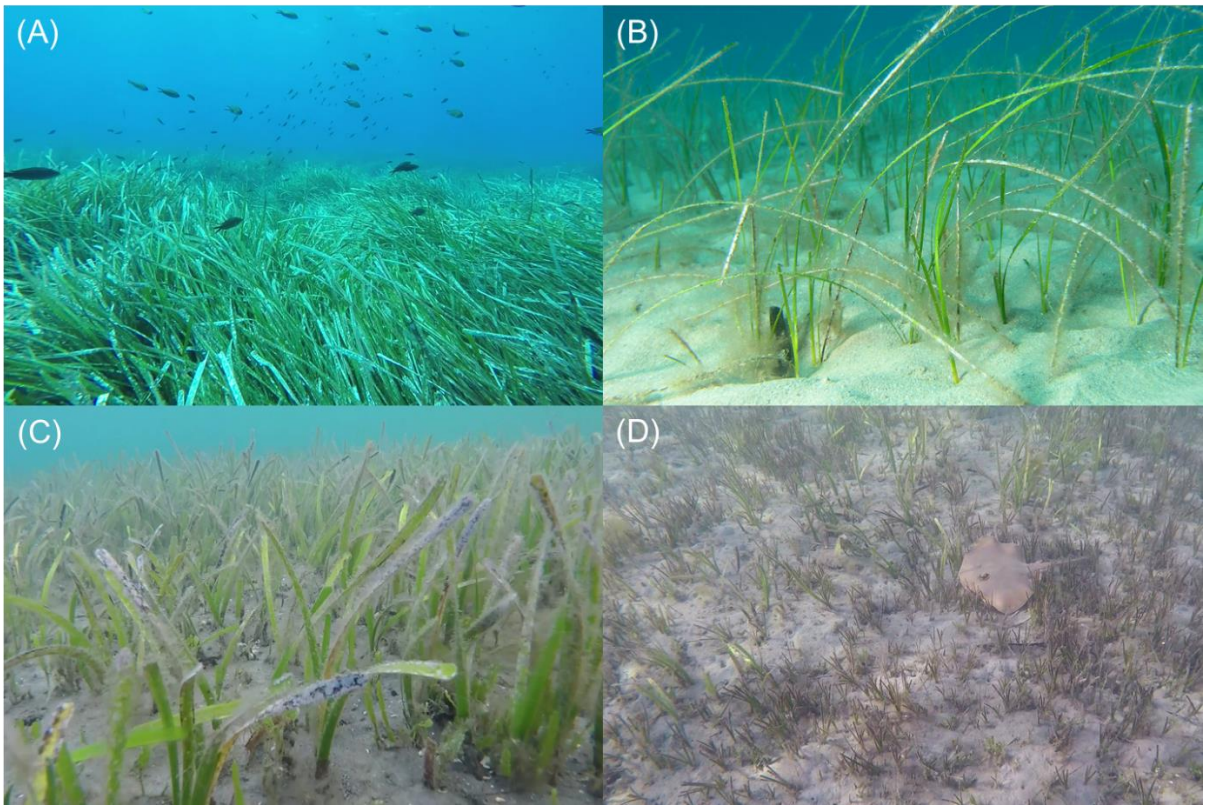

Figure 1.10 *Meadows of seagrass species used in this thesis. (A) Posidonia oceanica, (B) Cymodocea nodosa, (C) Posidonia australis, and (D) Zostera muelleri. Photo credit: Gabriele Procaccini (A,C,D) and Lázaro Marín-Guirao (B).*

Posidonia oceanica (L.) Delile is endemic to the Mediterranean Sea (see **Fig. 1.11A** for the species distribution) and forms large and dense monospecific meadows on rocks and sandy seabed ranging from shallow water (less than 1 m) down to 45-meter depth (Procaccini *et al.*, 2003). It ranks as one of the slowest-growing plants and among the longest-living plants on Earth, with single clones extending over kilometers and living for hundreds to thousands of years (Arnaud-Haond *et al.*, 2012).

Cymodocea nodosa (Ucria) Aschers. distributes throughout the Mediterranean Sea and extends also in nearby subtropical Atlantic areas (see **Fig. 1.11B** for *C. nodosa* distribution). *C. nodosa* is a relatively fast-growing species, commonly found in shallow waters in both sandy and mud substrates where it forms both monospecific and mixed meadows with other seagrass species (den Hartog, 1970; Guidetti *et al.*, 1998).

Posidonia australis Hooker f. is a slow-growing species found on sandy sediment between 1 to 15 m (Trautman and Borowitzka, 1999). This species distributes along the southern half of

Australia, from Shark Bay in Western Australia to Port Macquarie in New South Wales, and along the northern coast of Tasmania (**Fig. 1.11C**).

Zostera muelleri Irmisch ex Aschers. is a fast-growing species, commonly found in shallow water (< 4 m depth) on different sediments including fine sand, mud and others (Larkum, Kendrick and Ralph, 2018). *Z. muelleri* is found along the eastern coast of Australia, Tasmania Kangaroo Island, Lord Howe Island, and New Zealand (**Fig. 1.11D**).

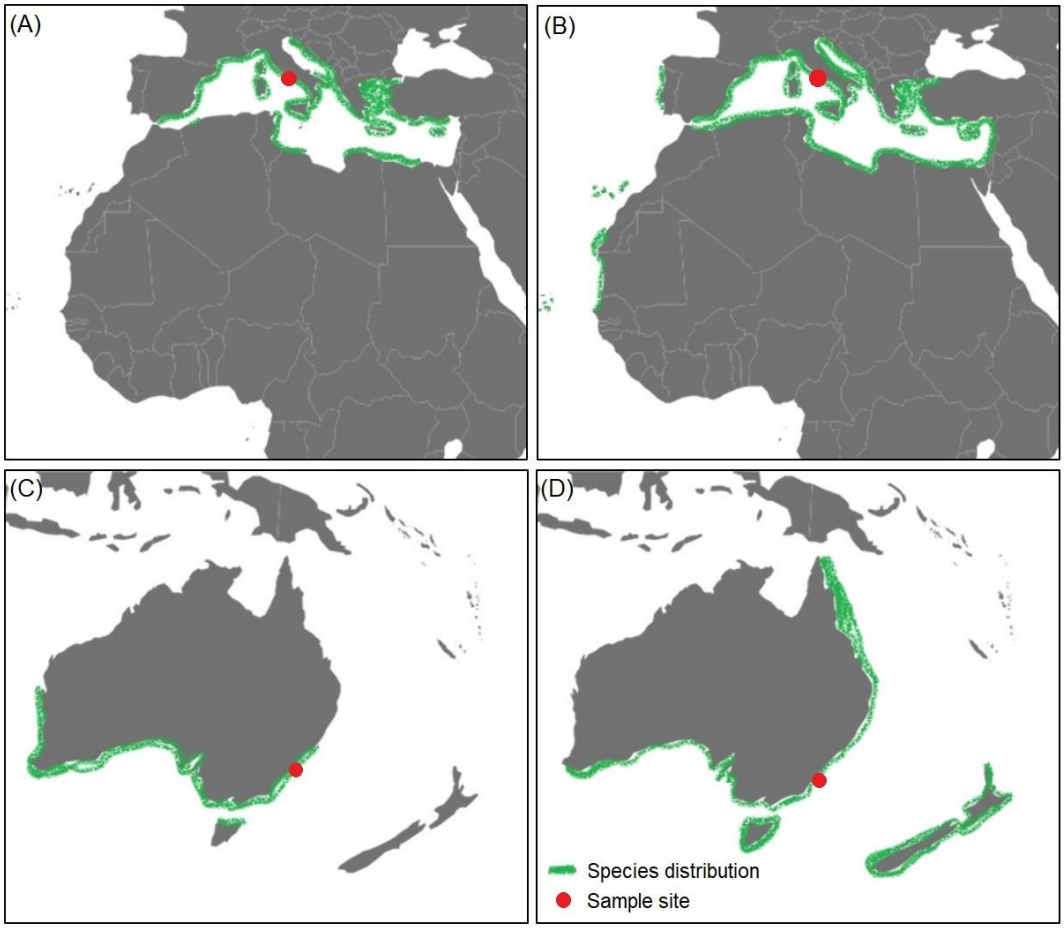

Figure 1.11 *Species distributions and sampling sites of four seagrass species used in this thesis. Information regarding species distribution ranges came from (1) Telesca et al., (2015) for Posidonia oceanica; (2) den Hartog (1970) for Cymodocea nodosa; (3) Trautman and Borowitzka (1999) for Posidonia australis; and (4) Waycott et al., (2004) for Zostera muelleri. According to species distributions, species thermal range and studied population thermal range for each species are as follow (i) 8 – 30℃ & 13 – 28℃ for P. oceanica, (ii) 8 – 30℃ & 13 – 28℃ for C. nodosa, (iii) 12 – 28℃ & 17 – 26℃ for P. australis, and 9 – 31℃ & 17 – 26℃ for Z. muelleri. Data regarding sea surface temperature were taken from World sea temperature of 2021 (available at https://www.seatemperature.org/).*

1.4.3 Thesis chapters and experiments

In **Chapter II**, I investigated the effect of thermal stress memory and the role of epigenetic modifications on two Southern hemisphere seagrass species (*P. australis* and *Z. muelleri*) using a two-heatwave experimental design on a mesocosm setup. Measurements were done across levels of biological organization including the molecular (gene expression), physiological (photosynthetic performances and pigments content) and organismal (growth) level aiming to provide the first evidence of thermal priming effect in seagrasses and explore potential roles of epigenetic modifications on thermal stress response and/or thermal stress memory in seagrasses.

In **Chapter III**, my goal was to identify molecular mechanisms that govern thermal stress memory in seagrasses. A two-heatwave mesocosm experiment was performed on two Mediterranean seagrass species (*P. oceanica* and *C. nodosa*). RNA-seq approach was applied in combination with other measurements (e.g. photo-physiology, morphology, pigment content) to explore molecular mechanisms potentially responsible for the formation and the activation of thermal stress memory in seagrasses. In addition, through RNA-seq data analysis, I also aimed at better understanding the involvement of epigenetic modifications at gene expression level with thermal stress response/ thermal stress memory in seagrasses.

In **Chapter IV**, with four seagrass species used in this thesis, I was able to study seagrass responses to warming at an across-hemisphere scale. Two comparable mesocosm experiments were conducted using four seagrass species from the northern (i.e. Mediterranean: *P. oceanica*, *C. nodosa*) and southern (i.e. Australia: *P. australis* and *Z. muelleri*) hemisphere. Plants were allowed to acclimatize to the mesocosm condition before being exposed to a simulated MHW. Plant responses (photo-physiology, morphology, and pigment content) to warming were measured at the end of the warming exposure to compare the response to warming between (*i*) hemispheres [*P. oceanica* versus *P. australis* (they both belong to the genus *Posidonia* with similar characteristics and ecological functions but happen to distribute in the two hemispheres) and (*ii*) between seagrasses with different life strategies [climax species (*P. oceanica* and *P. australis*) versus pioneer species (*C. nodosa* and *Z. muelleri*)].

In **Chapter V**, I aimed at deepening our understanding of the genetic basis of local adaptation to environmental variations in seagrasses. A field experiment was designed on *P. oceanica* meadows in a Mediterranean region that represents a naturally experimental site to study what might happen to seagrasses in the future. Samples were collected from Stagnone di Marsala, a

semi-enclosed coastal lagoon along the western coasts of Sicily (Italy) with geographically-neighboring *P. oceanica* meadows experiencing different environmental conditions (e.g. temperature and salinity). Environmental data and plant morphology were measured while the seagrass genome was screened by the mean of ddRADseq approach for SNP detection and outlier identification to identify signatures of local adaptation and micro-evolution in seagrasses.

Finally, in **Chapter VI**, I concluded by summarizing the main findings from this thesis and discussing future directions for seagrass research.

Chapter II – First evidence about the effect of thermal priming in seagrasses and the role of epigenetic modifications

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The work presented in this chapter has been published previously:

Hung Manh Nguyen, Mikael Kim, Peter J. Ralph, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini (2020) Stress memory in seagrasses: first insight into the effects of thermal priming and the role of epigenetic modifications. Frontiers in Plant Science 11:494. https://doi.org/10.3389/fpls.2020.00494

Special thanks

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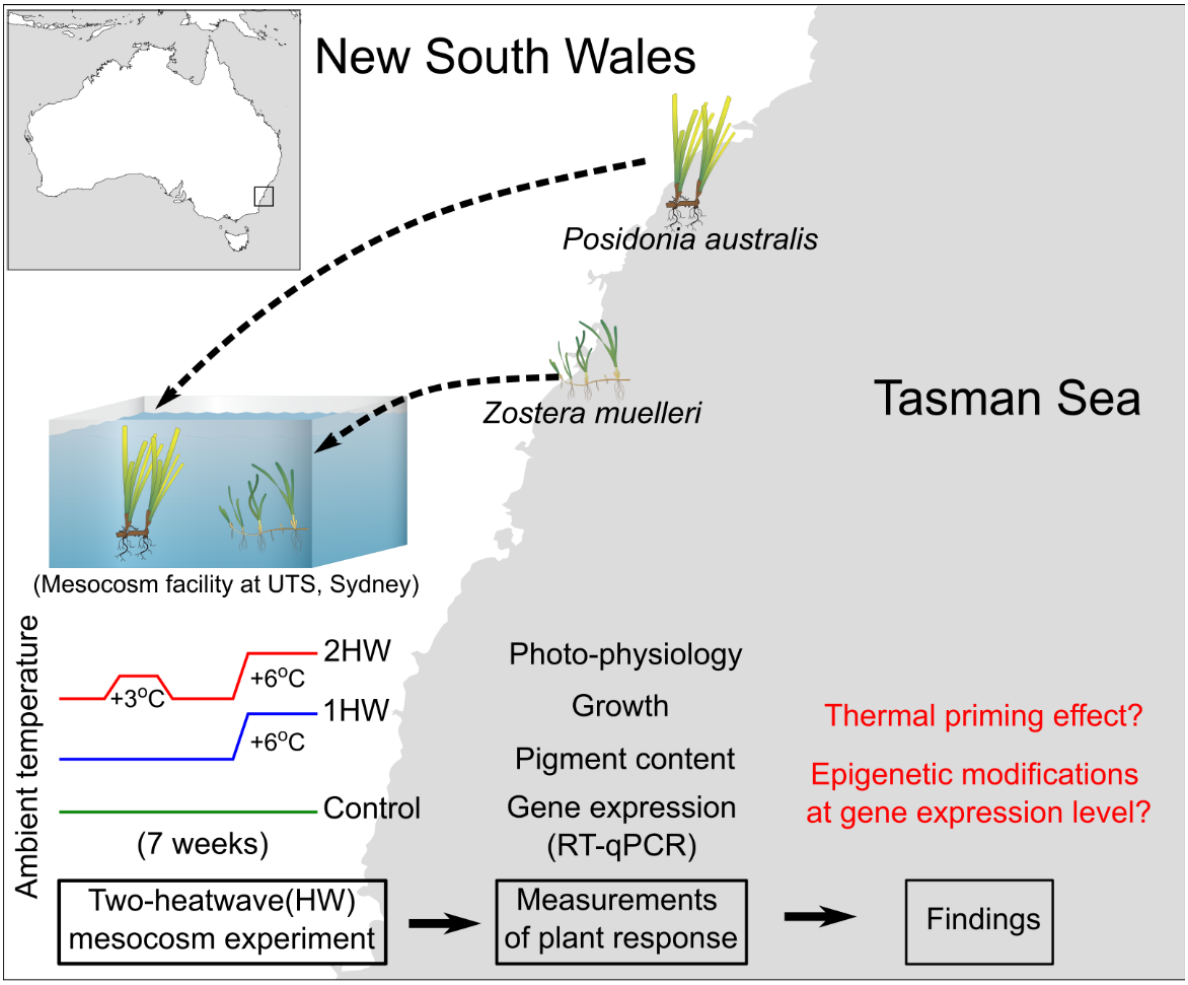

Figure 2.1 *Conceptual diagram illustrating the experiment presented in this chapter. Evidence of thermal priming and epigenetic modifications in two Australian seagrass species (Posidonia australis and Zostera muelleri). Symbols were taken from the IAN symbol libraries, available at https://ian.umces.edu/media-library/. Maps were taken from SimpleMappr, available at https://www.simplemappr.net/.*

2.1 Introduction

2.1.1 Thermal priming in terrestrial plants

The effect of thermal priming has been documented in several terrestrial plants such as Arabidopsis, rice, wheat, tomato, and so on (see reviews by Bäurle, 2016; Turgut-Kara, Arikan and Celik, 2020). Previous studies have shown that thermal priming can occur on different life stages of plants (from the seedling to the adult stage) and the outcome effects can vary among life stages, among species and even among different varieties within a single species (Xu *et al.*, 2006; Wang, Cai, *et al.*, 2014; Fan *et al.*, 2018). For instance, a study investigated the effect of thermal priming applied during early reproductive stages on two varieties of the winter wheat (*Triticum aestivum L.*) showing heat-primed plants were significantly better in preventing grain-yield damages caused by heat stress during the grain filling stage in comparison with non-primed plants (Fan *et al.*, 2018). Nevertheless, the effects of heat priming were also different between the two studied varieties (Fan *et al.*, 2018). Similarly, results from another study on two cool-season turfgrass species, including perennial ryegrass (*Lolium perenne* cv. Accent) and tall fescue (*Festuca arundinacea* cv. Barlexas), indicated that even both turfgrass species leaves benefited from the same heat-priming condition (i.e. heat acclimation pretreatment at 30℃ for 3 days in a growth chamber), however, the level of thermal priming effect varied between the two species as demonstrated by a greater membrane injury in perennial ryegrass leaves compared to tall fescue leaves at 46℃ (Xu *et al.*, 2006). In another case, heat responses in two rice subspecies (i.e. *Oryza sativa* ssp. *japonica* variety Nipponbare and *O. sativa* ssp. *indica* variety N22) were compared (Lin *et al.*, 2014). While the Nipponbare cultivars grow in temperate climates, the N22 cultivars are more adapted to subtropical climates. Very interestingly, results from Lin *et al.*, (2014) showed that the Nipponbare has a lower basal thermal tolerance but higher heat stress memory capacity, while the N22 had a higher basal thermal tolerance and a lower heat stress memory capacity. These contrasting characteristics in their thermal tolerance and their capacity to acquire thermal stress memory between the two sub-species suggest that the formation of heat stress memory may benefit from the rare exposure of the plants to heat stress in the past (Lin *et al.*, 2014).

It is known that the effect of thermal priming can be maintained for over several days after the condition returned to non-stress levels (Bäurle, 2016). Microarray analyses have identified a number of memory heat-inducible genes, which were maintained at very high expression levels for several days after the stressful condition ended (Stief *et al.*, 2014). Similarly, a study on the winter wheat showed that the thermal priming effect on early reproductive stages can last for

at least 15 days (Fan *et al.*, 2018). As suggested from other abiotic stress memories, the thermal priming effect may potentially last for even months or years, or in some cases, it can be transmitted to the next generations (Baldwin and Schmelz, 1996; Iqbal and Ashraf, 2007; Rendina González *et al.*, 2018). This sheds light on the need for more long-term studies on this particular topic.

Plants experiencing recurrent heat stress events can exhibit several mechanisms to better cope with the stress when it comes back. It has been demonstrated on tomatoes (*Solanum lycopersicum*) that heat priming improved the heat tolerance of the plants by increasing evaporation and decreasing leaf temperature (Zhou *et al.*, 2020). This study also witnessed that heat-primed plants developed some defense systems to protect themselves from multiple stresses [e.g. the accumulation of photo-protective pigment contents - carotenoids (Zhou *et al.*, 2020)]. Similarly, heat-primed winter wheat exhibited an improved tolerance to heat stress through increased photosynthetic capacity, stomatal conductance and chlorophyll contents in comparison with non-primed plants (Wang, Cai, *et al.*, 2014; Fan *et al.*, 2018). The improvement in heat tolerance of heat-primed plants also comes from an enhanced activity of antioxidant enzymes (superoxide dismutase, glutathione reductase and peroxidase) and reductions in reactive oxygen species and malondialdehyde production (Xu *et al.*, 2006; Wang, Cai, *et al.*, 2014; Fan *et al.*, 2018). In the case of two turfgrass species leaves (Xu *et al.*, 2006), heat primed plants were able to maintain relative water content and membrane thermos-stability of their leaves against the consequences of thermal stress damage. Additionally, heat primed leaves also showed lower membrane lipid peroxidation products than non-heat-acclimated leaves (Xu *et al.*, 2006). Additionally, the thermal priming effect has been shown to stabilize the ultrastructure of chloroplasts, hence contributing to protecting the chloroplast from damage related to temperature increase (Xu *et al.*, 2006). Recent work on *Achillea millefolium* confirmed the positive effect of thermal priming and suggested that thermal priming improved photosynthesis activities of heat-primed plants by enhancing stomatal conductance and synthesis of volatile and non-volatile secondary compounds with antioxidative characteristics, therefore maintaining the integrity of leaf membranes under stress. (Liu *et al.*, 2020). Exposures to repeated environmental stimuli (including warming) also result in genetic and epigenetic modifications that benefit the response of plants via quicker and more effective activation of specific cellular defenses when the stress comes back (see reviews by Liu *et al.*, 2015; He and Li, 2018; Chang *et al.*, 2020). For instance, a study on *A. thaliana* subjected to heat stress found that high accumulation of H3K4 methylation

(H3K4me3 and H3K4me2) persisted even after active transcription from the loci had subsided (Lämke *et al.*, 2016). This H3K4 methylation induces modified plant response by rapidly switching genes on or off following repeated stimulus (Ng *et al.*, 2003).

It is noteworthy that plants exposed to recurrent heat stress are not only more resistant to heat stress but also with other abiotic stress [e.g. salinity, drought, etc. see a review by Hossain *et al.*, (2018) for more examples]. In this case, heat-priming-induced cross-tolerance often leads to synergistic co-activation of multiple stress signaling pathways that include the involvements of reactive nitrogen species, reactive oxygen species, reactive carbonyl species, plant hormones as well as transcription factors (Hossain *et al.*, 2018).

2.1.2 The study

In this chapter, I aimed on investigating the effect of thermal priming in seagrasses. To this end, a two-heatwave experimental design was conducted on two Southern hemisphere seagrass species with different biological attributes and functional traits, *Posidonia australis* and *Zostera muelleri*. I hypothesized that plants pre-exposed to a stressful thermal event can perform better and are less impacted by subsequent heat stress than non-pre-heated plants. Plant responses were examined at different levels including morphology, photo-physiology and gene expression to assess heat-stress induced priming effects on the two seagrass species. Regarding gene expression, special attention was paid to the response of methylation-related genes to explore the potential involvement of epigenetic modifications on seagrass heat-stress memory.

2.2 Materials and Methods

2.2.1 Plant collection

Fragments of *P. australis* and *Z. muelleri*, bearing several connected shoots, were collected haphazardly at Port Stephens (PS) New South Wales (NSW), Australia (32°43'07.4"S 152°10'35.9"E) on the 19th of March 2019 and at Church Point (CP) NSW, Australia (33°38'46.8"S 151°17'11.9"E) on the 23rd of March 2019, respectively (see **Fig. 2.2**). Plant fragments were collected at a reciprocal distance > 25 m one from another in order to reduce the likelihood of sampling the same genotype twice. Both species were collected during low tide in shallow water (~70 cm), then plant fragments were transported immediately to the seagrass mesocosm facility at the University of Technology Sydney (UTS). Environmental conditions including salinity and water temperature at PS and CP were measured at the same time as plant collection to mimic the natural conditions at the mesocosm facility at UTS. Water temperature was ~25℃ at both sites while the salinity was slightly higher at PS (34.1 ppt) than

at CP (33.0 ppt). Rapid light curves were performed with a diving-PAM fluorometer (Walz GmbH, Germany) on three random plants at each site to define experimental light levels. These analyses showed that the saturating irradiance levels of plants in the field were approx. 350 µmol photons m^{-2} s^{-1} for both *P. australis* and *Z. muelleri* plants.

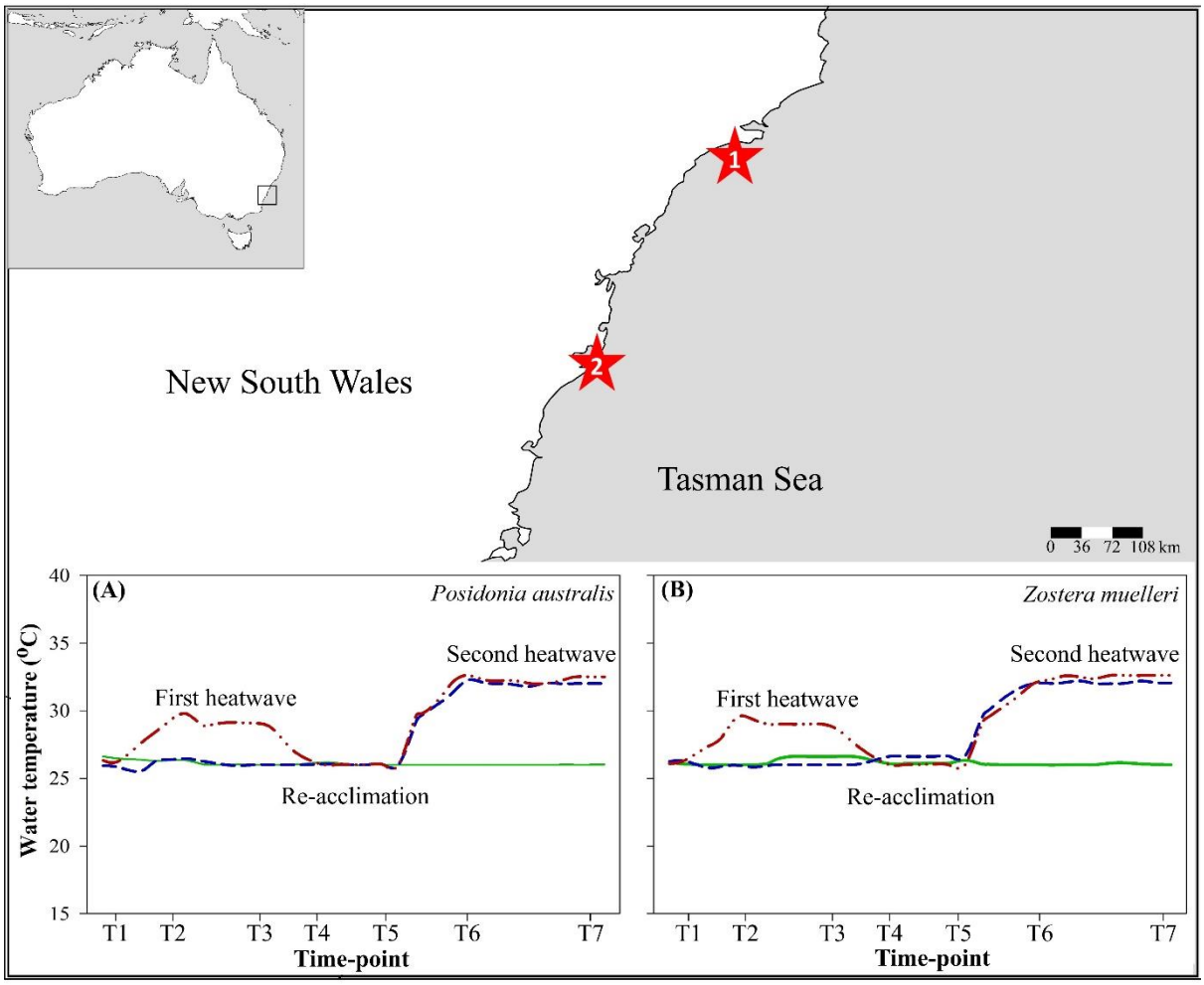

Figure 2.2 *Sample collection sites during low tides: (**1**) Collection site of Posidonia australis at Port Stephens, New South Wales, Australia, (**2**) Collection site of Zostera muelleri at Church Point, New South Wales, Australia. Thermal profile in experimental treatments during the course of the experiment (**A**, **B**): Green continuous lines: control; Blue dashed lines: Treatment 1-heatwave (1HW) and Red dashed lines with dots: Treatment 2-heatwave (2HW).*

2.2.2 Experimental setup

Once at UTS, for each species, eight-teen rhizome fragments bearing a similar number of shoots (i.e. 8-10 shoots) were carefully selected and separately planted into plastic trays filled with mini pebbles. Then, these trays were randomly allocated in six tanks (60-L aquaria for *P. australis* and 40-L aquaria for *Z. muelleri*) of the mesocosm facility (i.e. three trays per tank).

For each species, three experimental treatments including control (CT), treatment 1 heatwave (1HW) and treatment 2 heatwave (2HW) were conducted in parallel. Thus, for each treatment, two aquaria were considered as experimental replicates while six trays (plants) were treated as biological replicates. Each aquarium was equipped with an independent light source (Hydra FiftyTwo HD™, C2 Development, USA), two 55W-heaters, air and water pump to maintain circulation and homogeneity of seawater temperature. For both species, the irradiance level was set at 350 µmol photons m^{-2} s^{-1} at canopy height according to the saturating levels of plants from the fields (mentioned above) with a 12 h:12 h light:dark period. Light cycle started from 7:30 a.m., with light levels progressively increasing to the maximum irradiance at 12:30 p.m. and kept for 2 hours, before a progressive reduction until dark at 7:30 p.m. Water temperature was measured automatically every 30 min using iButton data logger (iButtonLink, USA) and manually checked twice a day using a digital thermometer (FLUKE 52II, USA). Throughout the experiment, purified water was added periodically to maintain the salinity level of 34 ppt and approximately 1/3 of seawater from each aquarium was renewed weekly to keep water quality consistent. See **Figure AII. 1** for a detailed description of the sampling regime and the experimental setup.

2.2.3 Experimental design

Water temperature was kept at 26℃ (~1℃ above the temperature in natural conditions at the time of the experiment, available at https://www.seatemperature.org/) in all aquaria during a 2-week acclimation period (**Fig. 2.2A,B**). Temperature was subsequently increased to 29℃ (heating rate 1℃ day^{-1}) in two aquaria of 2HW of each species and maintained for six days to simulate a MHW. Water temperature in these heated tanks was then reduced to control levels to allow heated plants to re-acclimate during a 1-week period before simulating a second, more intense and longer-lasting MHW (32℃ for 9 days; heating rate 1℃ day^{-1}). This second MHW was applied to four aquaria of each species, two pre-heated aquaria (2HW) and two non-pre-heated aquaria (1HW).

2.2.4 Chlorophyll *a* fluorescence

When chlorophyll is excited by photons (i.e. light), a part of the energy released by its subsequent de-excitation is used for photosynthesis while another part generates fluorescence. Therefore, there is an inverse relationship between photosynthetic yield and fluorescence yield. As a result, the measurement of fluorescence yield can be used for calculating photosynthetic quantum yield (Beer, Björk and Beardall, 2014). Pulse Amplitude Modulated (PAM) fluorometry uses synchronized pulses (on/off of 3 µs long and repeated at a frequency of 600

or 20000 Hz) that allows measuring only chlorophyll fluorescence while ignoring all background light of the same wavelength. PAM fluorometry is capable of measuring some components of photosynthesis such as efficiency of photosystem II (PSII) photochemistry, electron transport rates and impact of a compound on the electron transport chain, PSII reaction centres and thylakoid membrane (Schreiber *et al.*, 1995; Beer, Björk and Beardall, 2014). Especially, PAM fluorometry is a non-destructive method that allows repeated measurements of the same sample. To date, PAM fluorometry has been widely used to measure photosynthetic activities of various organisms from the terrestrial environment to the marine environment including seagrasses (Schreiber, 2004; Beer, Björk and Beardall, 2014).

The photo-physiological response of *P. australis* and *Z. muelleri* plants was determined using a diving-PAM fluorometer. Measurements included maximum quantum yield of PSII (Fv/Fm), effective quantum yield of PSII (ΔF/Fm') and non-photochemical quenching (NPQ). Fv/Fm was measured on night dark-adapted plants (i.e. at 7 am, before the start of the light cycle) and calculated following the equation $(F_m - F_0)/F_m$, where F_m is the maximal fluorescence yield induced by a saturation pulse (i.e. all PSII reaction centers are fully close) and F_0 is minimal fluorescence yield (i.e. all PSII reaction centers are open and all primary acceptors are fully oxidized) (Marín-Guirao, Ruiz, *et al.*, 2013). ΔF/Fm' was measured on light-adapted plants (i.e. at noon during the daily period of highest irradiance level) and calculated following the equation $(F_{m'} - F)/F_{m'}$, where $F_{m'}$ and F are the maximal and the background fluorescence yield of light-adapted plants, respectively (Marín-Guirao, Ruiz, *et al.*, 2013). NPQ reflects the photon energy diverted to heat via the xanthophyll cycle and other protective mechanisms and this is calculated as $(F_m - F_{m'})/F_{m'}$ (Maxwell and Johnson, 2000).

During the experiment, measurements were conducted on the second youngest leaf of five randomly selected plants from each treatment and each species at different time points along the course of the experiment (**Fig. 2.3**): end of the first acclimation period – experiment started (T1); beginning of the first heatwave (T2); end of the first heatwave (T3); beginning of the re-acclimation period (T4); end of the re-acclimation period (T5); beginning of the second heatwave (T6) and end of the second heatwave – experiment ended (T7). At each time point, selected leaves were marked to ensure ΔF/Fm' measurements were conducted at the same place where Fv/Fm measurements occurred. In addition, a custom-designed underwater leaf clip was employed to maintain a constant distance between the leaf and the fiber optic of the PAM fluorometer.

2.2.5 Plant growth

Plant growth measurements were done by adopting the leaf marking method (Zieman, 1974). In the middle of the second acclimation period between both simulated heatwaves, 5 randomly selected plants of each treatment were marked just above the ligule. These samples were then collected at the end of the 2nd heatwave (T7) for measuring leaf elongation (mm). Subsequently, newly grown leaf segments were dried at 70℃ for 24 hours and weighed to determine the growth as leaf biomass production (Dry weight).

2.2.6 Pigment content

Approximately 50 mm from the middle portion of the 2nd youngest leaf of *P. australis* and the whole 2nd youngest leaf of *Z. muelleri* was harvested from 5 randomly selected plants of each treatment at the end of the experiment (T7) for analyzing pigments content. Collected leaf samples were cleaned of epiphytes and kept on ice before fresh weights were measured. Samples were homogenized in liquid nitrogen using pestles and mortars, transferred into 1.5 mL tubes containing 1 mL of 100% methanol and stored in complete darkness at 4℃ for 8 hours before centrifugation. Absorbance of 200 µL of obtained solution was read at 470, 652, 665 and 750 nm using a microplate reader (TECAN Infinite® M1000 PRO, Switzerland) for calculations of the chlorophyll *a*, chlorophyll *b* and total carotenoid concentrations using equations from Wellburn (1994) after converting microplate readings into 1cm cuvette readings following Warren (2008). Finally, results were normalized to a milligram of fresh weight.

2.2.7 Quantitative Real-time PCR (qRT-PCR)

Primer design

Ten genes of interest (GOIs; **Table 2.1**) common to both species were chosen within three different categories including stress-related, photosynthesis-related and methylation-related genes.

Z. muelleri GOIs were newly designed using *Z. muelleri* database from AquaticPlantsDB® (Sablok *et al.*, 2018), while housekeeping genes (HKGs) were taken from previous studies (Schliep *et al.*, 2015; Pernice *et al.*, 2016; Kim *et al.*, 2018). For *P. australis*, however, no molecular resources are available to date, thus selected GOIs and HKGs were either newly designed or taken from previous studies on the congeneric species *P. oceanica*. Three photosynthesis-related genes (i.e. Photosystem II protein D1-psbA, Photosystem II protein D2-psbD and Rubisco large subunit-RBCL) and 4 HKGs were available in the literature (Serra *et*

al., 2012; Dattolo *et al.*, 2014; Marín-Guirao *et al.*, 2016). The rest of the primers were designed using a *P. oceanica* transcriptome database available at the National Center for Biotechnology Information (NCBI) (Marín-Guirao *et al.*, 2019).

Primers were designed using Primer3 v.0.4.0 (Koressaar and Remm, 2007; Untergasser *et al.*, 2012) with the following default settings: primer lengths: 18-22 bp, product sizes: 100-200 bp and Tm= 59-61℃. Primers were validated for their specificity firstly by checking PCR amplification on agarose gel electrophoresis (i.e. only single band, similar size as designed) and secondly by checking the melting curve for each RT-qPCR run. RT-qPCR efficiencies were assessed via a series of cDNA dilutions of 384, 81, 27, 9, 3, and 1 ng using a linear regression model (Pfaffl, 2001). The efficiency of each primer pair was then calculated with the following equation: $E\ (\%) = (10^{-1/\text{slope}} - 1)\times 100$ (Radonić *et al.*, 2004). Primers with efficiencies (*E*) within the range 90−110% and correlation coefficient > 0.95 were used in the study (**Table 2.1**).

RNA extraction and cDNA preparation

Three leaf samples, targeted in a similar way as for pigment content samples, were collected for RNA extraction at the end of each heatwave (T3 and T7). Epiphytes were carefully removed from plants and cleaned plant material was then immediately frozen in liquid nitrogen before being stored at -80℃ until RNA extraction. PureLink™ RNA Mini Kit (ThermoFisher, USA) was used to extract total RNA from both species. For *Z. muelleri*, extraction was done by following the manufacturer's instructions. For *P. australis*, to minimize the effects of phenolic compounds that can inhibit the extraction process, 2% (w/v) polyvinylpyrrolidone-40 (PVP) together with two glass beads were added to the lysis solution and vortexed at high speed at 4℃ for 10 min, all other steps were completed by following the manufacturer's instructions. During the extraction of total RNA, PureLink™ DNase Set (ThermoFisher, USA) was added to eliminate genomic DNA. The total RNA quantity and quality were assessed with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, USA). Then, cDNA was synthesized from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) according to the manufacturer's instructions. The resulting cDNA was diluted 1:20 prior to qRT-PCR assays.

RT-qPCR reaction

A 5 µL-final volume RT-qPCR reaction including 2.7 µL of iTaq™ Universal SYBR® Green Supermix (BIO-RAD, USA), 0.3 µL of 10 pmol µL-1 primers and 2 µL of diluted cDNA was

robotically prepared in a 384-well PCR plate (BIO-RAD, USA) via an Automated Liquid Handling Systems (EpMotion® 5075, Eppendorf, Germany). RT-qPCR assay was run in a Real-Time PCR Detection System (CFX384 Touch™, BIO-RAD) with the following conditions: 95℃ for 10 min, followed by 45 cycles of 95℃ for 30s, 60℃ for 30s and 68℃ for 30s. A melting curve from 60 to 95℃ was also included for each amplicon to check the specificity of each reaction.

All RT-qPCR reactions were performed in three technical replicates with three no-template negative controls. Additionally, three No Reverse Transcription (No-RT) controls were prepared for each primer's pair and included in each plate to ensure the absence effect of genomic DNA contamination (i.e., Cq value from No-RT sample was at least five cycles greater than the actual sample). Furthermore, an internal control assay was introduced in each plate to establish a reliable comparative result between different plates.

2.2.8 Gene expression analysis

Data from RT-qPCR reactions were analyzed with Bio-Rad CFX Manager v3.1 software (BIO-RAD, USA) and normalized relative quantities of amplification were used to determine the changes in the gene expression level of GOIs as described in a previous study (Kim *et al.*, 2018).

Before gene expression data analyses, three different algorithms were used to identify the best HKGs: NormFinder (Andersen, Jensen and Ørntoft, 2004), GeNorm (Vandesompele *et al.*, 2002) and BestKeeper (Pfaffl *et al.*, 2004) (**Appendix II**).

Relative quantities of genes of interest (GOIs) were first normalized using the two best housekeeping genes selected from three different algorithms (**Appendix II, Table AII.7**). Then, normalized data were used to determine gene expression levels of GOIs.

2.2.9 Statistical analysis

One-way analysis of variance (One-way ANOVA) is used to determine whether there are any statistically significant differences between the means of two or more independent groups by figuring out how much of the total variance comes from either the variance between groups (i.e. significant difference) or the variance within groups (i.e. not significant difference) (St»hle and Wold, 1989). In this study, one-way ANOVA performed with the statistical software SPSS v.20 was used to check for significant differences in plant growth and pigment content between treatments at the end of the 2nd heatwave (T7). Since these parameters greatly differ between the two species, each species was analyzed independently. Prior to the analysis, Levene's test

was used to check the homogeneity of variances and Shapiro–Wilk test was used to validate data normality. This approach (i.e. one-way ANOVA) was chosen because the data met assumptions that are required for a one-way ANOVA including (*i*) the three treatments were independent variable; (*ii*) measured variables were continuous; (*iii*) there was no significant outliers in the data; (*iv*) observations were independent; (*vi*) the data were normally distributed; and (*vi*) variances were homogeneous (St»hle and Wold, 1989). Moreover, a Tukey HSD post-hoc test (Abdi and Williams, 2010) was applied whenever significant differences were determined in order to define which of these groups differ from each other. There was only one special case of Chl *b*/*a* data from *P.* australis where the parametric assumptions were not met. In this situation, data were analyzed using Kruskal-Wallis test [i.e. also known as one-way ANOVA by ranks that is suitable for analyzing non-parametric data (Kruskal and Wallis, 1952)] together with the Bonferroni correction for multiple tests instead (**Table 2.3**).

At first, repeated measures ANOVA (St»hle and Wold, 1989) was considered to analyze photo-physiological and gene expression data because these variables are based on repeated observations. However, I was unable to have the data to meet the homogeneity of variances assumption for ANOVA even I had tried several transformation approaches (e.g. square-root transformation, log-transformation or Box-Cox transformation). Thus, Permutational Multivariate Analyses of Variance (PERMANOVA: a non-parametric multivariate statistical test) performed with Primer 6 v.6.1.16 and PERMANOVA + v.1.0.6 software package (PRIMER-E Ltd) (Anderson, Gorley and Clarke, 2008) was selected to analyze photo-physiological and gene expression results of GOIs. The basic principle of PERMANOVA is to divide different groups (i.e. treatments) into clusters using distance matrices, then statistically identify whether the centres of the clusters are different among others. Analyses were performed on the resemblance matrices (created using Bray Curtis similarity) with a selected number of permutations of 9999. Within the analyses, treatment was treated as a fixed factor while time was treated as a random factor. Finally, Pair-wise test was used to detect significant differences between treatments at each time point.

Principal component analysis [PCA (Wold, Esbensen and Geladi, 1987)] converts the correlations among groups into a 2D graph where the axes are ranked in order of importance, therefore, suitable to visualize the overall pattern of the data. Because of that, PCA was performed on normalized relative quantities of amplification of GOIs using the software PAST3 (Hammer, Harper and Ryan, 2001) to determine responsive patterns to heat stress between treatment at each time point for gene expression data. Additionally, data from all

measurements at T7 were analyzed altogether using a PCA to assess the overall difference in responses between the two seagrass species.

2.3 Results

2.3.1 Photo-physiological response

During the first heatwave (T2-T3), neither of the species showed significant differences in Fv/Fm between heated (2HW) and non-heated (CT and 1HW) plants (**Fig. 2.3A, B**), evidencing the absence of accumulated heat damage at the PSII level. In fact, the effective photochemical efficiency of PSII (ΔF/Fm') of heated plants was only slightly higher than that of control plants during this first heatwave (**Fig. 2.3**), being significant only in *Z. muelleri* (CT=1HW≠2HW). The level of photo-protection (NPQ) of heated plants also showed no signs of alteration during this first warming exposure as seen by the lack of significant differences in NPQ between heated and control plants of both species (CT=1HW=2HW).

Contrarily, during the more intense and longer-lasting second heatwave (T6-T7), heated plants (1HW and 2HW) of both species experienced a significant reduction in their maximum and effective photochemical capacity of PSII (Fv/Fm and ΔF/Fm') with respect to controls (**Fig. 2.3A-D**), that resulted in significant differences between treatments over time ($p_{perm} < 0.001$, **Table 2.2**). However, this heat-induced photochemical reduction was generally higher in non-preheated (1HW) than in preheated (2HW) plants of both species, and I found significant differences between non-preheated plants versus controls and preheated plants at T6 for both Fv/Fm and ΔF/Fm' (**Fig 2.3B**, D; CT=2HW≠1HW). The differences between 1HW and 2HW plants were clear at T7. In P. australis, the 2nd heatwave induced a 22% reduction in Fv/Fm and a 34% reduction in ΔF/Fm' of 1HW plants while these reductions were much smaller in 2HW plants (13% and 14%, respectively). Differences were significant for ΔF/Fm' (see **Fig 2.3C**, CT≠1HW≠2HW). Similarly, there was a significant reduction of 9% in Fv/Fm of *Z. muelleri*-1HW plants at T7, whereas there was only a slight reduction in Fv/Fm of 4% in *Z. muelleri*-2HW plants (**Fig. 2.3B**, CT≠1HW≠2HW). A similar trend was observed with ΔF/Fm' results from *Z. muelleri*. In respect to CT plants, the reduction in ΔF/Fm' in 1HW plants was more than double compared to that of 2HW plants (i.e. 14% and 6% respectively). Consequently, significant differences were detected between plants from the two heating treatments (1HW and 2HW) for Fv/Fm in the case of *Z. muelleri* (**Fig. 2.3B**; CT≠1HW≠2HW) and for ΔF/Fm' in the case of *P. australis* (**Fig. 2.3C**; CT≠1HW≠2HW).

Regarding non-photochemical quenching (NPQ), *Z. muelleri* interestingly showed no significant differences (p_{perm} = 0.9169, Pseudo-F = 0.5181, **Table 2.2**) among three treatments throughout the whole experiment (**Fig. 2.3E**). In contrast, *P. australis*-1HW plants significantly tripled their NPQ levels at T7 compared to CT and 2HW plants (**Fig. 2.3F**, Treatment (Time): p_{perm} < 0.001, Pseudo-F = 0.5181, **Table 2.2**, CT=2HW≠1HW).

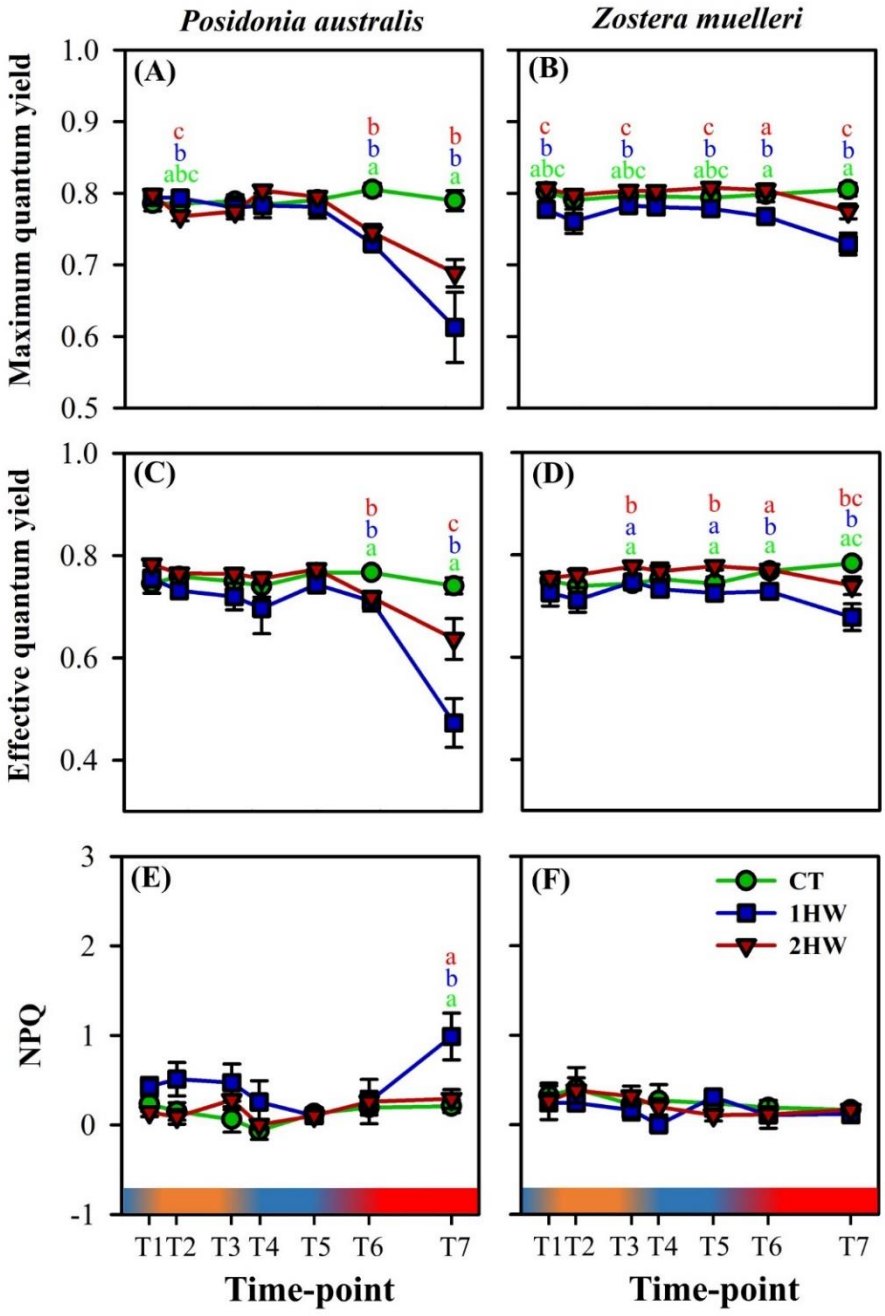

Figure 2.3 *Photo-physiological responses of Posidonia australis and Zostera muelleri:(A,B) Maximum quantum yield (Fv/Fm) were measured on dark-adapted plants; (C,D) Effective quantum yield (ΔF/Fm') and (E,F) Non-Photochemical Quenching (NPQ). CT: control, 1HW:*

1 heatwave treatment, 2HW: 2 heatwaves treatment. At each time point, different colors correspond to different treatments (green – CT, blue – 1HW, and red – 2HW) and different letters (a-c) indicate significant differences between treatments (e.g. in Fig. 2.3A: "a-green + b-blue + b-red" means CT≠1HW=2HW; Pair-wise comparison test, $p_{(perm)} < 0.05$). Data are mean ± SE, n=5. Gradient bars present water temperature changes in treatment 2HW throughout the experiment.

2.3.2 Plant growth response

Increased temperatures during the second heatwave (32℃) significantly reduced leaf elongation and leaf biomass production of both preheated (2HW) and non-preheated (1HW) *P. australis* plants (**Fig. 2.4A**, C; $p < 0.01$, **Table 2.3**). Growth reduction, however, was similar in 2HW plants (39%) and 1HW plants (40%).

In *Z. muelleri*, significant differences among treatments ($p < 0.05$, **Table 2.3**) were also detected for both leaf elongation and leaf biomass production measurements. During the second heatwave, leaf elongation rate decreased by 41% in 1HW plants while there was only a 16% reduction in the case of 2HW plants (**Fig. 2.4B**; CT=2HW≠1HW). It is interesting to note that while leaf biomass production decreased by 38% in 1HW plants, 2HW plants accumulated 6% more biomass than the CT plants during the second heatwave (**Fig. 2.4D**). This phenomenon led to a significant difference between 1HW versus 2HW plants in terms of leaf growth (**Fig. 2.4D**; CT=1HW=2HW, 1HW≠2HW).

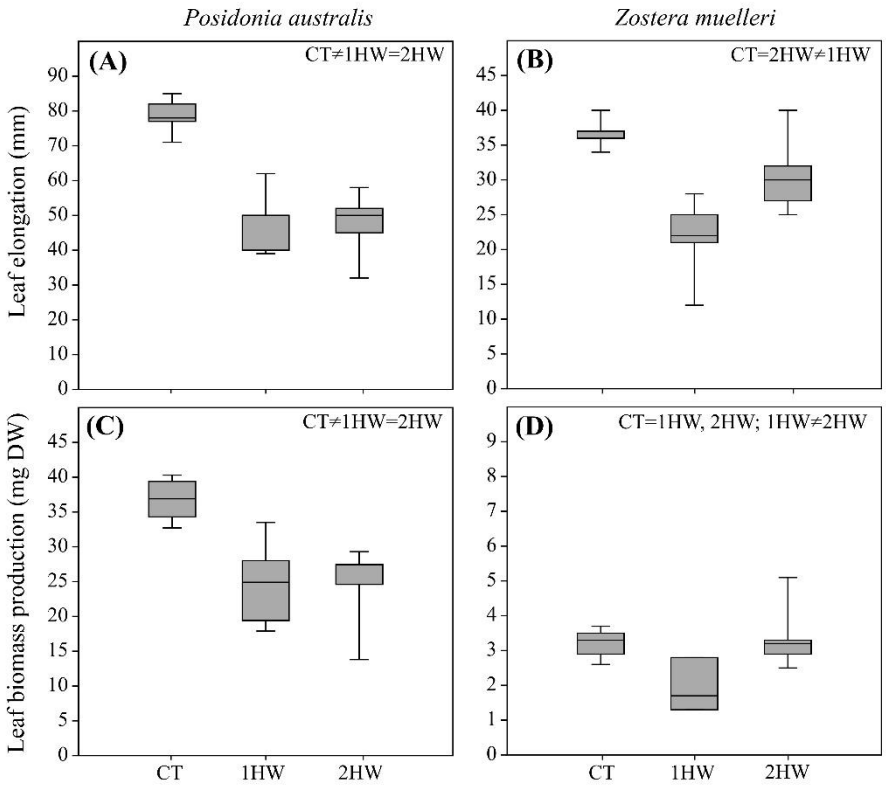

Figure 2.4 *Leaf elongation (a, b) and leaf biomass production (Dry weight; c, d) from control (CT), non pre-heated (1HW) and pre-heated (2HW) plants at the end of the second heatwave (T7). Tukey HSD post-hoc results are shown on the top of the graphs (Significant difference means $p < 0.05$). Data are mean ± SE, n = 5.*

2.3.3 Pigment content response

Chl *a* appeared as the most thermo-sensitive photosynthetic pigment in *P. australis* and in *Z. muelleri* (**Fig. 2.5A, B**). Interestingly, 2HW plants were able to maintain their Chl *a* contents similar as in CT plants, while 1HW plants suffered a strong reduction (41% and 28% for *P. australis* and *Z. muelleri*, respectively). Via Tukey HSD post-hoc test, a significant difference between 1HW plants and CT plants was found in *P. australis* (**Fig. 2.5A**). Both Chl *b* and Carotenoid content (**Table 3**) from 1HW *P. australis* plants were further impacted by elevated temperature during the second heatwave when compared to those from 2HW plants (**Fig. 2.5A**), although these differences were not statistically significant.

Temperature increase affected Chl *a* and Chl *b* contents differently in the two seagrass species, contributing to significant differences in Chl *b*/*a* ratios among experimental treatments ($p < 0.01$, Table 3). In *P. australis*, both 1HW and 2HW plants increased ~13% of Chl *b*/*a* ratios in respect to the CT plants (**Fig. 2.5A**). In contrast, only non-preheated (1HW) *Z. muelleri* plants increased their Chl *b*/*a* ratios (32% more than in CT plants) significantly, while preheated

plants kept their Chl *b*/*a* ratios comparable to control levels (0.28 and 0.29 in CT and 2HW plants, respectively; **Fig. 2.5B**).

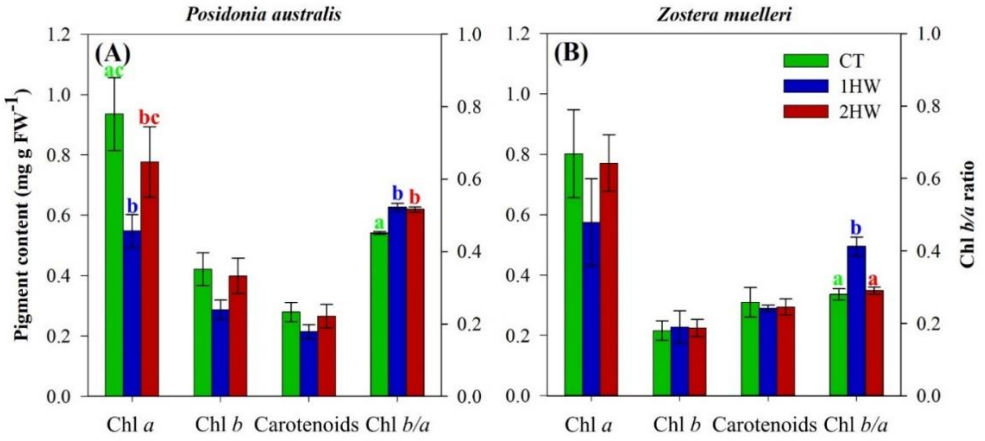

Figure 2.5 *Pigment relations at the end of the second heatwave (T7): Chlorophyll a (Chl a), Chlorophyll b (Chl b), Carotenoids and the Chlorophyll b/a molar ratio (Chl b/a) in P. australis (a) and Z. muelleri (b). CT = control plants; 1HW = non-pre-heated plants; 2HW = pre-heated plants. Different letters (a-c; green letters correspond with CT, blue letters correspond with 1HW and red letters correspond with 2HW treatment) indicate significant differences ($p < 0.05$) among treatments as derived from Tukey HSD post-hoc analyses. Error bars present ± SE, n = 5.*

2.3.4 Gene expression response

All selected primers were tested in the two species and some of them successfully worked on *both P. australis* and *Z. muelleri* (i.e. psbD and CAT, **Table 2.1**), indicating the presence of conservative genomic regions between the two different seagrass species belonging to different genera.

In general, during the first heatwave (T3), 2HW plants from both species showed up-regulation of all analyzed GOIs with respect to plants under control temperature (CT and 1HW). The difference, however, was significant only for 3 and 6 genes in *P. australis* and *Z. muelleri*, respectively (**Fig. 2.6A,C**).

At the end of the second heatwave (T7), all heated *P. australis* plants (1HW and 2HW) activated substantial molecular responses to compensate with extreme temperature changes, with 80% of the GOIs tested showing significant up-regulation (**Fig. 2.6B**). In *Z. muelleri*, while a similar number of significantly affected genes was observed at T3 and T7 (**Fig 2.6C**), the genes differentially expressed were different between the two time points. In both species,

results from both T3 and T7 evidenced that methylation-related genes were more responsive to the selected thermal treatments than stress-related and photosynthesis-related genes. Details about statistical analysis results from each GOIs at T3 and T7 can be found in **Table 2.4**.

Methylation-related GOIs

At T3, heat-primed plants of both species (2HW) showed significantly increased transcripts accumulation of ATX2 and ATXR7 (CT=1HW≠2HW). ASH2L was also highly up-regulated in treated plants (1HW and 2HW) although without significant differences among treatments (**Fig. 2.6A,C**). Also, a significant upregulation of SETD3 was found in *Z. muelleri* heated plants during the first heatwave (**Fig. 2.6C**).

At T7, most methylation-related GOIs showed significant up-regulations in 1HW and 2HW plants of both species (**Fig. 2.6B,D**). Significant differences between 1HW and 2HW *P. australis* plants were found for ATX2 and ATXR7 (**Fig. 2.6B**, 1HW>2HW). *Z. muelleri* plants followed a similar trend with 1HW plants showing higher gene expression levels than 2HW plants for all methylation-related GOIs, but being significant only for ASH2L and ATX2 (**Fig. 2.6D**).

Stress-related GOIs

At T3, during the first heatwave, all stress-related and photosynthesis-related GOIs were overexpressed in heat-primed plants (2HW), being significant (CT=1HW≠2HW) for CAT in *P. australis* plants (**Fig. 2.6A**) and for HSP90 in *Z. muelleri* plants (**Fig. 2.6C**).

At T7, during the second heatwave, the three stress-related GOIs (i.e. MSD, CAT, and HSP90) of *P. australis* showed similar and significant up-regulation in all heated plants (1HW and 2HW) (**Fig. 2.6B**, CT≠1HW=2HW). In contrast, CAT showed a significant difference between heat-primed and non-primed *Z. muelleri* plants (1HW>2HW, **Fig. 2.6D**).

Photosynthesis-related GOIs

At T3, all photosynthesis-related GOIs showed up-regulations in heated (2HW) plants for both species, although significant differences (CT=1HW≠2HW) were only detected in *Z. muelleri* plants for psbA and RBCL (**Fig. 2.6C**).

At T7, non-preheated *P. australis* plants (1HW) significantly increased their levels of gene expressions compared to CT plants (CT≠1HW in psbD), while preheated-plants (2HW) maintained or even decreased the expression levels of those genes, resulting in significant

differences between the two heated plants among all photosynthesis-related GOIs (1HW≠2HW, **Fig. 2.6B**). In contrast, in *Z. muelleri*, no significant difference was found between 1HW and 2HW plants for psbA and psbD (1HW=2HW, **Fig. 2.6B**). Moreover, even if no significant differences were detected between CT versus heated plants (CT=1HW=2HW), RBCL was expressed differently between 1HW and 2HW plants. As a consequence, the expression levels of RBCL were significantly different between the two heated treatments at T7 (1HW≠2HW).

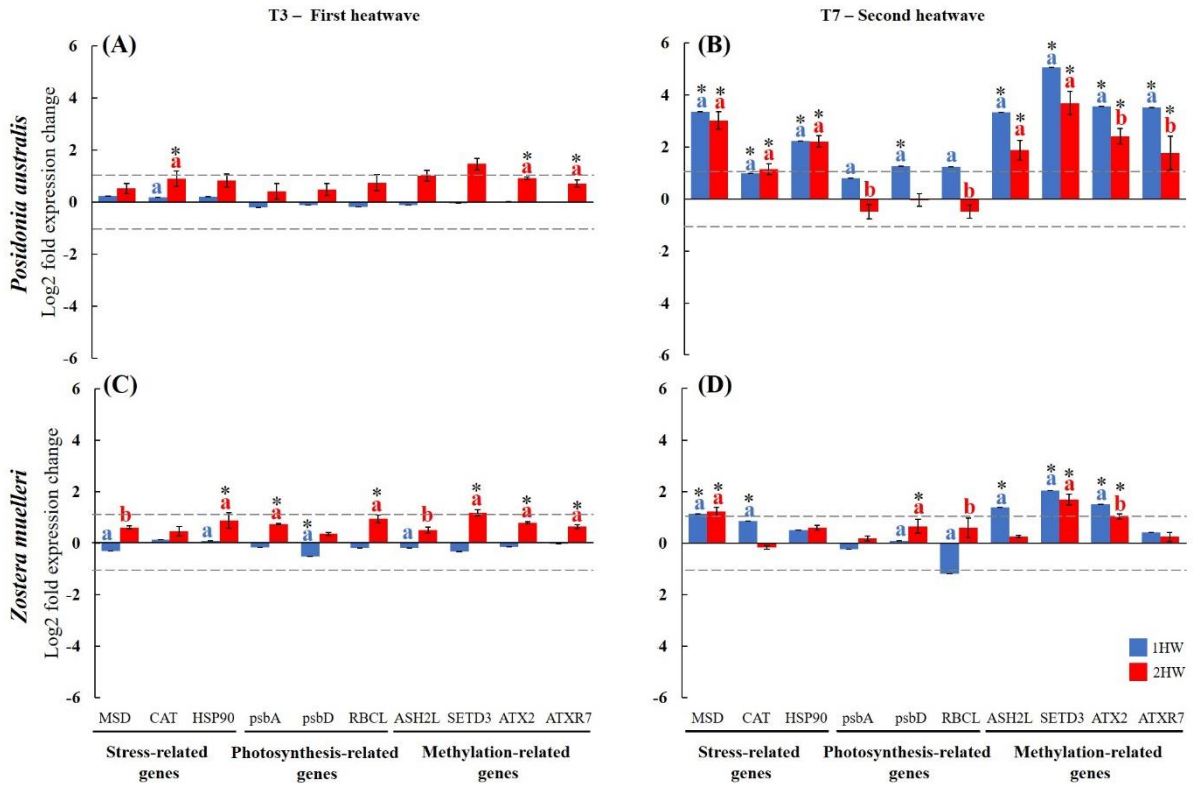

Figure 2.6 *Differential gene expression for GOIs at the end of the first (T3; left panels) and second heatwaves (T7; right panels), respectively. For P. australis (upper panels) and Z. muelleri (lower panels). Data is expressed as log2 Relative Quantification versus the control group. Data are mean, ±SE, n=3. Pair-wise results are presented on top of the column corresponding to a significant difference between control and treatments (asterisk) or between the two treatments (letters), p < 0.05. 1HW: 1 heatwave plants; 2HW: 2 heatwave plants.*

Principal component analyses (PCA) performed on gene expression results from both seagrass species demonstrated clearly that (*i*) at T3, heated plants (2HW) were separated from non-heated plants (CT and 1HW) while (*ii*) at T7, the two groups of plants experiencing heat stress (1HW and 2HW) were distant from CT plants, with 2HW plants showing more similarities to CT plants than to 1HW plants (**Fig. 2.7**). PCA results also highlighted methylation-related

genes were the main drivers differentiating 2HW plants at T3 and 1HW plants at T7. For instance, in *P. australis* at T3, ATX2 and ATXR7 together with CAT were the main drivers separating 2HW plants away from CT and 1HW plants along the PC1 axis responsible for 97.77% of this separation (**Fig 2.7A**). Whilst, in *Z. muelleri*, SETD3 and HSP90 mainly contributed to PC1, which was responsible for 86.42% of the separation between 2HW plants and the other two groups (**Fig. 2.7C**). At T7, in *P. australis* ATX2 and ATXR7 remained the strongest factors separating 1HW plants from 2HW and CT plants (**Fig. 2.7B**) while in *Z. muelleri*, ASH2L together with ATX2 and SETD3 separated 1HW plants from CT and 2HW plants along PC2 (23.8% of the variation) (**Fig. 2.7D**).

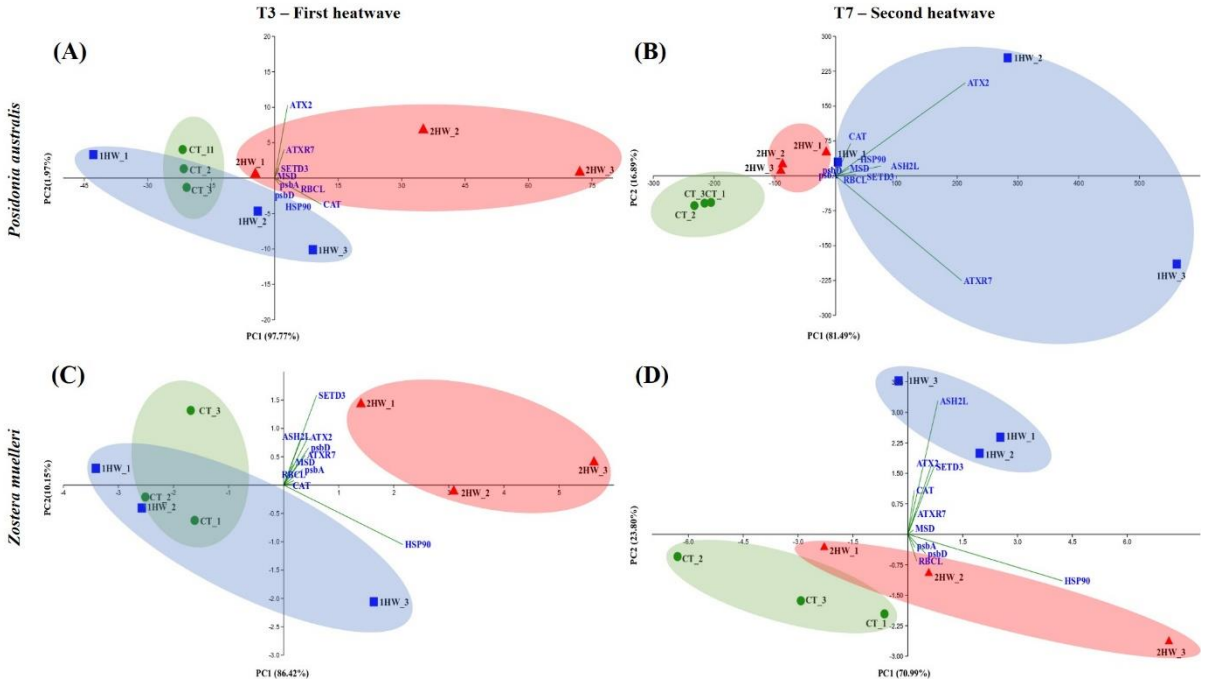

Figure 2.7 *PCAs conducted on gene expression data. Different colors correspond to different treatments (Green circle = Control-CT, Blue square = Treatment 1-heatwave-1HW, Red triangle = Treatment 2-heatwave-2HW).*

PCA results for both species and all analyzed plant variables at T7 showed similar results in both seagrass species with heated plants separated from control plants, reflecting the overall effects (i.e. molecular, physiological and organismal effects) of extreme temperature increase during the second heatwave (**Fig. 2.8**). Nonetheless, preheated plants (2HW) were closer to control plants than non-preheated ones, especially in the case of *Z. muelleri*. Additionally, control plants of both species were located within the same quadrant II of the PCA graph (**Fig.**

2.8), in accordance with their higher photochemical capacity (Fv/Fm; ΔF/Fm') and pigments content (Chl *a* and carotenoids). In contrast to controls, heated plants of the two species were separated along PC1 axis (responsible for 61.61% of total variance; **Fig. 2.8**), suggesting slight differences in the response of the two seagrass species to the experimental recurrent heatwave at T7.

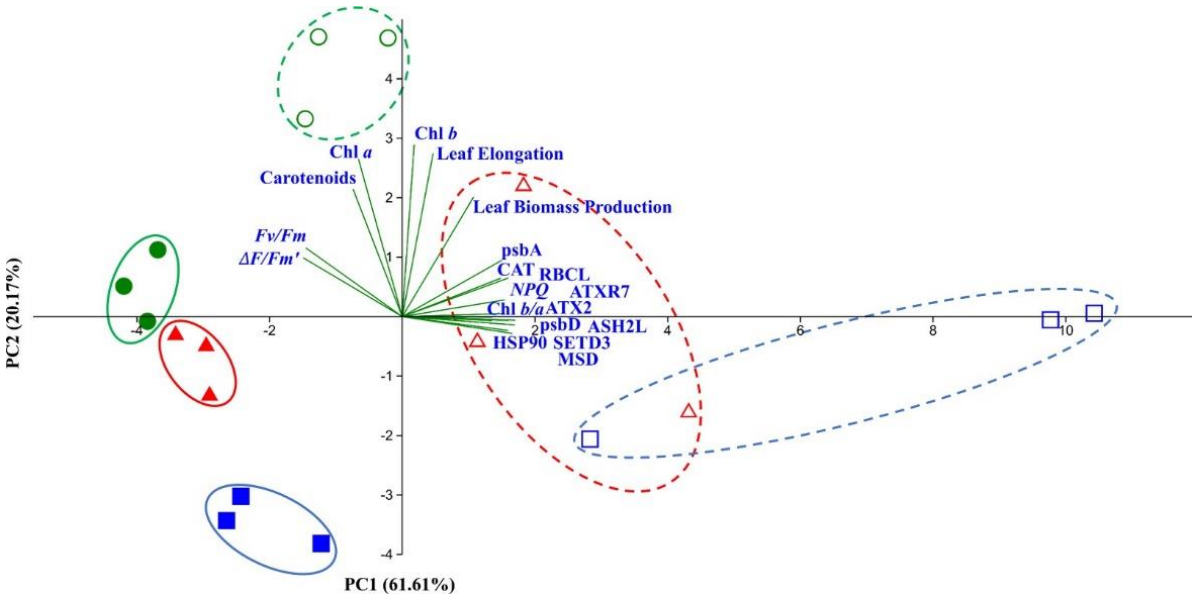

Figure 2.8 *PCA conducted on morphological, physiochemical and gene expression data at T7. Different colors and shapes correspond to different treatments (Green circle = Control-CT, Blue square = Treatment 1-heatwave-1HW, Red triangle = Treatment 2-heatwave-2HW) and species (filled = Zostera muelleri, un-filled = Posidonia australis).*

Table 2.1 *List of housekeeping genes and gene of interests used in this study. Pa: Posidonia australis, Zm: Zostera muelleri; E: Efficiency (%); R^2: Calibration coefficient.*

Gene category	Gene Name	Abbrev	Species	Forward primer (5' -> 3')	Reverse primer (5' -> 3')	Product size (bp)	E (%)	R^2	Accession number	Reference (note)
Stress-related	Heat Shock Protein 90	HSP90	Zm	GAGGGTTTGTGCAAGGTCAT	GTTGGCAGTCCACCCATACT	123	103.9	0.996	ZM251873	This study
			Pa	TCAAGGAGGTGTCACACGAG	CAGATGCTCCTCCCAGTCAT	134	109.8	0.997	PO008787	This study
	Catalase	CAT	Zm	AAGTACCGTCCGTCAAGTGG	CTGGGATACGCTCCCTATCA	169	100.5	0.999	ZM230093	This study
			Pa							Same as *Z. muelleri*
	Manganese superoxide dismutase	MSD	Zm	TTTTCGCCAAGAACAAAACC	TCTGCATGATCTCTCCGTTG	135	99.8	0.998	ZM212939	This study
			Pa	AATAATGCCGCTCAGCTTTG	ACCCAACCAGATCCAAACAG	176	98.0	0.994	PO035322	This study
Photosynthesis-related	Photosystem II protein D1	psbA	Zm	AAGCTTATGGGGTCGCTTCT	GTGCAGCAATGAAAGCGATA	134	100.4	0.999	ZM045788	This study
			Pa	GACTGCAATTTTAGAGAGACGC	CAGAAGTTGCAGTCAATAAGGTAG	136	100.9	0.999	KC954695	(Dattolo *et al.*, 2014)
	Photosystem II protein D2	psbD	Zm							Same as *P. australis*
			Pa	CCGCTTTTGGTCACAAATCT	CGGATTTCCTGCGAAACGAA	161	103.6	0.999	KC954696	(Dattolo *et al.*, 2014)
	Rubisco large subunit	RBCL	Zm	CCGAGACAACGGCTTACTTC	AGTCATCTCGCGTTCACCTT	175	100.1	1.000	ZM194765	This study
			Pa	GCTGCCGAATCTTCTACTGG	CACGTTGGTAACGGAACCTT	177	102.2	0.999	U80719.1	(Marín-Guirao *et al.*, 2016)
Housekeeping	Glyceraldehyde 3-phosphate dehydrogenase	GAPDH	Zm	CGGTTACTGTAGCCCCACTC	CAAAGGCTGGGATTGGTTTA	79	100.8	0.992	Zoma_C_c6252	(Kim *et al.*, 2018)
			Pa	AGGTTCTTCCTGCTTTGAATG	CTTCCTTGATTGCTGCCTTG	138	110.3	0.998	GO347079	(Serra *et al.*, 2012)
	Elongation factor 1-alpha	Ef1A	Zm	AAGCAAAGGCGTCACTTGAT	TCTGCTGCCTTCTTCTCCTC	82	103.4	0.989	Zoma_C_c59090	(Kim *et al.*, 2018)
			Pa	GAGAAGGAAGCTGCTGAAATG	GAACAGCACAATCAGCCTGAG	214	107.2	0.997	GO346663	(Serra *et al.*, 2012)
	β-tubulin	TubB	Zm	GGACAAATCTTCCGTCCAGA	TCCAGATCCAGTTCCACCTC	185	102.8	0.995	Zoma_Contig120	(Kim *et al.*, 2018)
	Actin	Actin	Zm	TAAGGTCGTTGCTCCTCCTG	ACTCTGCCTTTGCAATCCAC	104	95.3	0.993	Zoma_ZMF02257	(Kim *et al.*, 2018)
	18S ribosomal RNA	18S	Pa	AACGAGACCTCAGCCTGCTA	AAGATTACCCAAGCCTGTCG	200	93.0	1.000	AY491942.1	(Serra *et al.*, 2012)
	Ubiquitin	UBI	Pa	CACCCTCGCTGACTACAACA	TTTCTCAGCCTGACGACCTT	195	97.2	0.998	GO347694	(Serra *et al.*, 2012)
Methylation-related	ProteinSet1/Ash2 histone methyl transferase complex subunit ash-2	ASH2L	Zm	CTCAGACCCCCAATTCTCAA	GTGGAAGAGACGACGGTGAT	153	100.3	0.994	ZM248014	This study
			Pa	CTATCCTGCTGCCTCCATGT	TCAACTGCACCTTCAACTCG	174	108.1	0.992	SRP126951	This study
		SETD3	Zm	CGAACCTTCCTTTCTTGCTG	CCTCGGGTTGAGAATCAAAA	146	90.5	0.995	ZM228252	This study

	Histone-lysine N-methyltransferase setd3		Pa	TGGGCTTGTGAACTGTGGTA	CGAATGATTGAGTCGTCCAG	200	103.9	0.949	SRP126951	This study
	Histone-lysine N-methyltransferase ATX2	ATX2	Zm	ATCCCGTGAATGTGGAGAAG	ATACCAGGCACCGTCGATAG	161	97.2	0.992	ZM254823	This study
			Pa	CCAGATACAAAGCTGCACCA	GCATTGTCATCCCCTTGAGT	170	103.1	0.993	SRP126951	This study
	Histone-lysine N-methyltransferase ATXR7 isoform X1	ATXR7	Zm	CAGAGGATCAATCCCTCCAA	CTTTGCCCGAACTCTTTCAG	138	102.0	0.990	ZM256759	This study
			Pa	CGAGTAGGGTCGAATGTGGT	ATCCATCCAGTCACACACGA	149	105.2	0.973	SRP126951	This study

Table 2.2 *PERMANOVA analysis performed on photo-physiological measurements assessing the effect of increased seawater temperature among different treatments over time. Significant differences (p(perm) < 0.05) are in bold.*

Species	Measurement	Source	df	SS	MS	Pseudo-F	$p_{(perm)}$	Unique perms
Posidonia australis	Maximum quantum yield	Time	6	537.87	89.646	12.632	**0.0001**	9946
		Treatment(Time)	14	516.3	36.879	5.1968	**0.0001**	9925
	Effective quantum yield	Time	6	1468	244.66	15.354	**0.0001**	9947
		Treatment(Time)	14	1497.5	106.97	6.7128	**0.0001**	9928
	NPQ	Time	6	713.35	118.89	3.3991	**0.0045**	9945
		Treatment(Time)	14	1084.3	77.448	2.2142	**0.0129**	9907
Zostera muelleri	Maximum quantum yield	Time	6	33.814	5.6357	3.5623	**0.0037**	9932
		Treatment(Time)	14	122.68	8.7628	5.5389	**0.0001**	9930
	Effective quantum yield	Time	6	36.221	6.0368	1.2295	0.2884	9938
		Treatment(Time)	14	264.19	18.871	3.8433	**0.0002**	9924
	NPQ	Time	6	203.36	33.893	1.0434	0.3953	9944
		Treatment(Time)	14	235.6	16.828	0.51807	0.9169	9918

Table 2.3 *Results from One-way ANOVA analyses and Kruskal-Wallis test performed on plant growth and pigment content results. Significant differences (p < 0.05) are in bold.*

Species	Measurement	Statistical analysis	df	*F*	*p*
Posidonia australis	Biomass	One-way ANOVA	2	8.130	**0.006**
	Leaf growth	One-way ANOVA	2	22.459	**0.000**
	Chl *a*	One-way ANOVA	2	3.698	0.056
	Chl *b*	One-way ANOVA	2	2.161	0.158
	Chl *b*/*a*	Kruskal-Wallis test	2		**0.007**
	Carotenoids	One-way ANOVA	2	1.301	0.308
Zostera muelleri	Biomass	One-way ANOVA	2	4.959	**0.027**
	Leaf growth	One-way ANOVA	2	11.473	**0.002**
	Chl *a*	One-way ANOVA	2	0.893	0.435
	Chl *b*	One-way ANOVA	2	0.041	0.960
	Chl *b*/*a*	One-way ANOVA	2	16.767	**0.000**
	Carotenoids	One-way ANOVA	2	0.795	0.474

Table 2.4 *PERMANOVA analysis performed on gene expression levels of GOIs from different treatments. Significant differences (p(perm) < 0.05) are in bold.*

			Posidonia australis						***Zostera muelleri***				
GOI	**Source**	**df**	**SS**	**MS**	**Pseudo-F**	$p_{(perm)}$	**Unique perm**	**df**	**SS**	**MS**	**Pseudo-F**	$p_{(perm)}$	**Unique perm**
HSP90	Time	1	11370	11370	54.839	**0.0001**	9950	1	1367.2	1367.2	5.708	**0.023**	9946
	Treatment(Time)	4	9144.3	2286.1	11.026	**0.0001**	9928	4	2341.1	585.3	2.444	0.074	9952
CAT	Time	1	1654.8	1654.8	7.715	**0.0059**	9943	1	661.2	661.2	5.783	**0.032**	9932
	Treatment(Time)	4	3888.5	972.13	4.5323	**0.0052**	9944	4	2475.7	618.9	5.413	**0.009**	9945
MSD	Time	1	13097	13097	31.569	**0.0001**	9958	1	5659.8	5659.8	80.129	**0.000**	9956
	Treatment(Time)	4	11668	2917	7.0308	**0.0001**	9933	4	4383.4	1095.8	15.514	**0.000**	9937
psbA	Time	1	3337.6	3337.6	9.5367	**0.0009**	9939	1	1683.3	1683.3	9.690	**0.002**	9946
	Treatment(Time)	4	3082.5	770.63	2.2019	**0.0762**	9942	4	1797.0	449.2	2.586	**0.054**	9956
psbD	Time	1	6433.2	6433.2	23.064	**0.0001**	9930	1	446.1	446.1	2.997	0.099	9911
	Treatment(Time)	4	3889.9	972.47	3.4865	**0.0081**	9943	4	2147.8	536.9	3.607	**0.019**	9964
RBCL	Time	1	9830.1	9830.1	40.425	**0.0001**	9952	1	1219.8	1219.8	3.474	0.061	9948
	Treatment(Time)	4	5831.5	1457.9	5.9954	**0.0001**	9929	4	6060.9	1515.2	4.315	**0.004**	9938
ASH2L	Time	1	7703.7	7703.7	16.493	**0.0001**	9960	1	1789.8	1789.8	33.712	**0.000**	9945
	Treatment(Time)	4	12466	3116.6	6.6724	**0.0001**	9935	4	4312.0	1078.0	20.305	**0.000**	9954
SETD3	Time	1	8866.8	8866.8	13.863	**0.0001**	9953	1	217.0	217.0	1.685	0.196	9939
	Treatment(Time)	4	18997	4749.3	7.4254	**0.0001**	9923	4	10304.0	2576.0	19.997	**0.000**	9954
ATX2	Time	1	13600	13600	64.66	**0.0001**	9960	1	541.8	541.8	9.199	**0.002**	9953
	Treatment(Time)	4	14096	3523.9	16.754	**0.0001**	9942	4	5489.7	1372.4	23.301	**0.000**	9956
ATXR7	Time	1	5666.8	5666.8	14.48	**0.0001**	9951	1	128.4	128.4	1.680	0.226	9928
	Treatment(Time)	4	11148	2786.9	7.1212	**0.0001**	9942	4	1318.4	329.6	4.312	**0.015**	9950

2.4 Discussion

2.4.1 Thermal priming effect on seagrasses

This study provides for the first time some evidence of thermal priming effects in seagrasses. Looking at the photo-physiological results during the 2nd heatwave, it is clear that 2HW plants had been primed during the first heatwave (**Fig. 2.3**). From both seagrass species, the photochemical efficiency (Fv/Fm and ΔF/Fm') were higher (significantly in some cases) in preheated plants (2HW) with regard to non-preheated plants (1HW). Several studies from terrestrial plants have demonstrated that heat-primed plants had a higher photosynthetic rate in relation to the non-primed plants (Smillie and Gibbons, 1981; Wang, Cai, *et al.*, 2014; Wang, Vignjevic, *et al.*, 2014; Li *et al.*, 2015). Hence, photo-physiological results strongly support the induction of a thermal priming status on both studied seagrass species. Focusing on T7, while 2HW-*P. australis* plants were able to keep their NPQ values similar to CT plants, the 1HW-*P. australis* plants greatly increased their NPQ, which is a photo-protective mechanism commonly activated in stressed plants (Ashraf and Harris, 2013).

From a morphological perspective, significant differences were detected between non-primed (1HW) and heat-primed (2HW) *Z. muelleri* plants in terms of leaf elongation and leaf biomass production (**Fig. 2.4**). For both parameters, 1HW-*Z. muelleri* plants suffered a significant reduction with respect to 2HW plants and CT plants. This indicated that (*i*) 2HW plants were primed by the first heatwave, (*ii*) performed better during the second heatwave and (*iii*) were able to better maintain their growth as compared to that of the 1HW plants. This is similar to those found in terrestrial plants (Wang, Cai, *et al.*, 2014; Wang, Vignjevic, *et al.*, 2014) showing that primed *Triticum aestivum* L. better maintained their biomass compared to un-primed plants during a more severe heat stress. It is likely that the relatively slow growth rates of this climax species and the short marking time (i.e. growing period) compared to the pioneer species, did not allow for the detection of differences in growth between both heat treatments (1HW vs 2HW). A longer lasting growing period could be needed to detect effects at the plant growth level in slow-growing seagrass species.

In support of the hypothesis of thermal priming effects in seagrasses, large Chl *a* reductions were only detected in leaves of non-primed plants (1HW). This becomes more obvious in the Chl *b*/*a* ratios of *Z. muelleri* at the end of the second heatwave (T7). While heat-primed 2HW plants kept their Chl *b*/*a* ratios similar to the controls, non-primed 1HW plants experienced a significant increase in Chl *b*/*a* ratios as seen in previous studies (Almeselmani and Viswanathan, 2012; Niu *et al.*, 2017).

At the molecular level, there were some more findings about the acquisition of a thermal-priming status in both seagrasses. This is supported by a significantly lower expression level of some GOIs from 2HW plants compared to those from 1HW plants. In *P. australis* at T7, the expression levels of some methylation-related GOIs (i.e. ATX2 and ATXR7) and photosynthesis-related GOIs (i.e. psbD) were significantly higher in non-primed plants (1HW) in comparison with heat-primed plants (2HW) and control plants (CT). Similarly, evidence supporting the thermal priming hypothesis can be found in stress-related GOIs (i.e. CAT) and methylation-related GOIs (i.e. ASH2L and ATX2) in heated *Z. muelleri* plants.

In addition, PCA results at T7 (**Fig. 2.7B,D** and **Fig. 2.8**) further supported the successful effects of the thermal-priming treatment in both species as 2HW plants were clustered with CT plants while 1HW plants separated away from these two former groups in both studied species.

During the first heatwave (T3) the two species showed differences in gene expression. While a large amount of GOIs (i.e. 6/10) showed significant up-regulation in the case of *Z. muelleri*, only 3 GOIs were significantly up-regulated in the case of *P. australis*. An alternative to epigenetic modifications, the accumulation of protective molecules (i.e. HSPs) is also likely involved in facilitating a fast stress response and hence are also possible mechanisms underlying stress memory. At T3, only *Z. muelleri* activated HSP90 which is a well-known heat-protective molecule also involved in the heat stress response of different seagrasses (Marín-Guirao *et al.*, 2016; Tutar *et al.*, 2017; Mota *et al.*, 2018; Traboni *et al.*, 2018). Together, these differences between the two species suggest that *Z. muelleri* plants were, indeed, more prone to thermal priming and hence to acquire thermal tolerance after recurrent heat events than *P. australis* plants.

2.4.2 Evidence about the role of epigenetic modifications with thermal priming effect on seagrasses

This study also suggested the involvement of epigenetic modifications in response to thermal stress in seagrasses. This study confirmed recent transcriptomic discoveries in seagrasses showing the induction of genes involved in DNA and histone methylation [including some methylation-related GOIs in this study (i.e. ATX2 and SETD3)], in heated *P. oceanica* (Marín-Guirao *et al.*, 2017; Marín-Guirao *et al.*, 2019). Among methylation-related GOIs, ProteinSet1/Ash2 histone methyl transferase complex subunit ash-2 (ASH2L) and Histone-lysine N-methyltransferase ATX2 are known as being specifically involved in methylation and dimethylation at Lys4 of histone H3 (H3K4) (Wysocka *et al.*, 2003; Patel *et al.*, 2009).

Methylation status of H3K4 involves in changing chromatin structure during environmentally-induced transcriptional memory (D'Urso and Brickner, 2017) and plant stress response via activating or silencing gene expression (Shanker, 2016). ATXR7 belongs to the Trithorax family proteins that connect with seasonal memory in plants (Iwasaki and Paszkowski, 2014). Histone-lysine N-methyltransferase SETD3 is linked to H3K36 methyltransferase (Pontvianne, Blevins and Pikaard, 2010; Suzuki, Murakami and Takahata, 2017) which in plants has been suggested to play an important role in development and stress responses (Huang *et al.*, 2016). The regulations of the methylation-related GOIs in this study are consistent with previous works which highlighted the role of epigenetic modifications in seagrasses (Davey *et al.*, 2016; Marín-Guirao *et al.*, 2017; Duarte *et al.*, 2018; Marín-Guirao *et al.*, 2019) or in terrestrial plants (Chinnusamy and Zhu, 2009; Liu *et al.*, 2015; Rey *et al.*, 2016).

Appendix II

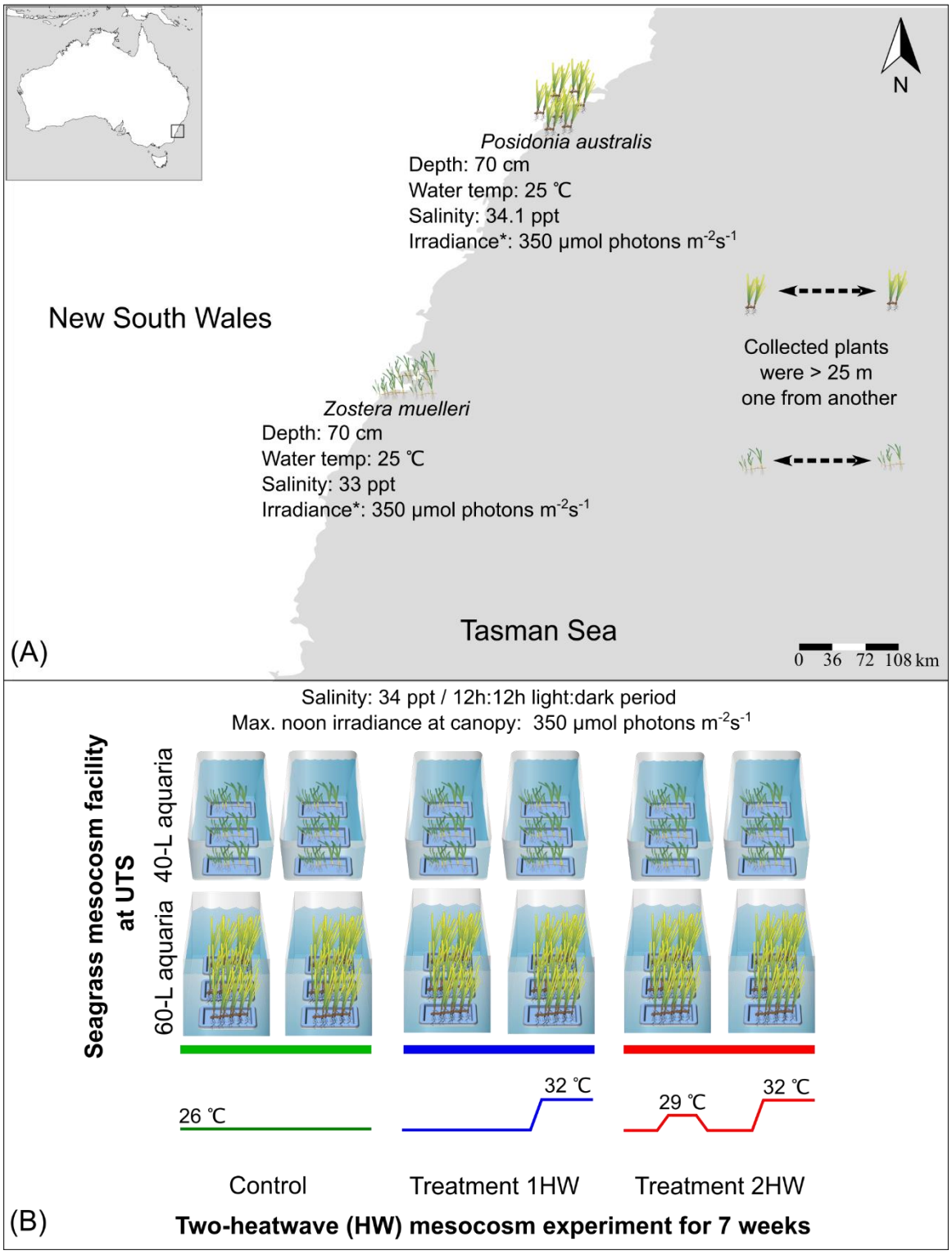

Figure AII.1 *Sampling regime (A) and experimental setup (B) of the experiment at UTS. Irradiance*: Saturating irradiance levels of plants in the field determined by rapid light curves using a PAM fluorometer.*

Table AII.1 *Results from NormFinder for the selection of best HKGs for P. australis*

Gene name	**Stability value**	**Best gene**	18S
GADPH	10.623	**Stability value**	7.817
ef1A	10.750		
18S	7.817	**Best combination of two genes**	ef1A and 18S
UBI	11.581	**Stability value for best combination of two genes**	6.718

Table AII.2 *Results from GeNorm for the selection of best HKGs for P. australis*

Gene name	**Normalization Factor**
GADPH	1.1532
ef1A	0.6867
18S	0.8256
UBI	1.5295

Table AII.3 *Results from Bestkeeper for the selection of best HKGs for P. australis*

	HKG 1	**HKG 2**	**HKG 3**	**HKG 4**
BestKeeper vs. coeff. of corr. [r]	0.934	0.916	0.975	0.855
***p*-value**	0.001	0.001	0.001	0.001
Regression Analysis: HKG vs. BestKeeper				
	GAPDH	**ef1A**	**18S**	**UBI**
	TG 1 vs. BK	TG 2 vs. BK	TG 3 vs. BK	TG 4 vs. BK
coeff. of corr. [r]	0.934	0.916	0.975	0.855
coeff. of det. [r^2]	0.872	0.839	0.951	0.731
intercept [CP]	-0.7048	1.9011	-4.8694	10.0904
slope [CP]	1.2312	0.6345	1.1933	1.0783
SE [CP]	±12.86	±7.576	±7.383	±17.821
p-value	0.001	0.001	0.001	0.001
Power [x-fold]	2.497351726	1.552399636	2.191571	2.07968769

Table AII.4 *Results from NormFinder for the selection of best HKGs for Z. muelleri*

Gene name	**Stability value**	**Best gene**	ef1A
Actin	36.326	**Stability value**	23.198
ef1A	23.198		
GADPH	65.052	**Best combination of two genes**	ef1A and Tub
Tub	34.808	**Stability value for best combination of two genes**	25.393

Table AII.5 *Results from GeNorm for the selection of best HKGs for Z. muelleri*

Gene name	**Normalisation Factor**
Actin	**0.6215**
ef1A	**0.8294**
GADPH	**2.5841**
Tub	**0.7508**

Table AII.6 *Results from Bestkeeper for the selection of best HKGs for Z. muelleri*

	HKG 1	**HKG 2**	HKG 3	**HKG 4**
BestKeeper vs. coeff. of corr. [r]	0.908	**0.911**	0.808	**0.912**
***p*-value**	0.001	**0.001**	0.001	**0.001**
Regression Analysis: HKG vs. BestKeeper				
	Actin	**ef1A**	GADPH	**Tub**
	TG 1 vs. BK	**TG 2 vs. BK**	TG 3 vs. BK	**TG 4 vs. BK**
coeff. of corr. [r]	0.908	**0.911**	0.808	**0.912**
coeff. of det. [r^2]	0.824	**0.83**	0.653	**0.832**
intercept [CP]	5.6725	**-15.682**	102.903	**-25.2161**
slope [CP]	0.5811	**0.9521**	1.8652	**0.971**
SE [CP]	±16.055	**±25.767**	±81.33	**±26.091**
p-value	0.001	**0.001**	0.001	**0.001**
Power [x-fold]	1.475458754	**1.965988342**	3.67041	**1.9868404**

Table AII.7 *Selections of best HKGs used in this study*

Species	**NormFinder (Stab. Value)**	**GeNorm (Norm. factor)**	**Bestkeeper [r]**
Posidonia australis	**18S** (7.817)	**ef1A** (0.6876)	GAPDH (0.934)
	GAPDH (10.623)	**18S** (0.8256)	**18S** (0.975)
	ef1A (10.750)	GAPDH (1.1532)	**ef1A** (0.916)
	UBI (11.518)	UBI (1.5295)	UBI (0.855)
Zostera muelleri	**ef1A** (23.198)	Actin (0.6215)	**Tub** (0.912)
	Tub (34.808)	**Tub** (0.7508)	**ef1A** (0.911)
	Actin (36.326)	**ef1A** (0.8294)	Actin (0.908)
	GAPDH (65.052)	GAPDH (2.5841)	GAPDH (0.808)

Chapter III – Molecular insights about the mechanisms governing thermal stress memory in seagrasses

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The work presented in this chapter has been included in the following manuscript:

Hung Manh Nguyen, Uyen V. T. Hong, Miriam Ruocco, Emanuela Dattolo, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Beneath the memory: new insights on molecular mechanisms governing thermal stress memory in seagrasses. *In preparation* targeting to Journal of Ecology.

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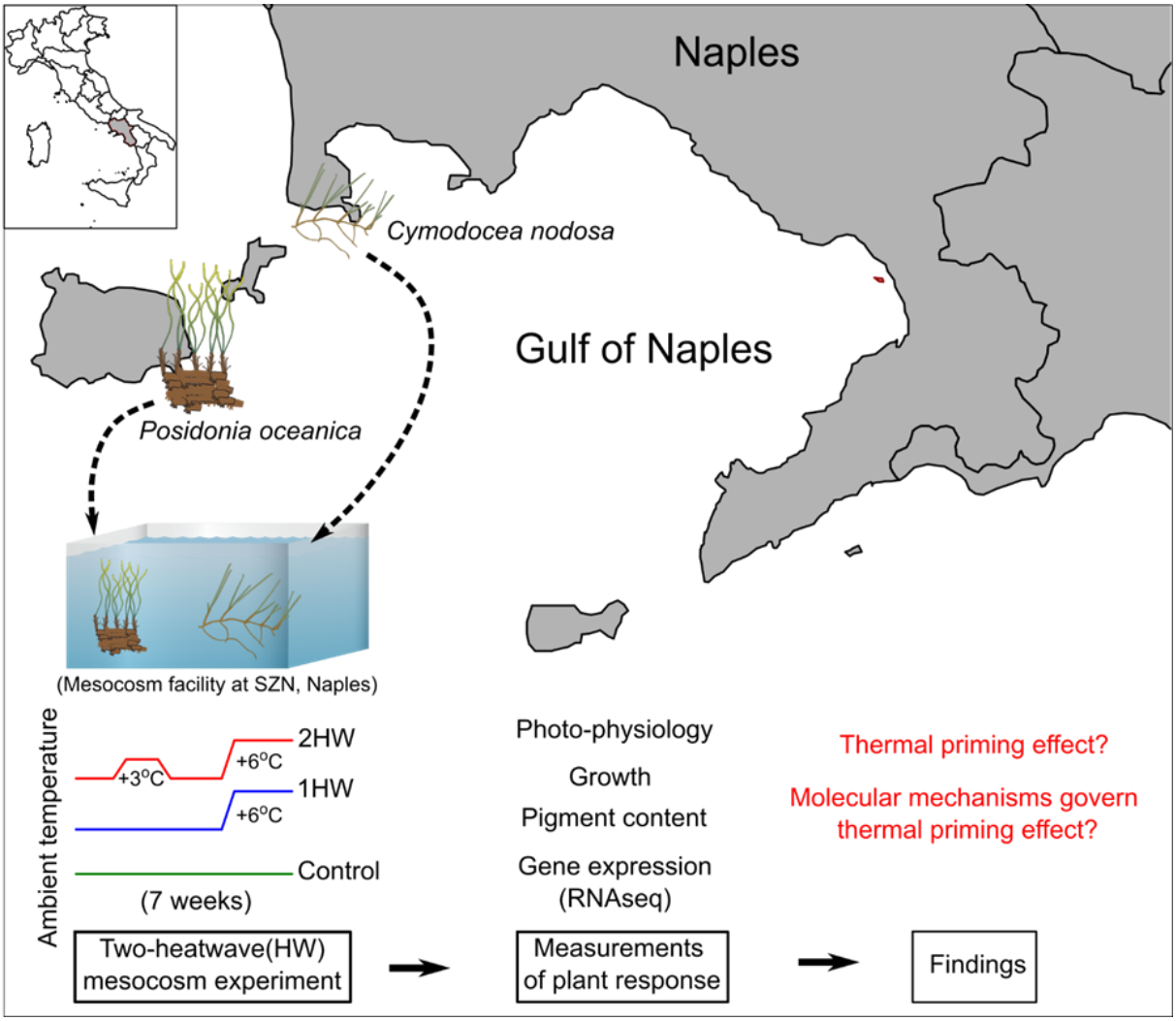


Figure 3.1 *Conceptual diagram illustrating the experiment presented in this chapter. Molecular mechanisms governing thermal stress memory in seagrasses. Symbols were taken from the IAN symbol libraries, available at https://ian.umces.edu/media-library/. Maps were taken from SimpleMappr, available at https://www.simplemappr.net/.*

3.1 Introduction

3.1.1 Molecular mechanisms of thermal priming and stress memory in response to warming

In plants, thermal stress memory can be divided into two phases including 'priming' (that refers to the first exposure of a plant to heat stress; 'thermal stress memory' is formed during this event) and 'memory' (that refers to the maintenance of 'thermal stress memory' and the activation of modified plant responses when the stimulus reoccurs) (Bäurle, 2016; Hilker *et al.*, 2016). Although the number of studies on thermal stress memory in terrestrial plants has increased recently (e.g. see reviews by Bruce *et al.*, 2007; Turgut-Kara, Arikan and Celik, 2020), the molecular mechanisms that underlie heat stress priming and heat stress memory still remain scarce (Bäurle, 2016).

At the transcriptional level, two mechanisms can be activated during the memory phase including (*i*) the maintenance of gene expression levels of those genes previously induced during the priming phase and (*ii*) the rapid regulation (induction or repression) of genes upon a recurrent stress event (Bäurle and Trindade, 2020). These two mechanisms allow primed plants to be more prepared and to respond in a faster manner to a subsequent stress event than non-primed plants (Hilker *et al.*, 2016; Bäurle and Trindade, 2020). The induction of HEAT SHOCK PROTEINs (HSPs) is among the fastest responsive processes that happen in plants when exposed to different abiotic stress factors, including thermal stress (Park and Seo, 2015). This process is governed by HEAT SHOCK TRANSCRIPTION FACTORs (HSFs), which are transcriptional factors responsible for the activation and orchestration of the heat stress response (Nover *et al.*, 2001). Previous studies have demonstrated that HSFs are involved in both phases of thermal stress memory (Bäurle, 2016; Olas *et al.*, 2021). For instance, among the 20 known members of the HSF family (Scharf *et al.*, 2012), HSFA1 occurs exclusively in the priming phase as a primary mediator of the heat priming process (Liu, Liao and Charng, 2011); whereas HSFA2 is required for the memory phase by maintaining high expression levels of several thermal stress memory-related genes (Schramm *et al.*, 2006; Charng *et al.*, 2007). It is important to note that some HSFs can act as positive regulators of heat stress responses while others can function as repressors (Nover *et al.*, 2001; Ikeda, Mitsuda and Ohme-Takagi, 2011). In addition to HSFs, many other genes are involved in thermal stress memory such as the stem cell regulators CLAVATA1 (CLV1), CLV3, HSP17.6A, HSP18.2, HSP21, HSP22, HSP101, FRUCTOSE BISPHOSPHATE ALDOLASE 6 (FBA6), PLASTIDIAL PYRUVATE KINASE 4 (PKP4), the plastid metalloprotease (FtsH6) among others (Wu *et al.*, 2013; Sedaghatmehr, Mueller-Roeber and Balazadeh, 2016; He and Li, 2018; Sharma *et al.*, 2019;

Olas *et al.*, 2021; Song *et al.*, 2021). In *Arabidopsis thaliana*, the expression of CLV1, CLV3 and HSP17.6A was significantly higher in primed plants with respect to non-primed plants, however, the level of expression of HSP17.6A gradually declined in the matter of hours after the stress started (Olas *et al.*, 2021). Also from Olas *et al.*, (2021), during the priming event, the induction of FBA6 gene was found not immediately but only after 2h from the beginning of warming. Nevertheless, when the warming event recurred, the overexpression of this gene was stronger and more quickly (already within 0.5h) in comparison with the response observed during the priming event (Olas *et al.*, 2021). Another study on the transcriptomics of *A. thaliana* under heat stress showed that HSP21 rapidly accumulated during the first exposure to warming (i.e. priming stimulus) and remained abundant during the subsequent memory phase ensuring a sustained thermal stress memory (Sedaghatmehr, Mueller-Roeber and Balazadeh, 2016). That study also showed that the accumulation of HSP21 was negatively controlled by the plastid-localized metalloprotease FtsH6 evidencing by the lack of a functional FtsH6 protein promoted HSP21 accumulation and increased the thermomemory capacity of primed plants (Sedaghatmehr, Mueller-Roeber and Balazadeh, 2016). Furthermore, the accumulation and persistence of histone H3 lysine 4 trimethylation and dimethylation (H3K4me3 and H3K4me2) was shown to be directly associated with the induction of memory-related genes including HSP18.2, HSP21 and HSP22 during both the priming and the memory phase (Lämke *et al.*, 2016; He and Li, 2018; Bäurle and Trindade, 2020). Interestingly, these H3K4me3 and H3K4me2 are dependent on functional HSFA2 even though HSFA2 itself appears not to be needed for the maintenance of those chromatin changes (Lämke *et al.*, 2016). Noteworthy, not only for thermal stress, H3K4 methylation is also involved in plant stress memory related with other abiotic stresses (Ding, Fromm and Avramova, 2012; Sani *et al.*, 2013; Hilker *et al.*, 2016). Besides H3K4 methylation, histone H3 lysine 27 trimethylation (H3K27me3) is also at the centre of the so-called 'epigenetic memory' in plants. However, the demethylation of H3K27me is, instead, related to HSP22 and HSP17.6C (Yamaguchi *et al.*, 2021). Lastly, the induction of DNA methylation-related genes such as DNA demethylase DEMETER (DME) and DNA methyltransferase (MET1) have been shown to provide not only 'with-in generation' stress memory but also 'heritable' stress memory to next generations (see reviews by Iwasaki and Paszkowski, 2014; Turgut-Kara, Arikan and Celik, 2020).

3.1.2 The cellular stress response concept

Cellular stress response (CSR) is a universal mechanism of cellular response to damage that environmental stressors impose on macromolecules (Kültz, 2005). This is a characteristic of

all cells regardless type of stress that causes such damage (Kültz, 2005). The CSR induces a specific set of proteins whose function is to prevent and/or repair macromolecular damage by cell cycle control, protein chaperoning, DNA/chromatin stabilization and repair, removal of damaged proteins, and certain aspects of metabolism (Kültz, 2003). The CSR plays a key role in determining the range of environmental changes an organism can tolerate, therefore, it can serve as an indicator for assessing the stress level experienced by organisms, including plants (Evans and Hofmann, 2012). The CSR can be divided into three different categories or thresholds that represent different stages of the severity and progression of stress (Evans and Hofmann, 2012). The first level is associated with protein denaturation and the increased synthesis of molecular chaperones (e.g. HSPs, see also Lindquist, 1986), that ultimately protect proteins from damage and re-establish unfolded proteins to their folded and functional conformations. The second level induces the expression of genes related to proteolysis, a biochemical process that removes irreversibly damaged proteins as an attempt to achieve protein homeostasis (proteostasis) when the stress progresses (e.g. see also Travers *et al.*, 2000). When the stress level is too extreme, the third level of CSR is activated and involved the induction of genes that prevent the replication of damaged DNA and/or stimulate programmed cell death pathways (e.g. see also Logan and Somero, 2011). In seagrass research, the CSR has been applied just in a previous study (Traboni *et al.*, 2018) to assess, through RT-qPCR gene expression analysis, thresholds for physiological function that underlie responses of *P. oceanica* to warming.

3.1.2 The study

In this chapter, I aimed at (*i*) confirming the effect of thermal priming on Mediterranean seagrasses, (*ii*) improving our understanding of how primed plants respond to warming at the molecular level, and (*iii*) digging into the molecular mechanisms that could govern the thermal-stress memory in seagrasses (both the priming and the memory phase). For this purpose, an *ad hoc* mesocosm experiment (with a heat-priming event and a heat-triggering event) was conducted using the two main Mediterranean seagrass species, *Posidonia oceanica* and *Cymodocea nodosa*. Plant responses were assessed at the end of the triggering event including photo-physiology, growth, pigments, and gene expression. As a methodological difference from the study presented in Chapter II (where RT-qPCR approach was applied), in this Chapter III, the study of gene expression was conducted through a high-throughput gene-expression profiling (i.e. RNA-seq approach). Moreover, the CSR concept was adopted to explore RNA-seq data and to compare the whole-transcriptome response to warming between heat-primed

and non-primed plants. In this way, it was hypothesized that: (*i*) heat-primed plants would perform better during the triggering event than non-primed plants, (*ii*) heat-primed plants would show, at the transcriptomic level and according to the CSR model, fewer evidences of heat stress than non-primed plants during the triggering event and, (*iii*) during the triggering event, heat-primed plants would display molecular evidence of the activation of the memory phase (i.e. stress memory-related genes) whereas non-primed plants would inform about the molecular mechanisms underlying the activation of the priming phase (i.e. priming-related genes).

3.2 Materials and Methods

3.2.1 Sample collection

Plant samples were collected haphazardly by SCUBA diving at the gulf of Naples, Italy on the 18th September 2019 including *Posidonia oceanica* (40°44.020'N, 13°58.039'E at 5−6 m depth) and *Cymodocea nodosa* (40°47.021'N, 14°04.404'E at 8-10 m depth). To ensure a highly diverse genotypic profile in experimental populations, each plant was sampled at a minimum distance of 10 m one from another. After collections, plants were kept in a dark cooler filled with natural seawater at ambient temperature and transported shortly (<2 hours) to a benthic mesocosm facility at Stazione Zoologica Anton Dohrn (SZN), Napoli, Italy. Environmental conditions (i.e. salinity, irradiance and water temperature) were measured at the time of plant sampling to mimic the natural conditions at the experimental setup.

3.2.2 Experimental setup

Once at the SZN mesocosm facility, plants bearing a similar number of shoots (~ 10 shoots) were carefully selected to standardize the experiment. Selected *P. oceanica* plants were transplanted onto plastic net cages (i.e. two fragments in each cage) similar to Ruocco, Marín-Guirao and Procaccini (2019), while each *C. nodosa* plant was individually mounted in a plastic container (32 × 23 × 10 cm) filled with natural sediments from the collection site as described in Marín-Guirao *et al.*, (2018). After the transplant, eighteen plant fragments from each species were randomly allocated into nine 500L-aquaria (i.e. each aquarium contained two *P. oceanica* plants and two *C. nodosa* plants) filled with filtered and UV-treated natural seawater from a close area. Plants were arranged inside each aquarium to avoid the consequences of crossed-species shading (see **Fig. 3.2**). In addition, the depth of the two species inside the tanks was adjusted accordingly to reproduce a similar light level to that existing at the collection sites (i.e. 300 µmol photons m^{-2} s^{-1} for *P. oceanica* and 200 µmol photons m^{-2} s^{-1} for *C. nodosa*

above the canopy, respectively). Three experimental treatments were selected including control, non-primed and heat-primed. For each species, each experimental treatment included six plants allocated in three tanks (i.e. two plants per tank). Details about the systems for controlling light, water temperature and water quality can be found in a previous study (Ruocco, Marín-Guirao and Procaccini, 2019). A 12h:12h light:dark photoperiod was applied to start from 7:00, then progressively increased to the maximum irradiance at 13:00 before a gradual reduction until dark at 19:00. In addition, the water temperature was measured automatically every 10 min using HOBO Pendant® Temperature/Light 64K Data Logger (Onset, USA) and manually checked twice a day with WTW Cond 3310 Set 1 (Xylem Analytics, Germany). Throughout the experiment, the salinity level was kept at 37.5 PSU as in natural conditions by adding purified water accordingly to compensate for evaporation. Furthermore, seawater was filtered by using both continuous mechanical filtrations and UV sterilizations and partly renewed (25−30%) weekly to sustain the water quality. An introductory video was made for this experiment and could be found on the website of Dr. Gabriele Procaccini's Laboratory (available at *https://gpgroupszn.wixsite.com/website*: EpicSea). See also **Figure AIII. 1** for a detailed explanation about the sampling regime and the experimental setup.

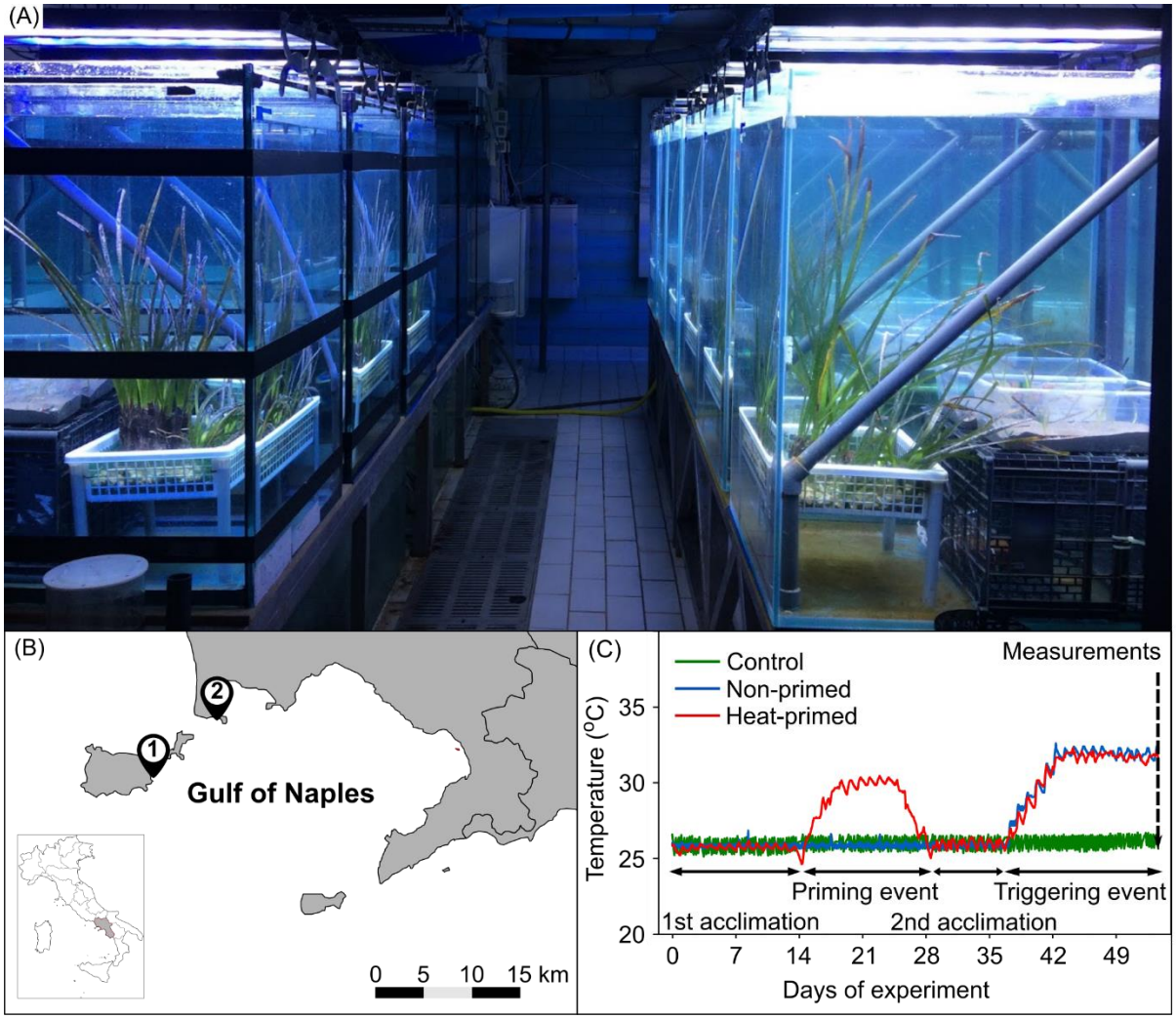


Figure 3.2 *(**A**) Benthic mesocosm system at Stazione Zoologica Anton Dohrn (Naples-Italy) used in this study. (**B**) Sample collection sites (**1**-Posidonia oceanica and **2**-Cymodocea nodosa) and (**C**) temperature profile during the experiment.*

3.2.3 Experimental design

Before the beginning of the experimental treatments, all plants were allowed to acclimatize to the mesocosm conditions for a two-week period at 26℃ (similar to the natural seawater temperature at the time of plant collection). During the whole experimental period, control aquaria were maintained at 26℃. In the priming event (**Fig. 3.2C**), seawater temperature in the three heat-primed aquaria was progressively increased up to 29℃ at a heating rate of 1℃ day^{-1} and maintained for 7 days. Subsequently, the temperature in these aquaria was lowered to 26℃ for a second one-week acclimation period (**Fig. 3.2C**). The non-primed aquaria were kept as in control temperature during the priming event. After the second acclimation phase, seawater temperature in both non-primed and heat-primed aquaria was gradually heated up to

32℃ at the same rate (i.e. 1℃ day^{-1}) and maintained constant for 11 days to simulate the triggering event. All plant responses were measured at the end of the triggering event (**Fig. 3.2C**). The aquarium was the true experimental replicate for each seagrass species and variable (n = 3), and measurements performed on plants from the same aquarium (i.e. '*pseudo'* replicates) were averaged to obtain an independent replicated value. Therefore, the number of replicates used in statistical tests was n = 3.

3.2.4 Chlorophyll *a* fluorescence measurement

A diving-PAM fluorometer (Walz, Germany) was used to assess the photo-physiological responses of *P. oceanica* and *C. nodosa*. Measurements included maximum quantum yield (Fv/Fm) of photosystem II (PSII) and effective quantum yield of PSII (ΔF/Fm') (a detailed explanation about the principle of PAM fluorometry as well as the information regarding the measurement of Fv/Fm and ΔF/Fm' can be found in section 2.2.4). Fv/Fm was measured on whole-night dark-adapted plants (around 6:00 - 7:00, before the light cycle started), while ΔF/Fm' was determined on light-adapted plants at noon (around 12:30 - 13:30). All measurements were performed on the same leaf portion (i.e. 10 cm above the ligule) of the 2nd youngest leaf of each plant to standardize the procedure. Selected leaves were marked to ensure the measurement of Fv/Fm and the related ΔF/Fm' happened at the same place. Moreover, a dedicated underwater leaf clip was used to maintain a constant distance between the leaf and the fiber optic of the PAM fluorometer.

3.2.5 Plant growth measurement

Plant growth was assessed through the leaf marking method (Zieman, 1974). Plants were marked with a needle above the ligule in the middle of the second acclimation phase (**Fig. 3.2C**). Samples were then collected at the end of the triggering event. Newly developed leaf segments were selected and gently cleaned of epiphytes. Subsequently, samples were dehydrated (70℃ for 24 hours) and weighted for measuring leaf biomass production (dry weight, mg DW).

3.2.6 Pigment content measurement

Samples were collected for pigment content measurements at the end of the triggering event (**Fig. 3.2C**). Approx. 5 cm of the middle portion of the 2nd youngest leaf of *P. oceanica* or the whole 2nd youngest leaf of *C. nodosa* were harvested from each plant. Collected materials were immediately cleaned of epiphytes, covered with aluminum foil and kept on ice in darkness until

further processing on the same day of sample collection (< 4 hours). Samples were first weighted before being homogenized in liquid nitrogen by using pestles and mortars. Homogenized samples were then transferred into 1.5 mL tubes filled with 1 mL of 100% methanol. Thenceforward, samples were kept in complete darkness at 4℃ for 8 hours before centrifugation. An aliquot (200 μL) of the solution was then used to measure the absorbance at 4 different wavelengths (i.e. 470, 652, 665, and 750 nm) in a microplate reader (TECAN Infinite® M1000PRO, Switzerland) to calculate chlorophyll *a*, chlorophyll *b*, and total carotenoids. Pigments were calculated using equations from Wellburn (1994) after converting microplate readings into 1-cm cuvette readings following Warren (2008). Finally, results were normalized to milligrams of fresh weight.

3.2.7 Statistical analysis

Two-way analysis of variance (Two-way ANOVA) is used to compare the means between groups that have been split on two independent variables (i.e. factors) and eventually define whether there are any interactions between the two independent variables on the dependent variable. In this study, data come from each measurement (i.e. photo-physiology, growth or pigment) are dependent variables that depend on two fixed factors including Species (2 levels: *P. oceanica* and *C. nodosa*) and Treatment (3 levels: control, non-primed and heat-primed). Moreover, the data met all the assumptions for ANOVA (as explained in section 2.2.9), thus, Two-way ANOVA was performed to assess seagrass responses to warming. Prior to analysis, homogeneity of variance assumption was checked by using Levene's test. Then, Shapiro-Wilk test was used to validate data normality. Finally, a Tukey HSD post-hoc test was applied to determine significant differences among treatments of each species when applicable. All statistical analyses were conducted in R-studio v.1.2.5033 (R Core Team, 2018). Graphs were plotted with R-studio using package ggplot2 (Wickham, 2009).

3.2.8 Transcriptome-wide sequencing and analysis

Sample collection, RNA isolation and RNA sequencing

Samples for RNA extraction were obtained from three randomly selected plants (n = 3 per treatment per species) at the end of the triggering event. For each replicate, a 5cm leaf segment from the middle part of the second youngest leaf or a whole leaf was sampled for *P. oceanica* and *C. nodosa*, respectively. Immediately after collection, samples were gently cleaned of

epiphytes and preserved in RNA later. Samples were first stored at 4℃ overnight before being stored at -20℃ until RNA extraction.

For both species, total RNA was extracted from each sample using Aurum™ Total RNA Mini Kit (BIO-RAD) following the manufacturer's instructions. RNA purity was assessed with a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific) and its integrity was checked on a 1% agarose gel electrophoresis. RNA was used only when Abs260nm/Abs280 nm ratios were > 1.8 and 1.8 < Abs260 nm/Abs230 nm ratios < 2. RNA integrity was double-checked by measuring the RNA integrity number (RIN) with a 2100 Bioanalyzer (Agilent Technologies, Inc.). Only high-quality (RIN > 6.5) RNA was used for RNA sequencing. RNA quantity was determined by Qubit® RNA BR assay kit using the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). In total, eighteen cDNA libraries (3 replicates × 3 treatments × 2 species) were prepared with 3' mRNA-Seq Library Prep Kit (Lexogen) and sequenced with an Ion Proton™ sequencer at the SZN Molecular Service.

Data filtering and transcriptome assembly

Raw sequencing reads were quality checked using FASTQC v.0.11.5 (Andrews, 2010) and trimmed for quality using Trimmomatic (Bolger, Lohse and Usadel, 2014). To avoid potential artifacts caused by sequencing errors, reads with a quality per bases below 15 Phred score and a minimum length < 50 bp for *P. oceanica* or below 20 Phred score and a minimum length < 25 bp for *C. nodosa* were excluded from the analysis. Thenceforth, a dataset of unique tags was obtained by collapsing all the trimmed reads using Cd-hit [90% identity (Li and Godzik, 2006)]. To re-evaluate the quality of the tags dataset, all the cleaned reads were mapped independently on the obtained dataset using the Bowtie2 aligner with default settings (Langmead and Salzberg, 2012). For each replicate, read count was computed by the eXpress software (Roberts *et al.*, 2011).

Differential expression analysis

To assess overall similarity across samples and their relationship, PCA analysis was conducted on read counts data for each species using an integrated web application iDEP [available online at http://bioinformatics.sdstate.edu/idep/ (Ge, Son and Yao, 2018)]. The analysis of differentially expressed genes (DEGs: non-primed vs. control and heat-primed vs. control) was performed using the edgeR package (Robinson, McCarthy and Smyth, 2010). Transcripts were considered as significantly differentially expressed when FDR-corrected p value ≤ 0.05. Additionally, a cut-off of > |1.5| was also applied for log2 fold change values (Log2FC).

Finally, DEGs were visualized using DiVenn 2.0 [available online at https://divenn.noble.org/index.php (Sun *et al.*, 2019)], while Venn diagrams (available at http://bioinformatics.psb.ugent.be/webtools/Venn/) were used to identify DEGs shared between non-primed and heat-primed plants and DEGs that were unique of each experimental group.

Gene expression response analysis

Functional annotation of DEGs was carried out through sequence similarity search against UniProtKB/Swiss-Prot database (downloaded on April 2020) using the BLASTx software (Camacho *et al.*, 2009) with an e-value cutoff of $1e^{-3}$. In addition, DEGs were also annotated with previous transcriptomes [Ruocco *et al.*, (2020) and Ruocco *et al.*, (2017) for *P. oceanica* and *C. nodosa*, respectively] using the BLASTn software (Camacho *et al.*, 2009) with an e-value cutoff of 0.05 and query cover ≥ 90% (only 1 best hit was selected for each alignment) to maximizing the number of functional-annotated DEGs.

The CSR was adopted to categorize gene expression responses into three different levels based on the severity of the stress experienced by experimental plants during the second MHW (as previously done in Traboni *et al.*, 2018). These three stress-level categories were assigned with colors: low (green), medium (yellow) and high (red). In addition, DEGs were categorized according to other specific pathways related to plant thermal stress response (e.g. photosynthesis, growth, and transcription factors), epigenetics and especially thermal stress memory-related genes as described below.

- The red category of DEGs included genes related to ultimate cell intervention to counteract the effects of extreme heat stress, such as DNA repair and apoptosis regulators [e.g. ATM serine/threonine kinase (ATM) and Apoptosis-inducing factor homolog A (aifA) (Lorenzo *et al.*, 1999; Su *et al.*, 2017)].
- The yellow category of DEGs was composed of genes coding for proteins implicated in protein aggregate tagging and removal, such as ubiquitination and proteolysis [e.g. BTB/POZ and TAZ domain-containing protein 2 (BT2) and BTB/POZ domain-containing protein NPY2 (NPY2) (Mazzucotelli *et al.*, 2006; Shu and Yang, 2017)].
- The green category of DEGs consisted of genes encoding proteins that take an active part in protein protection, re-folding and assembly, such as the heat shock protein family, molecular chaperones, and anti-reactive oxygen species [e.g. Heat shock protein

90-1 (HSP90-1) and Superoxide dismutase Cu-Zn (SOD1) (Sørensen, Kristensen and Loeschcke, 2003; Paridah *et al.*, 2016)].

- The photosynthesis category of DEGs was selected for photosynthesis-related genes [e.g. Photosystem II protein D1 (psbA) and Oxygen-evolving enhancer protein 1 (psbO) (Knoetzel *et al.*, 2002; Chang *et al.*, 2006)].
- The growth DEGs category was devoted to genes associated with plant growth and development [e.g. Auxin-responsive protein SAUR50 (SAUR50) and VQ motif-containing protein 22 (VQ22) (Hagen and Guilfoyle, 2002; Cheng *et al.*, 2012)].
- The transcription factor category of DEGs included genes related to plant transcription factors [e.g. GRF1-interacting factor 2 (GIF2) and Transcription factor TGA4 (TGA4) (Foley and Singh, 2004; Kim and Kende, 2004)].
- The epigenetic category of DEGs was devoted to epigenetic-related genes [e.g. Histone H3.3 (HTR4) and Histone-lysine N-methyltransferase ATXR2 (ATXR2) (Soppe *et al.*, 2002; Lee, Park and Seo, 2017)].
- Finally, the stress memory category of DEGs embraced genes known to be involved in thermal-stress memory [e.g. 18.2 kDa class I heat shock protein (HSP18.2) and Heat shock 22 kDa protein (HSP22) (Lämke *et al.*, 2016; Olas *et al.*, 2021)].

GO enrichment analysis

Two approaches were applied to maximize the number of Gene Ontology (GO) terms retrieved. First, all tags were loaded on Blast2GO v.5.2.5 to retrieve GO terms (e-value cutoff $1e^{-6}$) for DEGs with a positive BLAST hit. Then, all tags were annotated with previous transcriptomes [Ruocco *et al.*, (2020) and Ruocco *et al.*, (2017) for *P. oceanica* and *C. nodosa*, respectively] as described in the previous section to improve GO-terms assignment. GO terms retrieved from both methods were combined to build a dataset for each species that was subsequently used for GO enrichment analysis. Enriched GO terms of the biological process of DEGs were tested by Fisher Exact tests using package 'topGO' v. 2.42.0 (Alexa and Rahnenfuhrer, 2010) in R with a threshold FDR of 0.05. I used '*weight01*' method [this is a mixture of '*elim*' and '*weight*' method (Alexa, Rahnenführer and Lengauer, 2006)] that takes the GO hierarchy into account when calculating enrichment. In this way, the 'inheritance problem' (that can lead to false positives) can be avoided (Grossmann *et al.*, 2007). Finally, Venn diagrams were used to identify shared and unique GO terms among different contrasts.

3.3 Results

3.3.1 Photo-physiological response

The triggering event had no substantial effects on the photosynthetic performance of heat-primed *P. oceanica* plants, however, significantly impacted non-primed *P. oceanica* plants (**Table 3.1**, **Fig. 3.3A**). In detail, significant differences were detected between non-primed vs. control plants and between non-primed vs. heat-primed plants on maximum quantum yield (Fv/Fm) results (Turkey HSD *post-hoc* test, $p < 0.05$ & $p < 0.01$, respectively; **Fig. 3A**). In contrast, both heated *C. nodosa* plants (non-primed and heat-primed) were able to maintain their Fv/Fm values unaltered during the triggering event (**Fig. 3.3B**). As a result, a significant difference was detected for S×T interactions for Fv/Fm measurements (Two-way ANOVA, $p < 0.01$, **Table 3.1**).

On the other hand, the effective quantum yield (ΔF/Fm') of both species showed no significant warming-induced alternations (**Fig. 3.3C**,**D, Table 3.1**). Yet, warming induced a slight increment of 3% for heat-primed *C. nodosa* plants while, in the opposite, a slight decline of 3% for non-primed *P. oceanica* plants in respect to their controls (**Fig. 3.3C,D**).

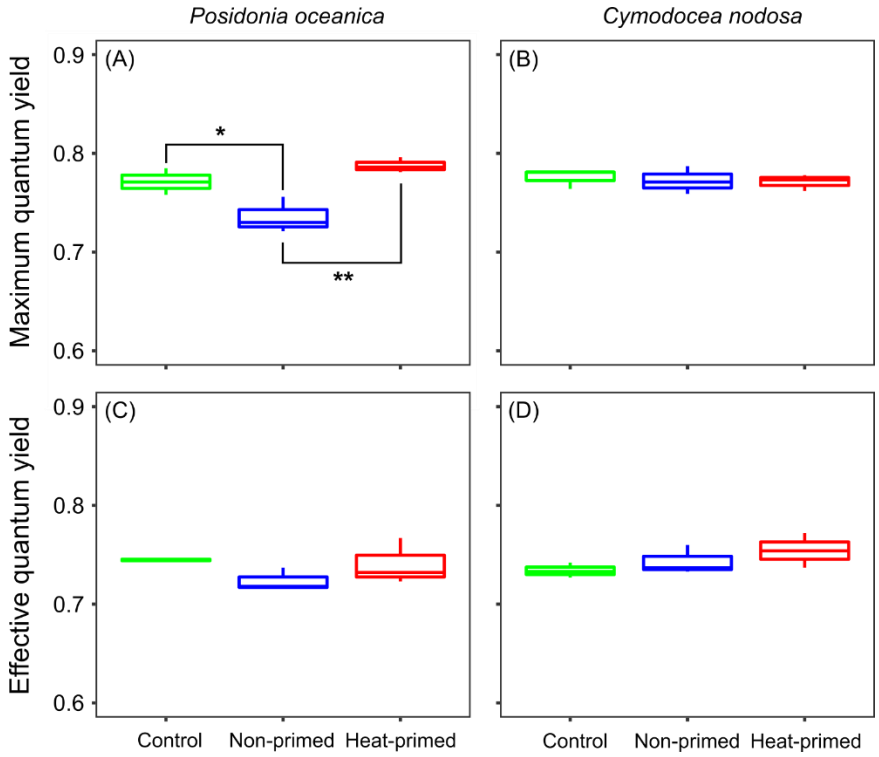


Figure 3.3 *Boxplots of photo-physiological responses of Posidonia oceanica (**A**,**C**) and Cymodocea nodosa (**B**,**D**) at the end of the triggering event. Asterisks indicate significant differences as results of Tukey HSD post-hoc test (*p < 0.05 & ** p < 0.01). n = 3.*

3.3.2 Growth response

Warming strongly influenced seagrass growth as evidenced by the significant differences detected for the Treatment factor (Two-way ANOVA, $p < 0.01$, **Table 3.1**). In *P. oceanica*, a substantial decline in growth was detected in heat-primed plants with respect to control and non-primed plants (Turkey HSD *post-hoc* test, $p < 0.01$ and $p < 0.001$, respectively, **Fig. 3.4A**). In *C. nodosa*, even if no significant differences were detected among treatments, it is important to note that both non-primed and heat-primed plants further increased their growth rates with respect to control plants. More importantly, heat-primed *C. nodosa* plants produced 27% more biomass during the triggering event than non-primed plants (**Fig. 3.4B**).

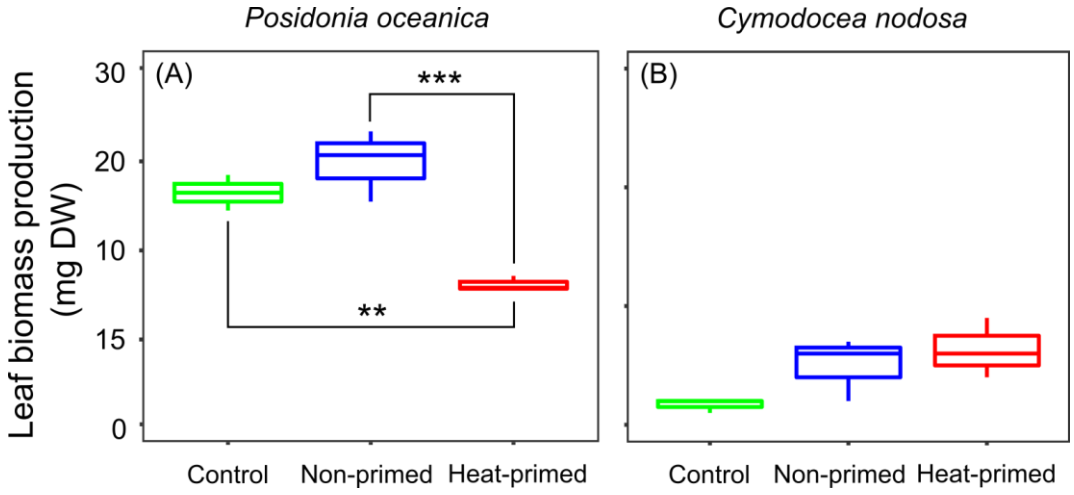


Figure 3.4 *Plant growth responses of Posidonia oceanica (**A**,**C**) and Cymodocea nodosa (**B**,**D**) at the end of the triggering event. Asterisks indicate significant differences as results of Tukey HSD post-hoc test (** $p < 0.01$ & *** $p < 0.001$). $n = 3$.*

3.3.3 Pigment response

The first obvious observation is that the concentration of pigment content was significantly higher in *C. nodosa* than in *P. oceanica* (Two-way ANOVA, Species: $p < 0.001$, **Table 3.1**). Second, warming negatively impacted the pigment content of *P. oceanica* plants while it enhanced the accumulation of leaf pigments in *C. nodosa* plants (**Fig. 3.5**).

In *P. oceanica*, warming reduced the pigment content of both non-primed and heat-primed plants. However, pigment content in heat-primed plants was approximately 33% higher than in non-primed plants (Chl *a*, Chl *b* and total carotenoids; **Fig. 3.5**), although these differences were not statistically significant.

In *C. nodosa*, the warming exposure increased Chl *a* (44%), Chl *b* (57%) and total carotenoids (44%) in heat-primed plants with regard to control plants. The change was significant for Chl *b* (Turkey HSD *post-hoc* test, $p < 0.05$, **Fig. 3.5D**). Likewise, the corresponding percentages for non-primed plants were 31% (n.s.), 23% (n.s.) and 29% (n.s.), respectively.

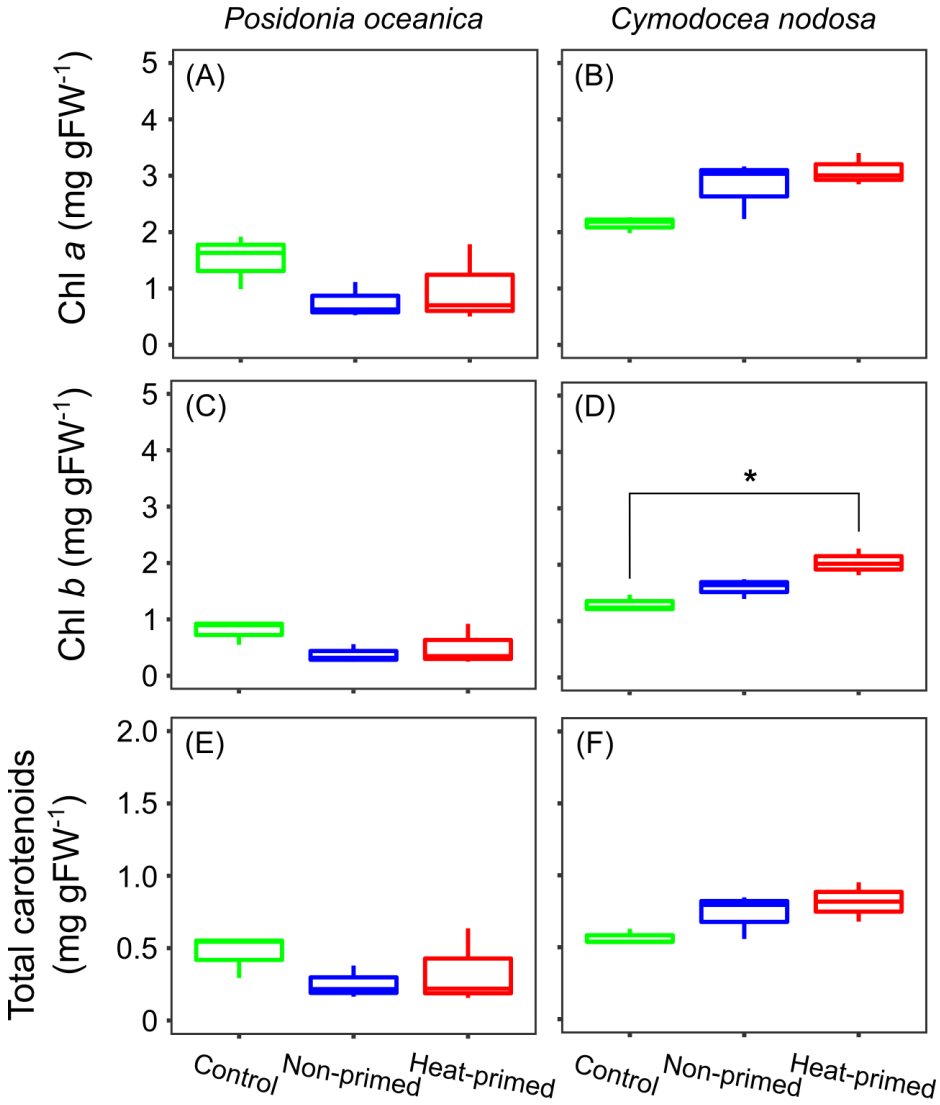


Figure 3.5 *Boxplots of leaf pigment content in P. oceanica (**A**,**C**,**E**) and C. nodosa (**B**,**D**,**F**) at the end of the triggering event. Asterisks indicate significant differences as results of Tukey HSD post-hoc test (*p < 0.05). n = 3.*

Table 3.1 *Two-way ANOVA performed on photo-physiological, plant growth and pigment responses of P. oceanica and C. nodosa at the end of the experiment. Significant codes: *** p < 0.001; ** p < 0.01; * p < 0.05; ns p > 0.05. df: degrees of freedom; MS: Mean Square; F: F-value.*

	Fv/Fm				**ΔF/Fm'**				**Leaf biomass production**			
Source of variation	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*
Species (S)	1	< 0.001	1.855	ns	1	< 0.001	1.230	ns	1	812.300	192.081	***
Treatment (T)	2	0.001	6.772	*	2	< 0.001	1.421	ns	2	30.800	7.280	**
S × T	2	0.001	6.985	**	2	< 0.001	1.854	ns	2	72.000	17.044	***
Residual	12	< 0.001			12	< 0.001			12	4.200		

	Chlorophyll *a*				**Chlorophyll *b***				**Total carotenoids**			
Source of variation	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*
Species (S)	1	11.387	58.918	***	1	5.274	99.622	***	1	0.561	22.652	***
Treatment (T)	2	0.114	0.588	ns	2	0.138	2.603	ns	2	0.011	0.446	ns
S × T	2	1.039	5.378	*	2	0.420	7.931	**	2	0.071	2.864	ns
Residual	12	0.193			12	0.053			12	0.025		

3.3.4 Transcriptome sequencing and assembly

The Ion Proton sequencing generated 69,267,359 and 64,422,853 single-end reads for *P. oceanica* and *C. nodosa*, respectively. Then, raw reads were quality-trimmed to obtain 53,421,823 (77%) and 52,436,340 (81%) cleaned reads. The cleaned reads were subsequently used for read collapsing to identify tags. As a result, after collapsing all cleaned reads using Cd-hit (90% identity), 675,032 and 835,468 tags were identified for *P. oceanica* and *C. nodosa*, respectively.

3.3.5 Global gene expression response

The overall profile of expression across different samples was explored through PCA analysis based on read counts (**Fig. AIII.1**). For *P. oceanica*, controls were segregated from heated plants (non-primed and heat-primed) along PC1 that accounts for 24% of the total variance, while PC2 (16% of variance) was responsible for the segregation between non-primed and heat-primed plants (**Fig. AIII.2A**). Regarding *C. nodosa*, the separation between control and heated plants was driven by PC1 (25% of variance) and PC2 (17% of variance), whereas non-primed and heat-primed plants were mainly separated along PC1 (**Fig. AIII.2B**). Additionally, it is worthy to note that for both species non-primed and heat-primed samples were partly overlapped (**Fig. AIII.2**).

The analysis of differential expression identified 2524 and 1796 DEGs (FDR-corrected *p* value ≤ 0.05 and log2FC > |1.5|) in *P. oceanica* and *C. nodosa*, respectively (**Fig. 3.6**).

In *P. oceanica*, the response of heated plants involved a great number of DEGs (i.e. 1628 and 1700 for non-primed and heat-primed plants, respectively), with half of them being unique of each treatment and the other half shared between non-primed and heat-primed (**Fig. 3.6A**). Among 824 DEGs exclusively belonging to non-primed plants, the number of up-regulated DEGs was more than twice the number of down-regulated DEGs (i.e. 575 vs. 249, **Fig. 3.6C**). In contrast, among the 896 DEGs uniquely identified in heat-primed plants, the number of down-regulated DEGs was 17% higher than the number of up-regulated DEGs (489 vs. 407, **Fig. 3.6C**). Finally, 804 DEGs were found in common between non-primed and heat-primed plants in which 612 DEGs were up-regulated and 192 DEGs were down-regulated (**Fig. 3.6C**).

In *C. nodosa*, the number of DEGs was 5-time higher in non-primed plants than in heat-primed plants (i.e. 1669 vs. 321 DEGs, **Fig. 3.6B**). More DEGs were found down-regulated than up-regulated in plants from both treatments (**Fig. 3.6D**). In detail, the number of down-regulated DEGs was 14-fold more abundant than up-regulated DEGs in heat-primed plants, while in non-primed plants this difference was only 3-fold (**Fig. 3.6D**).

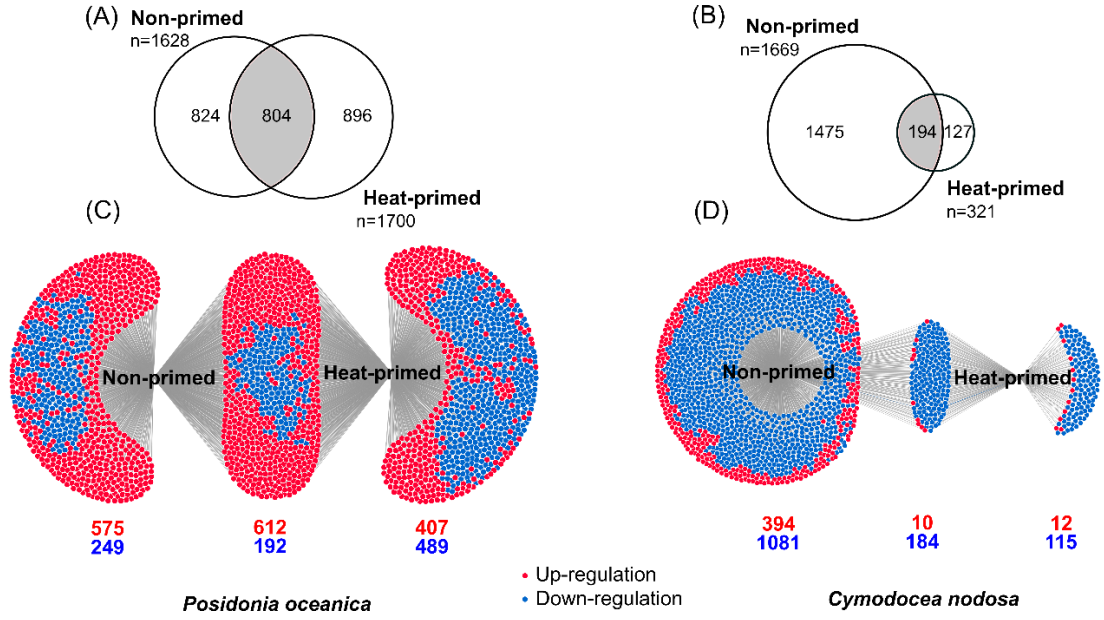


Figure 3.6 *Global gene expression responses of Posidonia oceanica (**A**,**D**) and Cymodocea nodosa (**B**,**D**). (**A**,**B**) Venn diagrams of differentially expressed genes (DEGs: FDR-corrected p value < 0.05 & log2FC > |1.5|) and (**C**,**D**) Divenn diagrams of DEGs (Red and blue numbers indicate the number of up-regulated and down-regulated DEGs, respectively).*

3.3.6 Responsive gene expression response

Functional annotation resulted in 903 DEGs with UniProt ID for *P. oceanica* plants (~36% total number of DEGs) and 320 DEGs for *C. nodosa* plants (~25% the total number of DEGs). Subsequently, annotated DEGs were classified into eight categories (as described previously in section 3.2.8) and considered as 'responsive DEGs'. As a result, 194 responsive DEGs were identified in non-primed and heat-primed *P. oceanica* plants covering all eight categories (**Fig. 3.7A, Table AIII.1**). In the same way, 78 responsive DEGs were assigned for non-primed and heat-primed *C. nodosa* plants. However, none of these responsive DEGs belonged to the red DEGs, growth DEGs and epigenetic DEGs categories (**Fig. 3.7B. Table AIII.2**). It is noteworthy that in both seagrass species, all detected DEGs from the stress-memory category were up-regulated (**Fig. 3.7**).

In *P. oceanica*, one red DEG (i.e. ATM gene) was exclusively up-regulated in non-primed plants while another red DEG (i.e. aifA gene) was down-regulated only in heat-primed plants (**Fig. 3.7A, Table AIII.1**). The number of up-regulated yellow-DEGs was the same (i.e. 5 DEGs) between non-primed and heat-primed plants, however, heat-primed plants down-regulated 3 more yellow-DEGs in comparison with non-primed plants (**Fig. 3.7A**). The responses of non-primed and heat-primed *P. oceanica* plants involved a large number of up-regulated green-DEGs and many of them were in common between the two groups (**Fig. 3.7A**). Nevertheless, the number of up-regulated green-DEGs found in heat-primed plants was only two-thirds the number detected in non-primed plants (**Fig. 3.7A**). Although warming did not result in any significant changes in the photosynthetic performance of *P. oceanica* plants (**section 3.3.1**), the treatment tended to lower the expression level of photosynthesis-related genes as evidenced by the fact that the number of down-regulated DEGs was ~2.6-fold and ~3.6-fold higher than the number of up-regulated photosynthesis DEGs in heat-primed and non-primed plants, respectively (**Fig. 3.7A**). Interestingly, in heat-primed plants, where the leaf biomass production was significantly inhibited (**section 3.3.2**), the number of down-regulated growth-DEGs was also more than 3-fold greater than in non-primed plants (**Fig. 3.7A**). Similarly, while non-primed and heat-primed plants both up-regulated 13 transcription factor-DEGs, the number of down-regulated DEGs from this category was 4-fold higher in heat-primed plants than in non-primed plants (**Fig. 3.7A**). Furthermore, warming induced significant changes in the level of expression of epigenetic-related genes in *P. oceanica* (**Fig. 3.7A**). However, the number of up-regulated epigenetic-DEGs was more than double in heat-primed plants with respect to non-primed plants (**Fig. 3.7A**). In addition to this, DEGs representing

Probable histone-arginine methyltransferase CARM1 and Protein arginine N-methyltransferase 5 were found exclusively in heat-primed plants (**Fig. 3.7A, Table AIII.1**). Especially, warming significantly induced in both non-primed and heat-primed plants the up-regulation of thermal stress memory-related genes including HSP18.2, HSP22 and FBA6 (**Fig. 3.7A, Table AIII.1**). Moreover, the number of HSP18.2- and FBA6-related DEGs detected was more abundant in non-primed plants than in heat-primed plants. When comparing the magnitude of up-regulation of HSP22-related DEGs, the expression level was around 1-fold higher in non-primed plants than in heat-primed plants (**Fig. 3.7A, Table AIII.1**).

In *C. nodosa*, no red-DEGs were detected in neither non-primed nor heat-primed plants while yellow-DEGs were exclusively found in non-primed plants (**Fig. 3.7B**). It is important to note that, among the five yellow-DEGs detected in non-primed plants, four of them were up-regulated (**Fig. 3.7B**). In addition, non-primed plants over-expressed 5 green-DEGs and down-expressed 13 green-DEGs. Similarly, heat-primed plants upregulated only 2 green-DEGs while the number of down-regulated green-DEGs was 4-fold higher (**Fig. 3.7B**). Similar to yellow-DEGs, photosynthesis-DEGs were only detected in non-primed plants, being 95% of them down-regulated (**Fig. 3.7B**). Corresponding with a dominance of down-regulated DEGs (as seen for green-DEGs and photosynthesis-DEGs), transcription factor-DEGs were mainly down-regulated non-primed and heat-primed plants. In detail, in non-primed plants, only 3 transcription factor-related DEGs were up-regulated while 16 transcription factor-related DEGs were down-regulated. Likewise, 13 down-regulated transcription factor-related DEGs were found in heat-primed plants, while no up-regulated DEGs were detected in this group (**Fig. 3.7B**). Lastly, DEGs from the induction memory category were only detected in non-primed *C. nodosa* plants, with two DEGs representative of the HSP22 gene family (**Fig. 3.7B, Table AIII.2**).

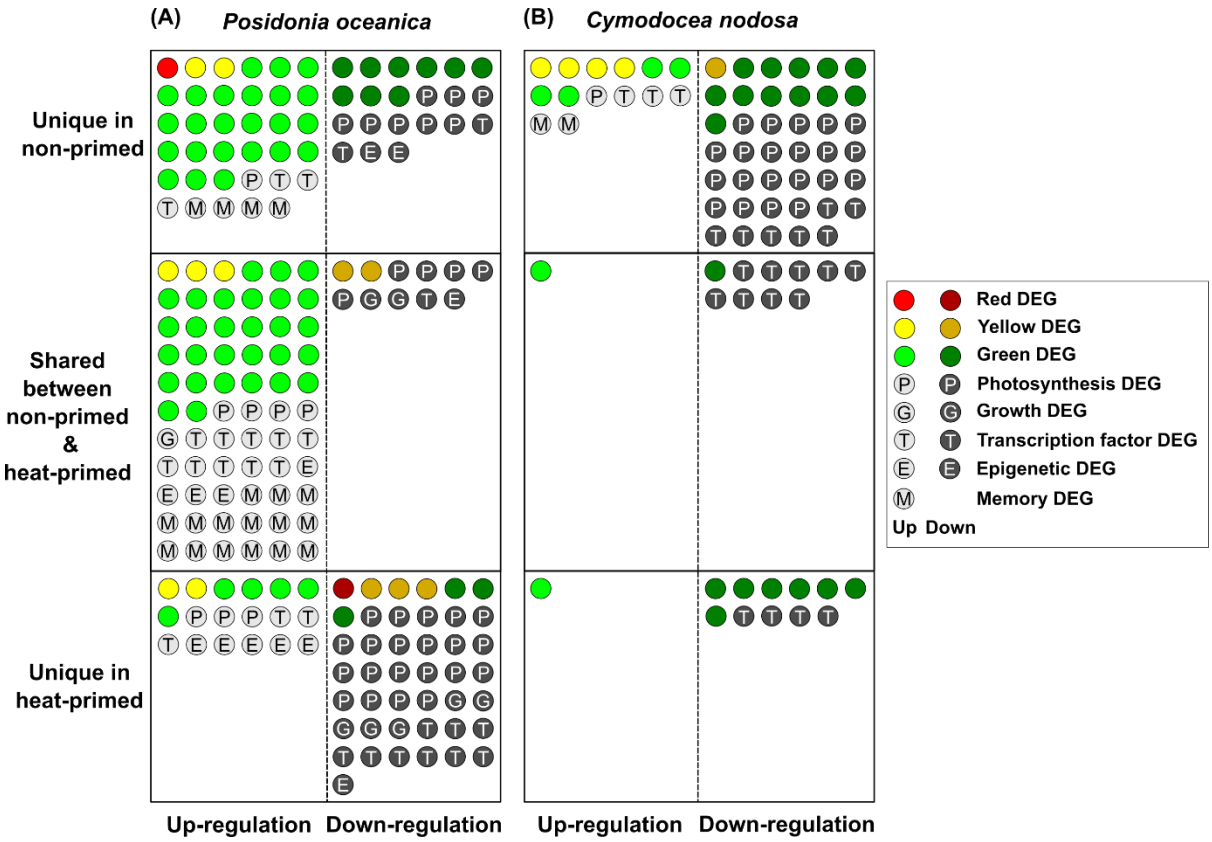


Figure 3.7 *Responsive DEGs of Posidonia oceanica and Cymodocea nodosa at the end of the triggering event.*

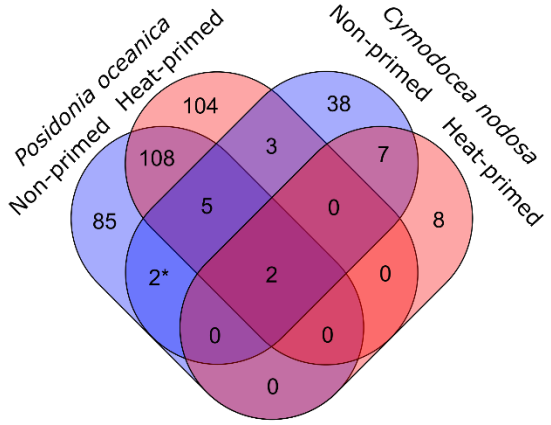
3.3.7 GO enrichment

A total of 167,504 tags in *P. oceanica* (25% of total tags) and 89,216 tags (11% of total tags) in *C. nodosa* were annotated with at least 1 GO term. These GO-annotated tags were subsequently used for GO enrichment analysis. Results of the enrichment analysis of the biological process GO terms (GO-BPs) are summarized in **Fig. 3.8 and Table AIII.3-6**. In *P. oceanica*, the number of enriched GO-BPs was 202 in non-primed plants (**Fig. 3.8, Table AIII.3**) and 222 in heat-primed plants (**Fig. 3.8, Table AIII.4**). Approximately 50% of those GO-BPs were shared between non-primed and heat-primed plants while the other 50% were unique for each group. In contrast, the number of GO-BPs in *C. nodosa* was three-time higher in non-primed plants (**Fig. 3.8, Table AIII.5**) than in heat-primed plants, which were enriched in only 17 GO-BPs (**Fig. 3.8, Table AIII.6**).

For both species, enriched GO-BPs terms related to plant thermal stress responses came from some major categories including response to heat, response to oxidative stress, transcription, sugars, fatty acid, signaling pathways involving the plant hormones, photosynthesis, protein modification, regulation of DNA damage and apoptotic process (**Fig. 3.8**).

In detail, protein refolding was found in common across different species and treatments (Region 2, **Fig. 3.8**). Despite that, only 1 GO-BP related to plant heat-stress response was enriched in heat-primed *C. nodosa* plants (i.e. positive regulation of transcription, DNA; Region 8, **Fig. 3.8**) while, in contrast, 11 GO-BPs related to response to oxidative stress, plant hormones, photosynthesis, and transcription were exclusively enriched in non-primed *C. nodosa* plants (Region 5, 3 and 38, **Fig. 3.8**).

In *P. oceanica*, non-primed and heat-primed plants shared many GO-BPs related to plant heat-stress response (Region 2, 5 and 108, **Fig. 3.8**). Notably, positive regulation of apoptotic process was found only in non-primed plants (Region 85, **Fig. 3.8**) while negative regulation of intrinsic apoptotic signaling pathway appeared exclusively in heat-primed plants (Region 104, **Fig. 3.8**). Specifically, enriched GO-BPs related to epigenetic modifications were detected only in heat-primed *P. oceanica* plants and included histone H3-R26 methylation and histone H3-R17 methylation (Region 104, **Fig. 3.8**).



Region	GO ID	GO Biological process
2	GO:0042026	protein refolding
2*		
5	GO:0042542	response to hydrogen peroxide
	GO:0072593	reactive oxygen species metabolic process
	GO:0009737	response to abscisic acid
	GO:0006457	protein folding
3	GO:0006979	response to oxidative stress
7		
38	GO:0031047	gene silencing by RNA
	GO:0009734	auxin-activated signaling pathway
	GO:0009693	ethylene biosynthetic process
	GO:0010206	photosystem II repair
	GO:0009686	gibberellin biosynthetic process
	GO:0009765	photosynthesis, light harvesting
8	GO:0045893	positive regulation of transcription, DNA
108	GO:0009751	response to salicylic acid
	GO:0080168	abscisic acid transport
	GO:0043516	regulation of DNA damage response, signal transduction by p53 class mediator
	GO:0042744	hydrogen peroxide catabolic process
	GO:0006986	response to unfolded protein
	GO:0050821	protein stabilization
	GO:0009939	positive regulation of gibberellic acid mediated signaling pathway
	GO:0006915	apoptotic process
	GO:0071588	hydrogen peroxide mediated signaling pathway
	GO:0009733	response to auxin
	GO:0009873	ethylene-activated signaling pathway
	GO:0009408	response to heat
	GO:0000302	response to reactive oxygen species
	GO:0009753	response to jasmonic acid
	GO:0061077	chaperone-mediated protein folding
	GO:0015979	photosynthesis
	GO:0006636	unsaturated fatty acid biosynthetic process
	GO:0009266	response to temperature stimulus
	GO:0010286	heat acclimation
104	GO:0034972	histone H3-R26 methylation
	GO:0019684	photosynthesis, light reaction
	GO:0009738	abscisic acid-activated signaling pathway
	GO:0006950	response to stress
	GO:0034971	histone H3-R17 methylation
	GO:0060416	response to growth hormone
	GO:0009926	auxin polar transport
	GO:2001243	negative regulation of intrinsic apoptotic signaling pathway
	GO:0009723	response to ethylene
	GO:0043617	cellular response to sucrose starvation
85	GO:0019430	removal of superoxide radicals
	GO:0010310	regulation of hydrogen peroxide metabolic process
	GO:2000378	negative regulation of reactive oxygen species
	GO:0070370	cellular heat acclimation
	GO:0043065	positive regulation of apoptotic process

Figure 3.8 *GO enrichment analysis of DEGs for Posidonia oceanica and Cymodocea nodosa at the end of the triggering event. Venn diagram represents the comparison between enriched GO terms among different contrasts (non-primed P. oceanica, heat-primed P. oceanica, non-primed C. nodosa and heat-primed C. nodosa). Inserted table shows a list of enriched GO terms (i.e. biological process) related to 'plant heat-stress response' and 'epigenetics' in each region of the Venn diagram.*

3.4 Discussion

Overall, this study provides valuable insights into the emerging field of thermal stress memory in seagrasses. For both *Posidonia oceanica* and *Cymodocea nodosa*, plant responses to warming at different levels of the biological organization agreed to insinuate an improved thermal tolerance in heat-primed plants. Molecular findings evidenced that while non-primed plants mainly overexpressed genes in response to warming, heat-primed plants primarily responded by repressing gene expression. This suggests a 'know-how' mechanism that heat-primed plants had acquired during the priming event and that eventually equipped these plants with the ability to induce more energy-effective responses when the thermal stress event recurred (i.e. the triggering event). In other words, results from this study support the existence of the so-call 'thermal stress memory' in the two Mediterranean seagrasses. Moreover, gene functional and GO enrichment analyses demonstrated the activation of genes related to

epigenetic modifications during the triggering event in both heat-primed and non-primed plants, suggesting an important role of epigenetic modifications not only in thermal stress response but also in thermal stress memory in seagrasses. Especially, this study provides, for the first time in seagrasses, some evidence at the level of gene expression that points to the likely involvement of thermal stress memory-related genes in such processes. Overall, these results contribute to broadening our understanding of the molecular basis of thermal stress memory in seagrasses.

3.4.1 Thermal priming effect on Mediterranean seagrasses

Under a triggering thermal stimulus, heat-primed *P. oceanica* plants were able to maintain their photo-physiological performance unaltered while, on the contrary, non-primed plants were negatively affected. This observation concurs with recent findings from other seagrass species (*Posidonia australis* and *Zostera muelleri* presented in **Chapter II**) as well as from terrestrial plants (Smillie and Gibbons, 1981; Wang, Cai, *et al.*, 2014; Li *et al.*, 2015) on demonstrating the positive effect of thermal priming on enhancing the photo-physiological tolerance of plants to warming. For *C. nodosa*, the fact warming did not alter the photosynthetic performance of heat-primed and non-primed plants can be explained by the higher tolerance of this species to warming (Olsen *et al.*, 2012; Marín-Guirao *et al.*, 2016, 2018; Savva *et al.*, 2018). Yet, evidence derived from growth and pigment measurements suggest that heat-priming treatment further benefited the species when subjected to increased temperatures.

In *P. oceanica*, only heat-primed plants significantly slowed down their growth rates during the triggering event, whereas the growth of non-primed plants was altered during the event. Regarding conservative strategies to withstand stressful warming conditions, it has been demonstrated that *P. oceanica* can activate an 'energy-saving' response by slowing down its growth to preserve the energy reserves needed for overwintering and to allocate resources for fuelling the heat-stress response (Marín-Guirao *et al.*, 2018). Not only limited to thermal stress, but a recent study also demonstrated that *P. oceanica* reduced its plant size for minimizing the negative effects of chronic light shortage (Ruocco *et al.*, 2020). In this way, the plant can persist a long period under stressful conditions and get a better chance to survive afterward (Ruocco *et al.*, 2020). In terrestrial plants (especially crop plants), the inhibition of cell proliferation and cell growth has been recognized as one of the key responses to non-lethal stress and provides the resultant plant with a better chance of survival (Holcik and Sonenberg, 2005; Kitsios and Doonan, 2011). Therefore, the significant reduction in leaf biomass production observed in the

heat-primed *P. oceanica* plants in this study can be understood as an energy-effective mechanism to cope with the triggering condition. This improved response can be a result of having acquired a thermo-primed status. This interpretation is further supported by the molecular analysis, as reflects the fact that several growth-related genes (e.g. SAUR50 and VQ22) were found uniquely down-regulated in heat-primed *P. oceanica* plants. This exclusive response suggests that heat-primed plants of this species actively managed their growth as a response to face stressful high temperatures.

Non-primed plants of the two studied seagrass species, *P. oceanica* and *C. nodosa*, showed a more comprehensive and intense activation of genes encoding for HSPs when compared to heat-primed plants. This suggests that non-primed plants required a larger number of HSPs to deal with warming than heat-primed plants, and hence, that they were experiencing a higher thermal stress level. Moreover, although HSPs are among the most common and rapid responsive mechanisms of plants to cope with adverse conditions (Vierling, 1991; Kiang and Tsokos, 1998), the production of HSPs itself is an energy-costly process (Nover *et al.*, 2001), which suggests that non-primed plants had to 'pay a higher cost' to endure the negative impacts of warming. This is also supported by the global gene expression patterns of both studied species, as the response to warming of heat-primed plants was dominated by a reduced gene activity (most genes were down-expressed) contrarily to the strong activation of genes displayed by non-primed plants. This together with the fact that, in both studied species, heat-primed plants actually performed better, at photosynthetic, pigment and growth levels, than non-primed plants strengthen the assumption about a universal 'energy-effectively responsive mechanism' as a mean of thermal stress memory to facilitate seagrasses' response with repeated warming events.

Gene functional analysis and GO enrichment analysis provided further findings supporting that non-primed plants suffered more during the triggering warming event than heat-primed plants. First, the response of non-primed *P. oceanica* plants required activation of ATM gene (a red DEG). This gene plays a crucial role in a protective mechanism for DNA damage repair in response to adverse environmental stresses (Garcia *et al.*, 2003; Su *et al.*, 2017). Hence, the triggering warming event could have induced serious DNA damage to non-primed *P. oceanica* plants but not to heat-primed plants. This is further sustained by results from GO enrichment analysis in which the positive regulation of apoptotic process was found only in non-primed plants. Second, only non-primed plants activated yellow genes (e.g. FTSH8 and At1g55760) whose functions are related to the removal of irreversibly damaged proteins (Gingerich *et al.*,

2005; Zaltsman, Ori and Adam, 2005). These molecular data provide important hints that would otherwise be hardly detected from other measurements (e.g. photo-physiology) and further support my conclusion that non-primed plants were more negatively impacted by the triggering warming event than primed plants.

It is also noticed that the response to warming of non-primed plants required the involvement of a higher number of biological processes than that of heat-primed plants. This again suggests that the response to warming of non-primed plants demanded more complex and more sophisticated mechanisms while in heat-primed plants the response was far simpler. This conclusion is in line with a recent finding on *P. oceanica* in which the response of plagiotropic shoots (the ones less affected by light shortage stress) required a simpler response associated with few important functions while that of orthotropic shoots (the ones more impacted by light shortage stress) involved a wide variety of processes (Ruocco *et al.*, 2020).

Molecular and biochemical responses are better stress predictors and better anticipate seagrass decline under stressful conditions than physiological and morphological responses (Macreadie *et al.*, 2014; Ceccherelli *et al.*, 2018). This is the likely reason for a more evident impact of warming at the growth and gene expression level than at the photo-physiological level observed in this study.

3.4.2 Molecular mechanisms underlying priming and memory phases in seagrasses

This study provides, for the first time in seagrasses, some results at the gene expression level of the involvement of some thermal stress memory-related genes in response to warming. Three genes including HSP18.2, HSP22 and FRUCTOSE-BISPHOSPHATE ALDOLASE 6 (FBA6) were found significantly up-regulated in both heat-primed and non-primed *P. oceanica* plants. In *Arabidopsis*, these genes have been widely identified as thermal stress memory-related genes (Shahnejat-Bushehri, Mueller-Roeber and Balazadeh, 2012; Stief *et al.*, 2014; Lämke *et al.*, 2016; Olas *et al.*, 2021). In particular, HSP18.2 was found substantially up-regulated during the memory phase (Shahnejat-Bushehri, Mueller-Roeber and Balazadeh, 2012; Lämke *et al.*, 2016). In this study, HSP18.2 was found overly-expressed in heat-primed *P. oceanica* plants confirming the role of this gene in inducing an improved response to warming during the memory phase in seagrasses. Moreover, HSP18.2 was found up-regulated also in non-primed *P. oceanica* plants, although with less intensity than in heat-primed plants, which may suggest the involvement of this gene also during the formation of thermal stress memory. Recently, when Olas *et al.*, (2021) studied thermal stress memory at the shoot apical meristem (SAM) of

Arabidopsis thaliana, they found that the gene FBA6 was involved in the formation of thermal stress memory but not in the memory phase. In my study, FBA6 was found up-regulated in mature leaf tissues of both heat-primed and non-primed *P. oceanica* plants, although the response could vary among different tissues (Ruocco *et al.*, 2020; Olas *et al.*, 2021). Therefore, the role of FBA6 gene in seagrasses may not be limited to the priming phase but it can also play a role during the memory phase. Lastly, HSP22 is known for its rapid activation in response to increased temperatures and the activation remains long after the priming event terminates (Stief *et al.*, 2014; Lämke *et al.*, 2016). In this study, both primed and non-primed *P. oceanica* plants significantly up-regulated this gene in response to warming, however, the magnitude of changes was much greater in non-primed plants than the others. In *C. nodosa*, HSP22-related DEGs were detected only in non-primed *C. nodosa* plants. Together, these results may suggest even HSP22 is functional in both phases, its role may be more crucial during the priming phase.

Despite several pieces of evidences supported the likely involvement of epigenetics in thermal stress memory in *P. oceanica*, no hints were detected in the transcriptomic response of *C. nodosa* plants. This can be due to the higher thermal tolerance of the species over *P. oceanica,* as evident by results from this and previous studies (Olsen *et al.*, 2012; Marín-Guirao *et al.*, 2016, 2018; Savva *et al.*, 2018). Therefore, the warming treatment applied in this study may not be sufficient enough for inducing heat stress in *C. nodosa* plants, which indeed, were favoured by the temperature increase used in the triggering treatment of my experiment. In consequence, warmed *C. nodosa* plants did not need to activate the epigenetic machinery for orchestrating a heat-stress response and/or the acquisition of a stress memory. This sheds light on the importance of investigating how different levels of thermal stress (in terms of intensity and duration) can affect the induction of a primed status in seagrass species with higher thermal affinity, such as *C. nodosa*. Anyway, a curious observation in my experiment was the fact that the pre-exposure of *C. nodosa* to warm temperatures predispose plants to better benefit from a subsequent exposure, even if this exposure also favoured non-pre-exposed plants. This indicates that plants were somehow trained during the pre-exposure, enhancing their positive responses during a subsequent warming exposure.

Warming induced the expression of ATXR2 gene in both heat-primed and non-primed *P. oceanica* plants. ATXR2 is known for its histone methyltransferase function and plays a key role in promoting the accumulation of histone H3 (Lee, Park and Seo, 2017). Histone H3 relates extensively in epigenetic-regulated responses of plants to several abiotic stresses (Boyko and

Kovalchuk, 2008; Kim *et al.*, 2008; Yuan *et al.*, 2013) but also in the epigenetic memory in plants (Ng and Gurdon, 2008; Lämke *et al.*, 2016). Warming facilitated the expression of genes related to chromatin remodelling proteins in both heat-primed and non-primed *P. oceanica* plants. Chromatin remodelling proteins play important roles in epigenetic control of plant stress responses (Boyko and Kovalchuk, 2008; Buszewicz *et al.*, 2016). Warming also caused significant down-regulation of HTR4 gene in both heat-primed and non-primed *P. oceanica* plants. In *Arabidopsis*, HTR4 is among six genes encoding for histone H3.3 (Okada *et al.*, 2005) while histone H3.3 is highly associated with histone modifications and DNA methylations (Stroud *et al.*, 2012; Wollmann *et al.*, 2017). Together, these results indicate the involvement of epigenetic modifications in thermal stress response [as seen in previous studies (Marín-Guirao *et al.*, 2017; 2019)] and especially in both phases of thermal stress memory in seagrasses.

Additionally, two epigenetic-related genes were found exclusively in heat-primed *P. oceanica* plants including CARM1 [that involves in histone H3 methylation and chromatin remodelling (Wysocka, Allis and Coonrod, 2006)] and PRMT5 [that methylates arginine residues in histone H4 (Ahmad, Dong and Cao, 2011)]. Moreover, two epigenetic-related biological processes were enriched only in these heat-primed plants, named histone H3-R26 methylation and histone H3-R17 methylation [both have been recently identified as parts of the epigenetic mechanism regulating pluripotency in mammal embryos (Wu *et al.*, 2009)]. These results suggest that epigenetic modifications were more actively involved in the response of heat-primed plants than in non-primed plants. In other words, epigenetic modifications may be more active during the memory phase than during the priming phase of thermal stress memory in seagrasses.

To conclude, the results presented in this chapter confirm the existence of thermal stress memory in seagrasses (as presented in Chapter II) in the Mediterranean seagrasses. The present study also confirms the involvement of epigenetic modifications (more particularly, DNA methylation and histone methylation) in seagrass stress response, as seen in terrestrial plants (see reviews by Chinnusamy and Zhu, 2009; Kinoshita and Seki, 2014; Liu *et al.*, 2015) and in Chapter II and other seagrass studies (e.g. see Marín-Guirao *et al.*, 2019; Ruocco *et al.*, 2019; Jueterbock *et al.*, 2020; Entrambasaguas *et al.*, 2021) and potentially in seagrass thermal stress memory. Especially, the identification of thermal stress memory-related genes (e.g. HSP18.2, HSP22 and FBA6) in this study provides a starting point for future studies to investigate deeper into the molecular mechanisms governing thermal stress memory in seagrasses. While I acknowledge a limitation of this study relating to a limited number of annotated genes, I

highlight an urgent need for more genomes for more species (including two species used in this study) to facilitate molecular research (including epigenetics and stress memory) in seagrasses. Finally, in this study, the molecular responses of seagrasses were examined only during the triggering event, future studies are encouraged to investigate other time-points (e.g. between the two warming events) and under different stressful conditions (e.g. different temperatures or in combination with other stressors) to further broaden our understanding in this important topic.

Appendix III

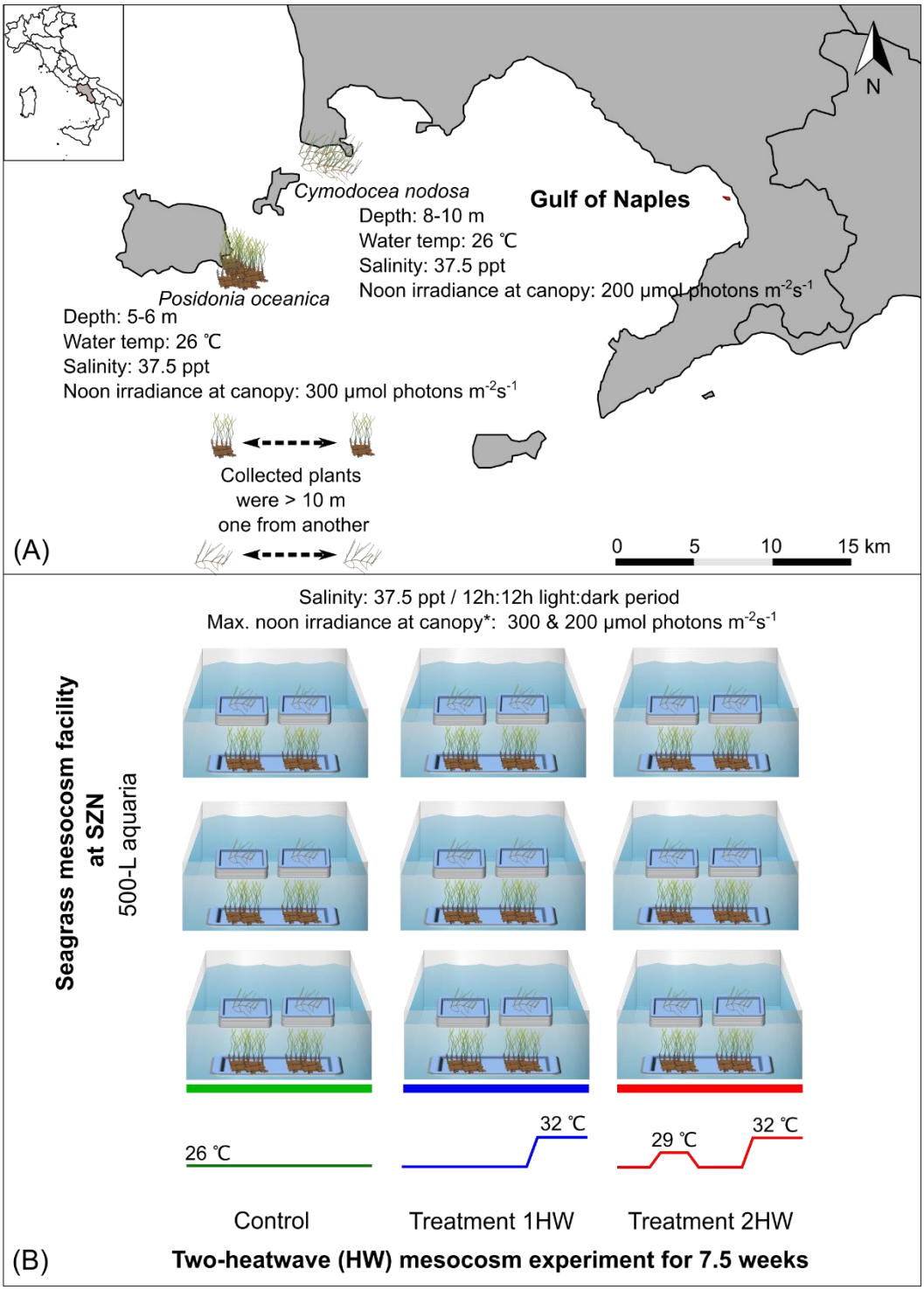


Figure AIII.1 *Sampling strategy (A) and experimental setup (B) of the experiment at SZN. Max. noon irradiance at canopy*: The light system was set for reproducing irradiance levels similar to the ones present in the field at the samping depth (i.e. 300 µmol photons m^{-2} $s^{-}1$ for P. oceanica and 200 µmol photons m^{-2} s^{-1} for C. nodosa, respectively).*

Figure AIII.2 *PCA analysis conducted on read counts of different biological replicates from each experimental treatment each species.*

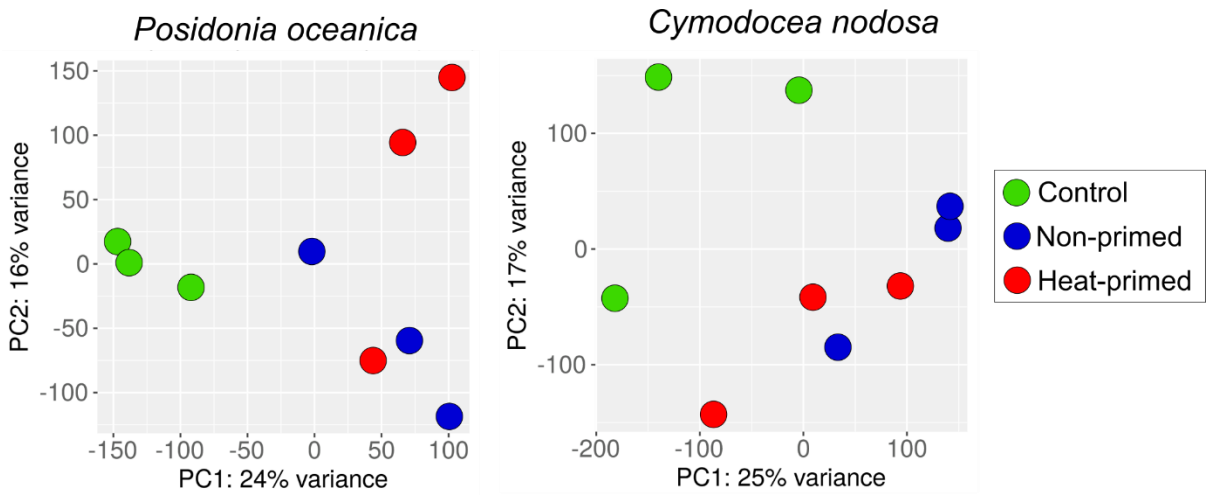


Table AIII.1 *List of functionally annotated sequences belongs to categories of interest for Posidonia oceanica. Query cover, Identity, E-value present BLAST results between a transcriptome generated in this study and a previous transcriptome from Ruocco et al., 2020. ****Bold*** *indicates annotation came directly from the sequence from this study while the rest came indirectly from Ruocco et al., 2020.*

SeqName	SeqName in Ruocco et al., 2020	Query cover (%)	Identity (%)	E-value	Log2FC		SwissProt ID	Description	Gene	Category
					Non-primed	Heat-primed				
1HEAT_XZTWC:07336:07532	se1_TR4154|c0_g2_i1	100	100	2.11E-92		-1.71	Q54NS9	Apoptosis-inducing factor homolog A	aifA	Red
2HEAT_XZTWC:01916:07871	pe2_TRINITY_DN37306_c0_g1_i2	100	98.707	5.74E-114	2.33		**Q9M3G7**	Serine/threonine-protein kinase ATM (EC 2.7.11.1) (Ataxia telangiectasia mutated homolog) (AtATM)	ATM	Red
CONTROL_XZTWC:07581:14645	se1_TR2242|c0_g2_i5	96	99	6.7E-98	-2.07	-2.71	Q9FMK7	BTB/POZ and TAZ domain-containing protein 1	BT1	Yellow
CONTROL_XDCTM:01403:02419	se2_TRINITY_DN23606_c1_g1_i6	97	98.883	7.68E-87		-5.13	Q94BN0	BTB/POZ and TAZ domain-containing protein 2	BT2	Yellow
CONTROL_XZTWC:04870:01878	pe1_TR43715|c6_g1_i11	97	99.648	4.26E-146		-1.83	Q94BN0	BTB/POZ and TAZ domain-containing protein 2	BT2	Yellow
CONTROL_XDCTM:10408:11035	pe1_TR43715|c6_g1_i11	95	99.497	5.24E-99		-1.70	Q94BN0	BTB/POZ and TAZ domain-containing protein 2	BT2	Yellow
CONTROL_XDCTM:02679:02243	pe2_TRINITY_DN34489_c1_g3_i6	98	99.621	5.09E-135	-2.08	-2.57	Q94BN0	BTB/POZ and TAZ domain-containing protein 2	BT2	Yellow
2HEAT_XZTWC:00151:04699	se3_TRINITY_DN56075_c0_g1_i6	99	100	2.31E-76		4.45	P93820	BTB/POZ domain-containing protein At1g04390	At1g04390	Yellow
CONTROL_XDCTM:08133:11084	se3_TRINITY_DN45386_c0_g1_i3	100	99.163	1.64E-119		1.80	O80970	BTB/POZ domain-containing protein NPY2	NPY2	Yellow
1HEAT_XZTWC:03817:05240	pe2_TRINITY_DN38786_c10_g11_i1	96	98.347	2.91E-117	1.77	1.55	Q9SRS9	E3 ubiquitin-protein ligase CHIP	CHIP	Yellow
2HEAT_XDCTM:09523:03978	pe1_TR31935|c1_g1_i2	100	99.632	1.84E-139	5.38		Q9M2P4	E3 ubiquitin-protein ligase SINAT2	SINAT2	Yellow
2HEAT_XZTWC:03518:06165	pe1_TR31935|c1_g1_i2	100	100	1.65E-93	7.02	5.54	Q9M2P4	E3 ubiquitin-protein ligase SINAT2	SINAT2	Yellow
1HEAT_XZTWC:10179:08330	pe2_TRINITY_DN33148_c2_g1_i2	97	98.491	5.21E-130	5.92	4.54	Q9M2P4	E3 ubiquitin-protein ligase SINAT2	SINAT2	Yellow
1HEAT_XDCTM:06790:01167	pe2_TRINITY_DN24006_c0_g1_i2	96	98.79	2.98E-122	1.55		Q9SKK0	EIN3-binding F-box protein 1	EBF1	Yellow
1HEAT_XZTWC:02867:04003	-	-	-	-	1.73	1.73	**Q9FHQ3**	15.7 kDa heat shock protein, peroxisomal (AtHsp15.7)	HSP15.7	Green
2HEAT_XZTWC:07350:12859	pe1_TR21712|c1_g1_i2	95	99.6	2.99E-127	1.63		Q652V8	16.0 kDa heat shock protein, peroxisomal	HSP16.0	Green
1HEAT_XZTWC:08943:08953	pe1_TR21712|c1_g1_i2	97	99.18	2.89E-122		1.52	Q652V8	16.0 kDa heat shock protein, peroxisomal	HSP16.0	Green
2HEAT_XZTWC:05518:05251	se3_TRINITY_DN38450_c0_g2_i5	94	99.63	7.02E-139	2.89	2.15	**P30221**	17.8 kDa class I heat shock protein		Green
1HEAT_XZTWC:10119:03107	pe1_TR34507|c2_g1_i4	98	93.75	1.04E-101	3.47		**P30221**	17.8 kDa class I heat shock protein		Green
2HEAT_XDCTM:08025:09671	-	-	-	-	3.78	2.82	**Q84Q77**	17.9 kDa class I heat shock protein (17.9 kDa heat shock protein 1) (OsHsp17.9A)	HSP17.9A	Green

2HEAT_XZTWC:05201:07360	-	-	-	-	5.39	5.19	**Q84Q77**	17.9 kDa class I heat shock protein (17.9 kDa heat shock protein 1) (OsHsp17.9A)	HSP17.9A	Green
2HEAT_XDCTM:06274:08828	pe2_TRINITY_DN36200_c1_g1_i2	92	97.748	4.72E-105	2.83	1.82	**Q84Q77**	17.9 kDa class I heat shock protein (17.9 kDa heat shock protein 1) (OsHsp17.9A)	HSP17.9A	Green
1HEAT_XDCTM:03606:04458	pe1_TR34507|c2_g1_i4	97	97.934	1.32E-115	3.52	2.14	P27879	18.1 kDa class I heat shock protein	HSP18.1	Green
1HEAT_XZTWC:07546:07432	pe1_TR34507|c2_g1_i4	98	100	4.53E-120	3.82		P27879	18.1 kDa class I heat shock protein	HSP18.1	Green
1HEAT_XZTWC:07626:10755	pe1_TR34507|c2_g1_i4	100	100	3.09E-106	4.94	3.78	P27879	18.1 kDa class I heat shock protein	HSP18.1	Green
1HEAT_XZTWC:07004:05472	pe1_TR34507|c1_g1_i1	97	99.288	9.15E-143	3.03	2.03	P27879	18.1 kDa class I heat shock protein	HSP18.1	Green
2HEAT_XDCTM:02291:07367	se2_TRINITY_DN21580_c0_g1_i3	94	99.451	1.34E-89	3.05		**P27879**	18.1 kDa class I heat shock protein (Fragment)	HSP18.1	Green
1HEAT_XDCTM:02067:05615	se3_TRINITY_DN38450_c0_g2_i5	100	100	9.75E-70	2.70		**P27879**	18.1 kDa class I heat shock protein (Fragment)	HSP18.1	Green
1HEAT_XZTWC:09969:09926	pe1_TR34507|c2_g1_i4	100	97.241	3.59E-64	5.42		**P05478**	18.5 kDa class I heat shock protein (HSP 18.5)	HSP18.5-C	Green
1HEAT_XZTWC:06171:10862	se2_TRINITY_DN23957_c0_g3_i2	99	96.863	1.04E-116	4.39		Q9C5R8	2-Cys peroxiredoxin BAS1-like, chloroplastic	At5g06290	Green
2HEAT_XDCTM:05631:10351	se2_TRINITY_DN23957_c0_g3_i2	99	95.547	2.85E-107	3.61		Q9C5R8	2-Cys peroxiredoxin BAS1-like, chloroplastic	At5g06290	Green
1HEAT_XZTWC:05890:04964	pe1_TR15054|c0_g1_i4	100	100	3.17E-147	2.82	2.40	Q05046	Chaperonin CPN60-2, mitochondrial	CPN60-2	Green
1HEAT_XZTWC:05072:01696	pe1_TR15054|c0_g1_i4	100	99.64	8.7E-143	2.50		Q05046	Chaperonin CPN60-2, mitochondrial	CPN60-2	Green
1HEAT_XDCTM:04587:10693	pe1_TR15054|c0_g1_i4	96	100	2.36E-133	2.98	2.82	Q05046	Chaperonin CPN60-2, mitochondrial	CPN60-2	Green
1HEAT_XZTWC:05152:04144	pe1_TR15054|c0_g1_i4	96	99.119	2.12E-113	3.04	2.79	Q05046	Chaperonin CPN60-2, mitochondrial	CPN60-2	Green
1HEAT_XDCTM:08455:08735	pe1_TR15054|c0_g1_i4	98	98.765	4.75E-120	2.58		**Q05046**	Chaperonin CPN60-2, mitochondrial (HSP60-2)	CPN60-2	Green
CONTROL_XZTWC:04776:09219	pe1_TR18160|c0_g1_i1	99	99.2	3.71E-126	-3.34		**P93735**	Early light-induced protein 1, chloroplastic	ELIP1	Green
1HEAT_XZTWC:07241:10933	se1_TR5910|c0_g1_i2	92	100	7.91E-66	5.64		**Q39818**	Heat shock 22 kDa protein, mitochondrial	HSP23.9	Green
2HEAT_XZTWC:05370:07872	pe2_TRINITY_DN35314_c0_g1_i1	97	99.628	2.43E-138	3.96	3.13	**Q39818**	Heat shock 22 kDa protein, mitochondrial	HSP23.9	Green
1HEAT_XDCTM:00417:04727	pe2_TRINITY_DN35314_c0_g1_i1	97	99.554	7.42E-113	4.48		**Q39818**	Heat shock 22 kDa protein, mitochondrial	HSP23.9	Green
CONTROL_XDCTM:02175:02538	pe1_TR28753|c3_g1_i1	95	97.727	4.45E-58	-5.56		O65719	Heat shock 70 kDa protein 3	HSP70-3	Green
1HEAT_XDCTM:06994:04722	se1_TR27496|c0_g1_i1	100	98.221	3.17E-137	2.19		P27322	Heat shock cognate 70 kDa protein 2	HSC-2	Green
2HEAT_XZTWC:05293:06051	se1_TR27496|c0_g1_i1	91	97.143	1.01E-43	1.96		P27322	Heat shock cognate 70 kDa protein 2	HSC-2	Green
2HEAT_XDCTM:02598:09357	pe1_TR4038|c4_g3_i1	100	100	5.91E-124	1.56		P36181	Heat shock cognate protein 80	HSC80	Green
2HEAT_XDCTM:07916:04215	pe2_TRINITY_DN19029_c0_g1_i1	95	98.016	3.93E-121	1.62		A2YWQ1	Heat shock protein 81-1	HSP81-1	Green
2HEAT_XZTWC:06763:02964	pe2_TRINITY_DN36706_c3_g1_i2	98	99.595	3.73E-126	3.40		**Q69QQ6**	Heat shock protein 81-2 (HSP81-2) (Heat shock protein 90)	HSP81-2	Green
1HEAT_XZTWC:02824:03215	pe2_TRINITY_DN36706_c3_g1_i2	99	97.63	5.35E-99	4.25	3.77	**Q07078**	Heat shock protein 81-3 (HSP81-3) (Gravity-specific protein GSC 381)	HSP81-3	Green
1HEAT_XDCTM:06309:04635	pe2_TRINITY_DN36706_c3_g1_i2	97	98.261	1.72E-51	3.82	3.26	**Q08277**	Heat shock protein 82	HSP82	Green

CONTROL_XZTWC:05968:05500	pe2_TRINITY_DN36706_c3_g1_i2	100	99.661	9.19E-153	3.68	2.71	**P36182**	Heat shock protein 82 (Fragment)	HSP82	Green
1HEAT_XZTWC:06030:08453	se1_TR26211|c0_g1_i3	100	98.864	3.39E-85	3.52		**P36182**	Heat shock protein 82 (Fragment)	HSP82	Green
1HEAT_XZTWC:08223:10085	pe2_TRINITY_DN36706_c3_g1_i2	100	97.993	5.61E-145	3.82	2.87	P27323	Heat shock protein 90-1	HSP90-1	Green
1HEAT_XDCTM:01628:06105	pe2_TRINITY_DN36706_c3_g1_i2	98	99.615	8.41E-133	3.86	3.06	P27323	Heat shock protein 90-1	HSP90-1	Green
2HEAT_XDCTM:06163:03421	pe1_TR43317|c0_g1_i3	100	99.64	8.7E-143	1.89	1.95	Q9SIF2	Heat shock protein 90-5, chloroplastic	HSP90-5	Green
1HEAT_XZTWC:07835:10647	pe1_TR23297|c1_g1_i7	96	99.184	8.14E-123	1.98	2.10	F4JFN3	Heat shock protein 90-6, mitochondrial	HSP90-6	Green
1HEAT_XZTWC:05387:09243	pe2_TRINITY_DN27773_c0_g1_i2	98	98.233	2.54E-138		1.68	F4JFN3	Heat shock protein 90-6, mitochondrial	HSP90-6	Green
2HEAT_XZTWC:07176:01722	pe2_TRINITY_DN27773_c0_g1_i2	99	98.551	1.14E-136		1.56	F4JFN3	Heat shock protein 90-6, mitochondrial	HSP90-6	Green
1HEAT_XDCTM:08850:10231	pe2_TRINITY_DN27773_c0_g1_i2	100	99.618	6.42E-134	1.72	1.53	F4JFN3	Heat shock protein 90-6, mitochondrial	HSP90-6	Green
1HEAT_XDCTM:06740:02404	pe2_TRINITY_DN27773_c0_g1_i2	97	99.592	1.73E-124	1.68	1.66	F4JFN3	Heat shock protein 90-6, mitochondrial	HSP90-6	Green
1HEAT_XZTWC:05338:03574	pe2_TRINITY_DN27773_c0_g1_i2	96	99.029	3.18E-101	2.50	2.04	F4JFN3	Heat shock protein 90-6, mitochondrial	HSP90-6	Green
2HEAT_XDCTM:04683:06936	pe2_TRINITY_DN27773_c0_g1_i2	99	99.203	3.74E-126		1.68	**F4JFN3**	Heat shock protein 90-6, mitochondrial (AtHSP90.6) (AtHsp90-6) (Heat shock protein 89-1) (Hsp89-1)	HSP90-6	Green
1HEAT_XDCTM:09865:02966	pe2_TRINITY_DN27773_c0_g1_i2	100	97.826	8.65E-133	1.55	1.50	**F4JFN3**	Heat shock protein 90-6, mitochondrial (AtHSP90.6) (AtHsp90-6) (Heat shock protein 89-1) (Hsp89-1)	HSP90-6	Green
1HEAT_XDCTM:02180:06620	pe1_TR6410|c0_g3_i1	99	99.615	8.23E-133	1.65	1.67	P48534	L-ascorbate peroxidase, cytosolic	APX1	Green
2HEAT_XZTWC:04880:11755	pe1_TR6410|c0_g3_i1	96	99.512	2.48E-102	1.89	1.86	P48534	L-ascorbate peroxidase, cytosolic	APX1	Green
1HEAT_XZTWC:06142:07215	pe1_TR6410|c0_g3_i1	99	100	2.43E-107	1.62		P48534	L-ascorbate peroxidase, cytosolic	APX1	Green
1HEAT_XZTWC:01481:05230	pe2_TRINITY_DN30916_c1_g1_i2	96	98.958	2.02E-144	1.57		**P48534**	L-ascorbate peroxidase, cytosolic (AP) (EC 1.11.1.11) (PsAPx01)	APX1	Green
1HEAT_XDCTM:07444:10930	se1_TR18757|c0_g2_i2	94	98.819	3.97E-126	2.04	1.63	Q38931	Peptidyl-prolyl cis-trans isomerase FKBP62	FKBP62	Green
1HEAT_XDCTM:10099:06885	se1_TR18757|c0_g2_i2	100	98.885	6.61E-134	2.10		**Q38931**	Peptidyl-prolyl cis-trans isomerase FKBP62 (PPIase FKBP62) (EC 5.2.1.8) (70 kDa peptidyl-prolyl isomerase) (FK506-binding protein 62) (AtFKBP62) (Immunophilin FKBP62) (Peptidylprolyl isomerase ROF1) (Protein ROTAMASE FKBP 1) (Rotamase)	FKBP62	Green
1HEAT_XZTWC:05228:08793	pe1_TR12110|c0_g1_i10	100	99.39	3.17E-80	3.34		**Q38931**	Peptidyl-prolyl cis-trans isomerase FKBP62 (PPIase FKBP62) (EC 5.2.1.8) (70 kDa peptidyl-prolyl isomerase) (FK506-binding protein 62) (AtFKBP62) (Immunophilin FKBP62) (Peptidylprolyl isomerase ROF1) (Protein ROTAMASE FKBP 1) (Rotamase)	FKBP62	Green
CONTROL_XZTWC:05935:05260	pe2_TRINITY_DN26575_c0_g1_i1	90	100	6.99E-98		-5.39	P32110	Probable glutathione S-transferase	HSP26-A	Green
1HEAT_XZTWC:10491:10583	pe2_TRINITY_DN26575_c0_g1_i1	95	99.474	5.02E-94		-3.96	P32110	Probable glutathione S-transferase	HSP26-A	Green

2HEAT_XDCTM:00735:08300	se1_TR1989|c0_g1_i2	99	99.242	2.34E-133		-1.51	**O24031**	Probable phospholipid hydroperoxide glutathione peroxidase (PHGPx) (EC 1.11.1.12)	GPXle-1	Green
2HEAT_XZTWC:04370:14780	pe2_TRINITY_DN19797_c0_g1_i1	99	98.864	1.24E-84	-1.77		**A2WXD9**	Photosystem II 22 kDa protein 1, chloroplastic (22 kDa protein of photosystem II 1) (Photosystem II subunit 1) (OsPsbS1)	PSBS1	Green
1HEAT_XZTWC:06035:07667	pe1_TR16231|c5_g2_i1	99	98.485	1.8E-129	-1.71		Q02060	Photosystem II 22 kDa protein, chloroplastic	PSBS	Green
CONTROL_XZTWC:07413:06993	pe1_TR16231|c5_g2_i1	95	98	5.05E-120	-1.56		Q02060	Photosystem II 22 kDa protein, chloroplastic	PSBS	Green
CONTROL_XZTWC:05645:03525	pe2_TRINITY_DN19797_c0_g1_i1	98	100	1.16E-146	-1.94		**Q02060**	Photosystem II 22 kDa protein, chloroplastic (CP22)	PSBS	Green
CONTROL_XZTWC:05819:01744	pe1_TR16231|c5_g2_i1	99	98.63	5.53E-145	-1.94		**Q02060**	Photosystem II 22 kDa protein, chloroplastic (CP22)	PSBS	Green
2HEAT_XZTWC:06476:11546	se1_TR12931|c1_g3_i3	99	99.662	9.23E-153	2.34		Q02028	Stromal 70 kDa heat shock-related protein, chloroplastic	HSP70	Green
1HEAT_XZTWC:09554:04396	pe2_TRINITY_DN36907_c0_g1_i4	100	94.086	1.02E-75		4.25	Q9SQL5	Superoxide dismutase [Cu-Zn]	SOD1	Green
2HEAT_XZTWC:01667:04098	se3_TRINITY_DN44315_c0_g1_i2	100	100	9.7E-122	2.22	2.28	Q9FMX0	Superoxide dismutase [Fe] 3, chloroplastic	FSD3	Green
1HEAT_XZTWC:00719:01832	pe1_TR34561|c0_g1_i3	96	99.074	9.3E-107	2.12	2.17	Q9FMX0	Superoxide dismutase [Fe] 3, chloroplastic	FSD3	Green
1HEAT_XDCTM:08563:02607	pe1_TR10122|c0_g2_i4	100	100	1.33E-73	1.90		Q8LGG8	Universal stress protein A-like protein	At3g01520	Green
2HEAT_XDCTM:08603:04980	se1_TR5934|c0_g1_i4	99	99.216	2.26E-128	1.63	1.59	Q8LGG8	Universal stress protein A-like protein	At3g01520	Green
CONTROL_XDCTM:10314:10840	pe2_TRINITY_DN32275_c0_g1_i2	95	99.479	1.09E-95	-2.31		Q8LGG8	Universal stress protein A-like protein	At3g01520	Green
CONTROL_XZTWC:09709:08456	pe2_TRINITY_DN32275_c0_g1_i2	94	100	9.81E-86	-2.71		Q8LGG8	Universal stress protein A-like protein	At3g01520	Green
1HEAT_XZTWC:10245:11993	pe2_TRINITY_DN38203_c5_g5_i8	93	100	3.23E-101	4.21	4.48	P05642	Cytochrome b6	petB	Photosynthesis
2HEAT_XDCTM:01600:09302	pe2_TRINITY_DN38203_c5_g5_i8	100	99.194	3.9E-58	5.02	4.80	P05642	Cytochrome b6	petB	Photosynthesis
CONTROL_XZTWC:03961:08168	-	-	-	-	-3.33		**Q9SDM1**	Chlorophyll a-b binding protein 1B-21, chloroplastic (LHCI type I CAB-1B-21) (LHCI-730 chlorophyll a/b binding protein) (Light-harvesting complex I 21 kDa protein)	LHC Ib-21	Photosynthesis
CONTROL_XZTWC:08325:14289	-	-	-	-		-1.52	**Q9SDM1**	Chlorophyll a-b binding protein 1B-21, chloroplastic (LHCI type I CAB-1B-21) (LHCI-730 chlorophyll a/b binding protein) (Light-harvesting complex I 21 kDa protein)	LHC Ib-21	Photosynthesis
CONTROL_XZTWC:09018:04851	-	-	-	-		-1.95	**Q9SDM1**	Chlorophyll a-b binding protein 1B-21, chloroplastic (LHCI type I CAB-1B-21) (LHCI-730 chlorophyll a/b binding protein) (Light-harvesting complex I 21 kDa protein)	LHC Ib-21	Photosynthesis
CONTROL_XDCTM:07475:10997	pe1_TR43279|c1_g1_i1	99	98.141	1.43E-130		-1.53	**Q9SDM1**	Chlorophyll a-b binding protein 1B-21, chloroplastic (LHCI type I CAB-1B-21) (LHCI-730 chlorophyll a/b binding protein) (Light-harvesting complex I 21 kDa protein)	LHC Ib-21	Photosynthesis

1HEAT_XDCTM:09767:04628	pe1_TR9242|c0_g1_i3	100	97.849	2.8E-86		-1.86	**P14275**	Chlorophyll a-b binding protein 1C, chloroplastic (LHCII type I CAB-1C) (LHCP) (Fragments)	CAB1C	Photosynthesis
CONTROL_XDCTM:01603:13047	-	-	-	-		-2.08	**P09756**	Chlorophyll a-b binding protein 3, chloroplastic (LHCII type I CAB-3) (LHCP)	CAB3	Photosynthesis
CONTROL_XDCTM:09785:03989	-	-	-	-		-1.97	**P27491**	Chlorophyll a-b binding protein 7, chloroplastic (LHCII type I CAB-7) (LHCP)	CAB7	Photosynthesis
CONTROL_XDCTM:02208:14698	-	-	-	-	-1.62		**P12469**	Chlorophyll a-b binding protein C, chloroplastic (LHCII type I CAB-C) (LHCP)	CABC	Photosynthesis
1HEAT_XDCTM:08093:05172	-	-	-	-		-1.79	**P12469**	Chlorophyll a-b binding protein C, chloroplastic (LHCII type I CAB-C) (LHCP)	CABC	Photosynthesis
1HEAT_XZTWC:03432:14127	-	-	-	-		4.16	**P10049**	Chlorophyll a-b binding protein type I, chloroplastic (CAB) (LHCP)		Photosynthesis
CONTROL_XDCTM:00321:12895	-	-	-	-		-2.14	**P10049**	Chlorophyll a-b binding protein type I, chloroplastic (CAB) (LHCP)		Photosynthesis
CONTROL_XDCTM:02733:14595	-	-	-	-		-2.05	**P10049**	Chlorophyll a-b binding protein type I, chloroplastic (CAB) (LHCP)		Photosynthesis
2HEAT_XZTWC:02969:07108	-	-	-	-		-1.50	**P92919**	Chlorophyll a-b binding protein, chloroplastic (allergen Api g 3)	LHC0	Photosynthesis
1HEAT_XZTWC:06378:08125	-	-	-	-		-2.90	**Q40459**	Oxygen-evolving enhancer protein 1, chloroplastic (OEE1) (33 kDa subunit of oxygen evolving system of photosystem II) (33 kDa thylakoid membrane protein) (OEC 33 kDa subunit)	PSBO	Photosynthesis
CONTROL_XZTWC:04206:02830	pe2_TRINITY_DN35250_c0_g1_i1	96	99.291	7.16E-144		-2.55	**Q40459**	Oxygen-evolving enhancer protein 1, chloroplastic (OEE1) (33 kDa subunit of oxygen evolving system of photosystem II) (33 kDa thylakoid membrane protein) (OEC 33 kDa subunit)	PSBO	Photosynthesis
1HEAT_XZTWC:08847:04424	pe1_TR21342|c5_g1_i3	98	99.291	7.05E-144		-3.94	P16059	Oxygen-evolving enhancer protein 2, chloroplastic	PSBP	Photosynthesis
CONTROL_XZTWC:10036:12367	pe1_TR21342|c5_g1_i3	96	98.367	1.76E-119		-2.14	P16059	Oxygen-evolving enhancer protein 2, chloroplastic	PSBP	Photosynthesis
CONTROL_XDCTM:04811:14549	se1_TR21378|c0_g3_i1	92	98.969	1.49E-94		-1.69	**P16059**	Oxygen-evolving enhancer protein 2, chloroplastic (OEE2) (23 kDa subunit of oxygen evolving system of photosystem II) (23 kDa thylakoid membrane protein) (OEC 23 kDa subunit)	PSBP	Photosynthesis
1HEAT_XZTWC:01887:10915	pe2_TRINITY_DN38102_c5_g4_i1	100	99.432	2.62E-86	1.52		Q3BAP0	Photosystem I P700 chlorophyll a apoprotein A1	psaA	Photosynthesis
1HEAT_XDCTM:00870:10820	pe2_TRINITY_DN38102_c5_g4_i1	91	99.32	8.82E-71	5.70	5.57	Q3BAP0	Photosystem I P700 chlorophyll a apoprotein A1	psaA	Photosynthesis
2HEAT_XZTWC:08191:12405	pe2_TRINITY_DN10451_c0_g1_i1	94	98.897	4.27E-136	-2.28		Q41385	Photosystem I reaction center subunit XI, chloroplastic	PSAL	Photosynthesis
2HEAT_XZTWC:00636:01903	-	-	-	-	-1.74		**Q9SUI4**	Photosystem I reaction center subunit XI, chloroplastic (PSI-L) (PSI subunit V)	PSAL	Photosynthesis
CONTROL_XDCTM:07701:10873	pe2_TRINITY_DN44549_c0_g1_i1	97	98.885	1.88E-134		-4.13	Q949Q5	Photosystem I subunit O	PSAO	Photosynthesis
CONTROL_XZTWC:06762:02326	-	-	-	-		-2.13	**Q949Q5**	Photosystem I subunit O (PSI-O)	PSAO	Photosynthesis

1HEAT_XZTWC:09226:05866	-	-	-	-		-2.86	**Q949Q5**	Photosystem I subunit O (PSI-O)	PSAO	Photosynthesis
1HEAT_XDCTM:07535:00898	-	-	-	-		-1.53	**Q949Q5**	Photosystem I subunit O (PSI-O)	PSAO	Photosynthesis
2HEAT_XZTWC:07875:07028	-	-	-	-	-2.88	-2.97	**P10690**	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XDCTM:06242:02772	-	-	-	-	-2.63	-2.07	**P10690**	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XDCTM:06366:02854	-	-	-	-	-3.07		**P10690**	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XDCTM:04751:03338	-	-	-	-		-3.26	**Q40163**	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XZTWC:06401:05435	-	-	-	-	-3.13	-2.50	**P10690**	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XZTWC:05880:08444	pe1_TR25089|c0_g1_i4	100	98.529	2.37E-27		-2.55	P06183	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XZTWC:09384:07010	-	-	-	-	-3.03	-2.97	**P10690**	Photosystem II 10 kDa polypeptide, chloroplastic	PSBR	Photosynthesis
CONTROL_XDCTM:04370:04711	pe1_TR38420|c0_g1_i2	97	98.876	2.74E-86	-4.15		**P06183**	Photosystem II 10 kDa polypeptide, chloroplastic (Light-inducible tissue-specific ST-LS1 protein)	PSBR	Photosynthesis
CONTROL_XDCTM:02773:01271	pe1_TR1642|c0_g1_i1	99	99.262	8.59E-138	-1.70		**O49347**	Photosystem II core complex proteins psbY, chloroplastic (L-arginine-metabolizing enzyme) (L-AME) [Cleaved into: Photosystem II protein psbY-1, chloroplastic (psbY-A1); Photosystem II protein psbY-2, chloroplastic (psbY-A2)]	PSBY	Photosynthesis
1HEAT_XZTWC:02211:13650	-	-	-	-		2.27	**A4QJB1**	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	psbC	Photosynthesis
CONTROL_XDCTM:07638:09179	-	-	-	-	-2.58	-3.14	**Q06GN4**	Photosystem II CP47 reaction center protein (PSII 47 kDa protein) (Protein CP-47)	psbB	Photosynthesis
1HEAT_XZTWC:07367:03420	-	-	-	-	2.91	1.56	**Q9S3W5**	Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	psbA	Photosynthesis
1HEAT_XZTWC:06415:13629	se3_TRINITY_DN56615_c1_g3_i1	99	100	1.2E-115		2.17	**P0C407**	Photosystem II reaction center protein I (PSII-I) (PSII 4.8 kDa protein)	psbI	Photosynthesis
2HEAT_XDCTM:00601:13504	-	-	-	-	-1.66		**Q09FU7**	Photosystem II reaction center protein J (PSII-J)	psbJ	Photosynthesis
CONTROL_XDCTM:02969:04286	pe1_TR44656|c0_g1_i1	100	99.242	2.32E-133	-1.76	-2.12	O65695	Auxin-responsive protein SAUR50	SAUR50	Growth
CONTROL_XDCTM:09709:12968	pe1_TR44656|c0_g1_i1	94	100	1.58E-109		-2.60	O65695	Auxin-responsive protein SAUR50	SAUR50	Growth
CONTROL_XZTWC:05864:09359	pe1_TR44656|c0_g1_i1	98	99.099	4.38E-110		-2.82	O65695	Auxin-responsive protein SAUR50	SAUR50	Growth
CONTROL_XZTWC:08580:12865	pe1_TR44656|c0_g1_i1	96	99.07	3.33E-106	-2.43	-2.53	O65695	Auxin-responsive protein SAUR50	SAUR50	Growth
1HEAT_XZTWC:04230:11504	pe1_TR44656|c0_g1_i1	90	98.947	2.47E-92		-2.41	O65695	Auxin-responsive protein SAUR50	SAUR50	Growth
CONTROL_XZTWC:06103:10079	pe1_TR44656|c0_g1_i1	100	100	1.35E-99		-5.02	**O65695**	Auxin-responsive protein SAUR50 (Protein SMALL AUXIN UP RNA 50)	SAUR51	Growth
1HEAT_XZTWC:04118:02840	pe2_TRINITY_DN44620_c0_g1_i1	92	99.517	5.62E-104	3.90	3.52	Q9SGU2	Auxin-responsive protein SAUR71	SAUR71	Growth
1HEAT_XZTWC:09621:03282	pe2_TRINITY_DN36503_c4_g1_i1	96	100	7.32E-113		-2.89	Q9LIE6	VQ motif-containing protein 22	VQ22	Growth
1HEAT_XZTWC:08871:05210	pe2_TRINITY_DN37910_c1_g1_i2	99	99.02	2.07E-154	1.70	1.90	Q6K7E6	Ethylene-responsive transcription factor 1	EREBP1	Transcription

1HEAT_XDCTM:07671:03519	pe2_TRINITY_DN37910_c1_g1_i2	92	99.115	2.85E-112	1.89	1.94	Q6K7E6	Ethylene-responsive transcription factor 1	EREBP1	Transcription
1HEAT_XDCTM:08791:12681	pe2_TRINITY_DN37910_c1_g1_i2	93	99.383	1.58E-78	2.31	2.36	Q6K7E6	Ethylene-responsive transcription factor 1	EREBP1	Transcription
1HEAT_XZTWC:06185:08134	pe1_TR35092|c1_g1_i1	94	99.515	7.02E-103	2.94	2.50	Q9SUE3	Ethylene-responsive transcription factor CRF4	CRF4	Transcription
2HEAT_XZTWC:04111:07127	se3_TRINITY_DN49988_c0_g1_i1	96	98.673	4.49E-110	2.66		P42736	Ethylene-responsive transcription factor RAP2-3	RAP2-3	Transcription
CONTROL_XZTWC:05876:10119	pe1_TR45330|c1_g1_i5	93	98.678	3.62E-111	1.93		Q9MAL9	GRF1-interacting factor 2	GIF2	Transcription
2HEAT_XDCTM:10323:08203	pe1_TR21358|c1_g1_i3	96	100	3.44E-85	2.84	3.05	A2XK30	Homeobox-leucine zipper protein HOX32	HOX32	Transcription
1HEAT_XZTWC:05713:05415	pe1_TR32158|c0_g1_i1	96	99.582	3.69E-121	3.90	2.73	Q9LV58	Multiprotein-bridging factor 1c	MBF1C	Transcription
1HEAT_XZTWC:07081:13499	se1_TR158|c0_g1_i1	96	99.565	9.95E-117	3.83	2.45	**Q9LV58**	Multiprotein-bridging factor 1c	MBF1C	Transcription
1HEAT_XDCTM:05607:03488	pe1_TR32158|c0_g1_i1	99	99.065	1.15E-105	3.27	2.20	Q9LV58	Multiprotein-bridging factor 1c	MBF1C	Transcription
1HEAT_XZTWC:07891:11671	pe1_TR32158|c0_g1_i1	100	99.537	1.92E-108	4.17	2.81	Q9LV58	Multiprotein-bridging factor 1c	MBF1C	Transcription
1HEAT_XDCTM:04452:05341	pe1_TR32158|c0_g1_i1	98	100	2.05E-87	3.55	2.67	Q9LV58	Multiprotein-bridging factor 1c	MBF1C	Transcription
1HEAT_XZTWC:09250:09078	pe1_TR32158|c0_g1_i1	92	99.359	3.3E-75	3.54		Q9LV58	Multiprotein-bridging factor 1c	MBF1C	Transcription
CONTROL_XDCTM:00334:02917	pe2_TRINITY_DN20059_c0_g1_i1	95	99.602	8.43E-128	-2.27	-3.60	O04681	Pathogenesis-related genes transcriptional activator PTI5	PTI5	Transcription
CONTROL_XZTWC:07391:12173	pe2_TRINITY_DN20059_c0_g1_i1	97	98.565	3.2E-101		-4.01	O04681	Pathogenesis-related genes transcriptional activator PTI5	PTI5	Transcription
CONTROL_XDCTM:03875:15012	pe1_TR45585|c1_g1_i4	100	100	4.73E-73		-3.29	Q93WU9	Probable WRKY transcription factor 51	WRKY51	Transcription
CONTROL_XDCTM:05787:12196	pe1_TR45585|c1_g1_i4	92	100	1.89E-56		-3.86	Q93WU9	Probable WRKY transcription factor 51	WRKY51	Transcription
CONTROL_XZTWC:02582:13223	pe2_TRINITY_DN35522_c0_g2_i1	99	99.27	9.49E-65		-5.55	Q93WU9	Probable WRKY transcription factor 51	WRKY51	Transcription
1HEAT_XDCTM:04608:04863	se3_TRINITY_DN54798_c0_g1_i1	96	99.524	4.22E-105		2.07	F4K933	Protein EFFECTOR OF TRANSCRIPTION 2	ET2	Transcription
2HEAT_XZTWC:00603:05792	pe1_TR37474|c0_g1_i9	94	98.851	1.67E-83		2.87	Q9ASX9	Transcription factor bHLH144	BHLH144	Transcription
1HEAT_XZTWC:04862:06378	pe2_TRINITY_DN32561_c0_g1_i1	100	99.468	1.68E-93		-5.21	Q2HIV9	Transcription factor bHLH35	BHLH35	Transcription
CONTROL_XZTWC:01000:12308	pe2_TRINITY_DN34192_c0_g2_i1	100	99.561	4.35E-115		-3.68	Q9SK55	Transcription factor JUNGBRUNNEN 1	JUB1	Transcription
1HEAT_XZTWC:01129:08865	pe2_TRINITY_DN34192_c0_g2_i1	96	98.598	5.54E-104		-1.83	Q9SK55	Transcription factor JUNGBRUNNEN 1	JUB1	Transcription
CONTROL_XZTWC:07666:01743	pe1_TR16893|c0_g1_i1	100	98.851	1.55E-83	-3.13		Q9LDE1	Transcription factor MYB108	MYB108	Transcription
CONTROL_XZTWC:03237:09000	se1_TR29691|c0_g1_i1	99	98.864	3.44E-85		-3.52	Q42379	Transcription factor MYB7	MYB7	Transcription
CONTROL_XDCTM:09965:11294	pe1_TR37097|c1_g1_i3	99	98.83	2E-82	-3.17		Q39162	Transcription factor TGA4	TGA4	Transcription
1HEAT_XDCTM:07437:08126	pe1_TR19275|c0_g1_i3	92	99.539	5.86E-109		2.32	Q6NQH4	Transcription initiation factor TFIID subunit 13	TAF13	Transcription
CONTROL_XZTWC:02472:02276	pe2_TRINITY_DN22187_c0_g1_i1	100	99.052	5.23E-104		-2.54	Q9XEC3	WRKY transcription factor 42	WRKY42	Transcription
1HEAT_XZTWC:09363:04947	pe1_TR28721|c2_g1_i9	95	98.712	1.7E-114		3.83	F4JGB7	Chromatin remodeling protein At4g04260	At4g04260	Epigenetic
2HEAT_XDCTM:06039:09160	pe1_TR28721|c2_g1_i9	97	98.684	9.65E-112		5.96	F4JGB7	Chromatin remodeling protein At4g04260	At4g04260	Epigenetic

1HEAT_XZTWC:08153:02251	pe1_TR28721|c2_g1_i9	97	99.522	4.17E-105	3.75	3.87	F4JGB7	Chromatin remodeling protein At4g04260	At4g04260	Epigenetic
2HEAT_XDCTM:01717:13354	pe1_TR28721|c2_g1_i9	93	100	1.8E-93		6.05	F4JGB7	Chromatin remodeling protein At4g04260	At4g04260	Epigenetic
1HEAT_XDCTM:00156:10280	pe1_TR28721|c2_g1_i9	100	98.947	6.12E-93		6.19	F4JGB7	Chromatin remodeling protein At4g04260	At4g04260	Epigenetic
CONTROL_XDCTM:08869:04220	pe2_TRINITY_DN37047_c3_g1_i1	98	100	4.89E-130	-1.98		P59169	Histone H3.3	HTR4	Epigenetic
CONTROL_XDCTM:05649:13623	pe2_TRINITY_DN37047_c3_g1_i1	96	99.587	8.07E-123	-2.70	-1.60	P59169	Histone H3.3	HTR4	Epigenetic
CONTROL_XZTWC:03781:04673	pe1_TR2207|c1_g1_i2	97	99.588	2.23E-123	-2.26		P59169	Histone H3.3	HTR4	Epigenetic
CONTROL_XDCTM:03780:03572	se3_TRINITY_DN49241_c1_g1_i9	100	98.45	1.05E-126	2.05	2.08	Q5PP37	Histone-lysine N-methyltransferase ATXR2	ATXR2	Epigenetic
1HEAT_XZTWC:02620:05688	se3_TRINITY_DN49241_c1_g1_i9	100	99.19	6.07E-124	2.73	2.63	Q5PP37	Histone-lysine N-methyltransferase ATXR2	ATXR2	Epigenetic
1HEAT_XZTWC:06511:04549	se3_TRINITY_DN49241_c1_g1_i9	99	99.015	1.41E-99	2.12	2.36	Q5PP37	Histone-lysine N-methyltransferase ATXR2	ATXR2	Epigenetic
1HEAT_XZTWC:00723:03953	pe1_TR29932|c3_g1_i4	90	98.052	2.59E-71		1.80	Q7XI75	Probable histone-arginine methyltransferase CARM1	CARM1	Epigenetic
CONTROL_XZTWC:00620:09521	pe1_TR22231|c0_g1_i23	94	99.095	1.65E-109		-3.65	Q6YXZ7	Protein arginine N-methyltransferase 5	PRMT5	Epigenetic
2HEAT_XDCTM:00634:11814	-	-	-	-	3.41		**P27880**	18.2 kDa class I heat shock protein	HSP18.2	Memory
1HEAT_XZTWC:07159:03512	se1_TR23518|c0_g1_i1	97	99.643	7.05E-144	3.98	3.09	P27880	18.2 kDa class I heat shock protein	HSP18.2	Memory
2HEAT_XZTWC:05437:10419	se1_TR23518|c0_g1_i1	97	99.278	1.51E-140	4.04	3.33	P27880	18.2 kDa class I heat shock protein	HSP18.2	Memory
1HEAT_XZTWC:07273:05496	se1_TR23518|c0_g1_i1	100	99.27	6.67E-139	3.64	2.92	P27880	18.2 kDa class I heat shock protein	HSP18.2	Memory
1HEAT_XZTWC:06420:03088	se1_TR23518|c0_g1_i1	94	99.087	5.81E-109	4.51	3.80	P27880	18.2 kDa class I heat shock protein	HSP18.2	Memory
1HEAT_XZTWC:07863:04952	pe1_TR16747|c0_g1_i1	99	99.2	1.34E-125	2.49		P27880	18.2 kDa class I heat shock protein	HSP18.2	Memory
1HEAT_XZTWC:09120:04492	pe1_TR28865|c4_g3_i2	99	99.653	2.53E-148	4.33	3.51	P46254	Heat shock 22 kDa protein, mitochondrial	HSP22	Memory
CONTROL_XZTWC:09220:05009	pe2_TRINITY_DN35314_c0_g1_i1	99	99.625	1.09E-136	4.04	3.31	P46254	Heat shock 22 kDa protein, mitochondrial	HSP22	Memory
1HEAT_XZTWC:01163:07763	pe1_TR28865|c4_g3_i2	96	98.381	4.94E-120	4.20	3.07	P46254	Heat shock 22 kDa protein, mitochondrial	HSP22	Memory
1HEAT_XDCTM:07045:11531	pe1_TR28865|c4_g3_i2	96	100	6.8E-103	3.68	2.87	P46254	Heat shock 22 kDa protein, mitochondrial	HSP22	Memory
CONTROL_XDCTM:06216:03587	pe2_TRINITY_DN35249_c0_g1_i1	97	96.887	8.48E-118	1.92		Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XDCTM:08845:07941	pe2_TRINITY_DN35249_c0_g1_i1	95	96.735	3.82E-111	1.99	1.57	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XDCTM:09577:07720	pe2_TRINITY_DN35249_c0_g1_i1	99	98.394	3.74E-121	2.26	1.95	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XZTWC:07736:03939	pe2_TRINITY_DN35249_c0_g1_i1	99	98.79	2.86E-122	2.20	2.24	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XDCTM:05596:10155	pe2_TRINITY_DN35249_c0_g1_i1	97	99.119	7.57E-113	2.02	2.01	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
CONTROL_XZTWC:04092:04216	pe2_TRINITY_DN35249_c0_g1_i1	96	99.541	4.38E-110	2.81	2.10	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XDCTM:03540:05305	pe2_TRINITY_DN35249_c0_g1_i1	99	100	4.31E-115	2.32		Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XDCTM:06970:11182	pe2_TRINITY_DN35249_c0_g1_i1	92	98.837	2.16E-82	2.11	1.68	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory
1HEAT_XDCTM:03851:12773	pe2_TRINITY_DN35249_c0_g1_i1	95	99.412	5.75E-83	2.44	1.94	Q9SJQ9	Fructose-bisphosphate aldolase 6, cytosolic	FBA6	Memory

Table AIII.2 *List of functionally annotated sequences belongs to categories of interest for Cymodocea nodosa. Query cover, Identity, E-value present BLAST results between a transcriptome generated in this study and a previous transcriptome from Ruocco et al., 2017.* ****Bold*** *indicates annotation came directly from the sequence from this study while the rest came indirectly from Ruocco et al., 2017.*

SeqName	SeqName in Ruocco et al., 2017	Query cover (%)	Identity (%)	E-value	Log2FC		SwissProt ID*	Description	Gene Name	Category
					Non-primed	Heat-primed				
HEAT2_XZTWC:07469:05776	c39881_g1_i4	95	99	1.04E-79	2.25		**Q8W585**	ATP-dependent zinc metalloprotease FTSH 8, chloroplastic (AtFTSH8) (EC 3.4.24.-)	FTSH8	Yellow
HEAT1_XDCTM:04342:06111	c38700_g1_i5	94	100	1.7E-103	2.75		Q680K8	BTB/POZ domain-containing protein At1g55760	At1g55760	Yellow
HEAT1_XZTWC:04586:01888	c38700_g1_i5	98	100	6.7E-123	2.83		Q680K8	BTB/POZ domain-containing protein At1g55760	At1g55760	Yellow
HEAT2_XDCTM:05911:12078	c38700_g1_i5	99	100	7.6E-107	2.92		**Q680K8**	BTB/POZ domain-containing protein At1g55760	At1g55760	Yellow
CONTROL_XDCTM:02563:12826	c46417_g2_i8	99	100	3.7E-84	-3.51		Q5U430	E3 ubiquitin-protein ligase UBR3	Ubr3	Yellow
HEAT1_XDCTM:09244:02954	c33622_g1_i2	99	100	2.6E-132	1.80		**O65282**	20 kDa chaperonin, chloroplastic (Chaperonin 10) (Ch-CPN10) (Cpn10) (Chaperonin 20) (Protein Cpn21)	CPN20	Green
HEAT1_XZTWC:01486:07238	-	-	-	-	4.49		**Q94IB9**	Alkaline ceramidase (AlkCDase) (Alkaline CDase) (AtACER) (EC 3.5.1.-) (Acyl-CoA independent ceramide synthase 1) (AtCES1) (Alkaline ceramidase YPC1) (AtYPC1) (Alkaline dihydroceramidase ACER) (Alkaline phytoceramidase) (aPHC)	ACER	Green
HEAT2_XZTWC:07695:02094	-	-	-	-	-1.98		**P49315**	Catalase isozyme 1 (EC 1.11.1.6) (Fragment)	CAT1	Green
HEAT1_XDCTM:03936:03445	c42206_g2_i12	100	99	1.5E-124		2.70	Q0E3C8	Chaperone protein ClpB3 mitochondrial	CLPB3	Green
HEAT1_XDCTM:00449:12512	c43114_g1_i6	96	99	1.58E-72	3.56		Q9QYI4	DnaJ homolog subfamily B member 12	Dnajb12	Green
HEAT1_XZTWC:05752:06119	c35563_g1_i1	95	100	2.24E-81	2.70		Q7TQ20	DnaJ homolog subfamily C member 2	Dnajc2	Green
HEAT1_XDCTM:01316:02614	c40832_g1_i1	97	100	2.1E-102		-2.11	**P09189**	Heat shock cognate 70 kDa protein	HSP70	Green
HEAT2_XDCTM:01244:02379	c41895_g1_i4	98	100	3E-116	2.89	2.43	**Q9SIF2**	Heat shock protein 90-5, chloroplastic (AtHSP90.5) (AtHsp90-5) (Heat shock protein 88-1) (Hsp88-1) (Hsp90C) (Protein EMBRYO DEFECTIVE 1956) (Protein chlorate-resistance 88)	HSP90-5	Green
CONTROL_XZTWC:07438:10301	c45581_g3_i4	99	100	4.7E-109	-4.92		**Q96520**	Peroxidase 12 (Atperox P12) (EC 1.11.1.7) (ATP4a) (PRXR6)	PER12	Green
CONTROL_XDCTM:05391:12060	c45581_g4_i3	99	99	1.4E-109	-2.75		**O80822**	Peroxidase 25 (Atperox P25) (EC 1.11.1.7)	PER25	Green

CONTROL_XDCTM:05685:03747	c45581_g3_i4	99	100	5.9E-144	-4.78		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
CONTROL_XDCTM:08525:02737	c45581_g3_i4	100	100	3E-121	-5.36		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
CONTROL_XZTWC:03921:06003	c45581_g3_i4	93	100	1.1E-115	-5.35		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
CONTROL_XZTWC:10479:08040	c45581_g3_i4	96	100	1.7E-108	-5.17		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
HEAT1_XDCTM:04421:03208	c45581_g4_i3	100	99	5.5E-129	-2.78		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
HEAT2_XDCTM:05232:05606	c45581_g3_i4	91	100	1.68E-98	-4.17		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
HEAT2_XZTWC:04337:12532	c45581_g4_i3	92	100	5.2E-114	-3.31		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
HEAT2_XZTWC:07645:03158	c45581_g4_i3	98	100	9.2E-132	-3.03		A7QEU4	Peroxidase 5	GSVIVT00037159001	Green
CONTROL_XZTWC:10297:05485	c45581_g3_i4	100	100	4.17E-57	-6.33		**A7QEU4**	Peroxidase 5 (EC 1.11.1.7)	GSVIVT00037159001	Green
CONTROL_XDCTM:06323:11619	c40832_g4_i1	97	99	2.4E-117		-1.88	P22953	Probable mediator of RNA polymerase II transcription subunit 37e	HSP70-1	Green
CONTROL_XDCTM:09342:01942	c40832_g4_i1	99	100	3.2E-126		-2.07	P22953	Probable mediator of RNA polymerase II transcription subunit 37e	HSP70-1	Green
CONTROL_XZTWC:07812:06963	c40832_g4_i1	97	98	1E-131		-1.72	P22953	Probable mediator of RNA polymerase II transcription subunit 37e	HSP70-1	Green
HEAT1_XZTWC:05083:09473	c40832_g4_i1	100	99	2.4E-117		-1.98	P22953	Probable mediator of RNA polymerase II transcription subunit 37e	HSP70-1	Green
CONTROL_XDCTM:01143:09587	c47286_g5_i1	94	99	1.7E-98		-3.67	Q9SL05	Protein PROTON GRADIENT REGULATION 5 chloroplastic	PGR5	Green
CONTROL_XDCTM:06519:06025	c47286_g5_i2	99	100	8.2E-117		-4.67	Q9SL05	Protein PROTON GRADIENT REGULATION 5 chloroplastic	PGR5	Green
CONTROL_XDCTM:09664:01812	c47286_g5_i1	94	99	5.37E-88	-2.90	-4.04	Q9SL05	Protein PROTON GRADIENT REGULATION 5 chloroplastic	PGR5	Green
HEAT2_XZTWC:07269:11599	-	-	-	-	-2.86		**Q09WY7**	Cytochrome b6	petB	Photosynthesis
HEAT2_XZTWC:08086:04429	-	-	-	-	-2.21		**P27518**	Chlorophyll a-b binding protein 151, chloroplastic (LHCII type II CAB-151) (LHCP)	CAB-151	Photosynthesis
HEAT2_XDCTM:08489:02291	-	-	-	-	-8.18		**P10707**	Chlorophyll a-b binding protein 1D (LHCII type I CAB-1D) (LHCP) (Fragment)	CAB1D	Photosynthesis
CONTROL_XZTWC:04674:08513	c33886_g1_i3	90	97	6.3E-40	-2.48		P27522	Chlorophyll a-b binding protein 8 chloroplastic	CAB8	Photosynthesis
CONTROL_XDCTM:09473:06574	c24150_g1_i2	93	100	1.5E-119	-2.31		**P15192**	Chlorophyll a-b binding protein type 2 member 2 (Chlorophyll a-b binding protein type II 2) (CAB) (LHCP) (Fragment)	N/A	Photosynthesis
CONTROL_XZTWC:07731:04713	c24150_g1_i2	100	99	2.8E-142	-2.10		**P15192**	Chlorophyll a-b binding protein type 2 member 2 (Chlorophyll a-b binding protein type II 2) (CAB) (LHCP) (Fragment)	N/A	Photosynthesis
HEAT2_XZTWC:06411:10171	-	-	-	-	-2.15		**P10049**	Chlorophyll a-b binding protein type I, chloroplastic (CAB) (LHCP)	N/A	Photosynthesis
HEAT1_XZTWC:05173:13229	-	-	-	-	-2.01		**Q3AUT5**	Photosystem I P700 chlorophyll a apoprotein A1 (EC 1.97.1.12) (PsaA)	psaA	Photosynthesis
HEAT2_XDCTM:07220:04267	-	-	-	-	-4.39		**Q9MTN8**	Photosystem I P700 chlorophyll a apoprotein A1 (EC 1.97.1.12) (PSI-A) (PsaA)	psaA	Photosynthesis
HEAT2_XDCTM:06091:09288	-	-	-	-	-1.97		**Q3BAM0**	Photosystem I reaction center subunit IX (PSI-J)	psaJ	Photosynthesis
HEAT1_XZTWC:08542:07257	-	-	-	-	2.34		**Q949Q5**	Photosystem I subunit O (PSI-O)	PSAO	Photosynthesis
CONTROL_XDCTM:06487:01345	-	-	-	-	-3.09		**A4GYQ5**	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	psbC	Photosynthesis

CONTROL_XZTWC:03793:08760	-	-	-	-	-3.10		**A6H5G7**	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	psbC	Photosynthesis
HEAT1_XZTWC:02948:11221	-	-	-	-	-2.94		**A4QJB1**	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	psbC	Photosynthesis
HEAT2_XDCTM:07837:07415	-	-	-	-	-1.97		**A6YG77**	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	psbC	Photosynthesis
HEAT2_XZTWC:00419:03879	-	-	-	-	-2.15		**Q3C1I3**	Photosystem II CP43 reaction center protein (PSII 43 kDa protein) (Protein CP-43)	psbC	Photosynthesis
CONTROL_XDCTM:01448:02816	-	-	-	-	-2.29		**Q49CA8**	Photosystem II D2 protein (PSII D2 protein) (EC 1.10.3.9) (Photosystem Q(A) protein)	psbD	Photosynthesis
CONTROL_XZTWC:01914:06862	-	-	-	-	-2.22		**Q9TNF7**	Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	psbA	Photosynthesis
HEAT1_XDCTM:00151:11039	-	-	-	-	-1.63		**Q9TNF8**	Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	psbA	Photosynthesis
HEAT1_XZTWC:10459:05633	-	-	-	-	-3.17		**Q9TNF8**	Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	psbA	Photosynthesis
HEAT2_XDCTM:07550:08135	-	-	-	-	-4.59		**A6MMS4**	Photosystem II protein D1 (PSII D1 protein) (EC 1.10.3.9) (Photosystem II Q(B) protein)	psbA	Photosynthesis
HEAT2_XDCTM:06964:06900	-	-	-	-	-4.02		**Q11A00**	Photosystem II protein D1 1 (PSII D1 protein 1) (EC 1.10.3.9) (Photosystem II Q(B) protein 1)	psbA	Photosynthesis
HEAT1_XDCTM:01584:03968	c44857_g7_i5	99	100	1.4E-119		-2.73	Q6K7E6	Ethylene-responsive transcription factor 1	EREBP1	Transcription
HEAT2_XZTWC:01271:06340	c44732_g5_i3	93	100	2.3E-107	-1.61		Q8H0T5	Ethylene-responsive transcription factor ERF073	ERF073	Transcription
CONTROL_XDCTM:06846:05436	c45948_g3_i10	94	100	1.1E-115	1.60		P0DI14	Floral homeotic protein APETALA 1	AP1	Transcription
CONTROL_XDCTM:10236:08905	c43610_g2_i5	96	98	7.3E-123		-2.45	Q7XBH4	Myb-related protein Myb4	MYB4	Transcription
CONTROL_XZTWC:05421:04147	c43610_g2_i5	97	99	4.75E-78		-4.92	Q7XBH4	Myb-related protein Myb4	MYB4	Transcription
HEAT1_XDCTM:03127:11471	c43287_g1_i5	92	100	3.8E-105	-1.62		Q7GCL7	NAC domain-containing protein 74	NAC074	Transcription
CONTROL_XDCTM:05463:07444	c47569_g1_i14	96	100	1.3E-104	-2.70	-3.62	P92973	Protein CCA1	CCA1	Transcription
CONTROL_XDCTM:05793:12085	c47569_g1_i14	100	99	4.59E-78	-2.82	-3.12	P92973	Protein CCA1	CCA1	Transcription
CONTROL_XZTWC:06359:08798	c47569_g1_i14	99	98	4.49E-73	-2.64	-3.43	P92973	Protein CCA1	CCA1	Transcription
HEAT1_XZTWC:02100:11789	c47569_g1_i14	92	99	2.16E-71	-2.91	-4.90	P92973	Protein CCA1	CCA1	Transcription
HEAT1_XZTWC:06895:11166	c47569_g1_i14	96	99	2.06E-71	-2.95	-3.52	P92973	Protein CCA1	CCA1	Transcription
CONTROL_XDCTM:05855:11900	c47569_g2_i2	100	100	3.7E-110	-2.94	-3.49	Q6R0H1	Protein LHY	LHY	Transcription
CONTROL_XDCTM:09981:10979	c47569_g2_i2	100	99	4.55E-78		-4.06	Q6R0H1	Protein LHY	LHY	Transcription
CONTROL_XZTWC:03631:04811	c47569_g2_i2	95	100	9.7E-132	-2.18		Q6R0H1	Protein LHY	LHY	Transcription

CONTROL_XZTWC:05078:10295	c47569_g2_i2	97	98	4.9E-104	-3.25	-3.91	Q6R0H1	Protein LHY	LHY	Transcription
CONTROL_XZTWC:05737:12495	c47569_g2_i2	100	99	1.4E-88	-3.29		Q6R0H1	Protein LHY	LHY	Transcription
CONTROL_XZTWC:06662:01051	c47569_g2_i2	92	100	1.43E-83	-3.18	-3.26	Q6R0H1	Protein LHY	LHY	Transcription
HEAT1_XDCTM:06049:13569	c47569_g1_i14	100	99	5.64E-98	-2.41	-4.34	**Q6R0H1**	Protein LHY (MYB-related transcription factor LHY) (Protein LATE ELONGATED HYPOCOTYL)	LHY	Transcription
HEAT2_XZTWC:04669:07409	c46952_g3_i10	99	100	1.58E-77	-5.60		Q700D9	Putative Myb family transcription factor At1g14600	At1g14600	Transcription
HEAT2_XZTWC:01767:11808	-	-	-	-	2.45		**Q3E811**	Regulator of rDNA transcription protein 15	RRT15	Transcription
HEAT2_XDCTM:08400:00801	c46952_g3_i11	91	98	5.01E-57	-3.53		Q940D0	Two-component response regulator ARR1	ARR1	Transcription
HEAT2_XZTWC:09857:03696	c46952_g3_i7	99	100	1.3E-104	-4.60		Q9LTH4	Transcription factor BOA	BOA	Transcription
HEAT2_XDCTM:06451:07826	c41581_g1_i2	96	100	1.2E-130	2.63		**Q9LJG8**	ASIL2	ASIL2	Transcription
HEAT2_XDCTM:00817:07522	c37274_g1_i3	95	99	5.37E-88	2.83		P46254	Heat shock 22 kDa protein mitochondrial	HSP22	Memory
HEAT2_XZTWC:07172:01481	c37274_g1_i3	100	100	1.4E-119	2.40		P46254	Heat shock 22 kDa protein mitochondrial	HSP22	Memory

Table AIII.3 *List of enriched GO-BPs (FRD < 0.05) from non-primed Posidonia oceanica. GO-BPs relate to thermal stress response are in* ***bold****.*

GO ID	GO - Biological process	FDR	GO ID	GO - Biological process	FDR
GO:0009408	**response to heat**	< 1e-30	GO:0033384	geranyl diphosphate biosynthetic process	0.00201
GO:0046686	response to cadmium ion	< 1e-30	GO:0033386	geranylgeranyl diphosphate biosynthetic process	0.00201
GO:0051131	chaperone-mediated protein complex assembly	3.80E-21	GO:0055080	cation homeostasis	0.00211
GO:0042542	**response to hydrogen peroxide**	6.40E-21	GO:0010081	regulation of inflorescence meristem growth	0.00233
GO:0061077	**chaperone-mediated protein folding**	2.90E-15	GO:0090408	phloem nitrate loading	0.00233
GO:0006970	response to osmotic stress	3.80E-15	GO:0045337	farnesyl diphosphate biosynthetic process	0.00233
GO:0046688	response to copper ion	7.90E-15	GO:0009737	**response to abscisic acid**	0.00239
GO:0080167	response to karrikin	3.50E-14	GO:0010193	response to ozone	0.00239
GO:0046685	response to arsenic-containing substance	3.90E-13	GO:0042938	dipeptide transport	0.00246
GO:0045471	response to ethanol	8.40E-13	GO:0008340	determination of adult lifespan	0.00256
GO:0006463	steroid hormone receptor complex assembly	4.20E-12	GO:0045037	protein import into chloroplast stroma	0.00264
GO:0010286	**heat acclimation**	4.90E-12	GO:0006801	superoxide metabolic process	0.00281
GO:0010187	negative regulation of seed germination	1.10E-11	GO:0009915	phloem sucrose loading	0.00311
GO:0040024	dauer larval development	1.70E-11	GO:0040011	locomotion	0.00341
GO:0009651	response to salt stress	2.00E-11	GO:0010167	response to nitrate	0.00342
GO:0071277	cellular response to calcium ion	3.00E-11	GO:0015692	lead ion transport	0.00376
GO:0030850	prostate gland development	3.60E-11	GO:0006542	glutamine biosynthetic process	0.00384
GO:0009409	response to cold	4.20E-11	GO:0042939	tripeptide transport	0.00412
GO:0042026	**protein refolding**	1.90E-10	GO:0009792	embryo development ending in birth or egg hatching	0.00423
GO:0009735	response to cytokinin	7.60E-10	GO:0009715	chalcone biosynthetic process	0.00472
GO:0055114	oxidation-reduction process	9.10E-10	GO:0010497	plasmodesmata-mediated intercellular transport	0.00511
GO:0090332	stomatal closure	2.00E-09	GO:0051973	positive regulation of telomerase activity	0.00521
GO:0070370	**cellular heat acclimation**	6.00E-09	GO:0009751	**response to salicylic acid**	0.00538
GO:0009611	response to wounding	7.40E-09	GO:0043508	negative regulation of JUN kinase activity	0.00543
GO:0009644	response to high light intensity	8.90E-09	GO:0080024	indolebutyric acid metabolic process	0.00569
GO:0030521	androgen receptor signaling pathway	1.20E-08	GO:0019637	organophosphate metabolic process	0.00577
GO:0007566	embryo implantation	1.50E-08	GO:0006094	gluconeogenesis	0.00589
GO:0045446	endothelial cell differentiation	1.60E-08	GO:0009306	protein secretion	0.00593
GO:0009908	flower development	2.10E-08	GO:0033673	negative regulation of kinase activity	0.00616
GO:0046677	response to antibiotic	2.30E-08	GO:1901140	p-coumaryl alcohol transport	0.0062
GO:0009816	defense response to bacterium	4.10E-08	GO:0002536	respiratory burst involved in inflammatory response	0.00698
GO:0031111	negative regulation of microtubule polymerization or depolymerization	6.10E-08	GO:0045581	negative regulation of T cell differentiation	0.00701
GO:0000302	**response to reactive oxygen species**	7.50E-08	GO:0001649	osteoblast differentiation	0.00735
GO:0006597	spermine biosynthetic process	1.50E-07	GO:0042790	nucleolar large rRNA transcription by RNA polymerase I	0.00738

GO:0031503	protein-containing complex localization	2.50E-07	GO:0060776	simple leaf morphogenesis	0.00758
GO:0006457	**protein folding**	3.80E-07	GO:0072593	**reactive oxygen species metabolic process**	0.00789
GO:0019464	glycine decarboxylation via glycine cleavage system	4.50E-07	GO:1900366	negative regulation of defense response to insect	0.00816
GO:0006986	**response to unfolded protein**	9.40E-07	GO:0030194	positive regulation of blood coagulation	0.00876
GO:0009414	response to water deprivation	9.70E-07	GO:0010189	vitamin E biosynthetic process	0.00915
GO:0010157	response to chlorate	1.80E-06	GO:0007623	circadian rhythm	0.00917
GO:0006096	glycolytic process	1.80E-06	GO:0042214	terpene metabolic process	0.00948
GO:0050821	**protein stabilization**	2.40E-06	GO:0019430	**removal of superoxide radicals**	0.00951
GO:0048769	sarcomerogenesis	2.80E-06	GO:0046967	cytosol to endoplasmic reticulum transport	0.01137
GO:0030241	skeletal muscle myosin thick filament assembly	2.80E-06	GO:0031665	negative regulation of lipopolysaccharide-mediated signaling pathway	0.01137
GO:0071786	endoplasmic reticulum tubular network organization	3.80E-06	GO:0009733	**response to auxin**	0.01144
GO:0006611	protein export from nucleus	3.90E-06	GO:0045676	regulation of R7 cell differentiation	0.01207
GO:0000413	protein peptidyl-prolyl isomerization	4.20E-06	GO:0006879	cellular iron ion homeostasis	0.0131
GO:0006098	pentose-phosphate shunt	5.00E-06	GO:2001240	negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	0.01316
GO:0042744	**hydrogen peroxide catabolic process**	6.10E-06	GO:0006855	drug transmembrane transport	0.01423
GO:0009845	seed germination	9.30E-06	GO:0010019	chloroplast-nucleus signaling pathway	0.01424
GO:0009699	phenylpropanoid biosynthetic process	9.40E-06	GO:0080164	regulation of nitric oxide metabolic process	0.01428
GO:0030240	skeletal muscle thin filament assembly	1.30E-05	GO:0071353	cellular response to interleukin-4	0.01466
GO:0046661	male sex differentiation	1.40E-05	GO:0031540	regulation of anthocyanin biosynthetic process	0.01505
GO:0050900	leukocyte migration	1.60E-05	GO:0006662	glycerol ether metabolic process	0.01623
GO:0010075	regulation of meristem growth	2.40E-05	GO:0051402	neuron apoptotic process	0.01693
GO:0009853	photorespiration	2.40E-05	GO:0071287	cellular response to manganese ion	0.01747
GO:0009934	regulation of meristem structural organization	2.50E-05	GO:0050908	detection of light stimulus involved in visual perception	0.01831
GO:0034340	response to type I interferon	3.30E-05	GO:0071492	cellular response to UV-A	0.01874
GO:0009854	oxidative photosynthetic carbon pathway	6.20E-05	GO:0045041	protein import into mitochondrial intermembrane space	0.01917
GO:0010259	multicellular organism aging	6.30E-05	GO:0009399	nitrogen fixation	0.02004
GO:0048366	leaf development	7.40E-05	GO:0000187	activation of MAPK activity	0.02019
GO:0008295	spermidine biosynthetic process	8.10E-05	GO:0010162	seed dormancy process	0.02258
GO:0001666	response to hypoxia	8.40E-05	GO:0009646	response to absence of light	0.02314
GO:0006919	activation of cysteine-type endopeptidase activity involved in apoptotic process	8.50E-05	GO:0030317	flagellated sperm motility	0.0236
GO:0046653	tetrahydrofolate metabolic process	9.40E-05	GO:0034440	lipid oxidation	0.02398
GO:0006954	inflammatory response	9.60E-05	GO:0051092	positive regulation of NF-kappaB transcription factor activity	0.02411
GO:0015773	raffinose transport	9.60E-05	GO:0006826	iron ion transport	0.02491
GO:0019216	regulation of lipid metabolic process	9.60E-05	GO:0018171	peptidyl-cysteine oxidation	0.02514
GO:0080168	**abscisic acid transport**	9.60E-05	GO:0080027	response to herbivore	0.02612
GO:0009753	**response to jasmonic acid**	0.00011	GO:0006807	nitrogen compound metabolic process	0.02671
GO:0006563	L-serine metabolic process	0.00013	GO:0010506	regulation of autophagy	0.02685
GO:0040035	hermaphrodite genitalia development	0.00026	GO:1902402	signal transduction involved in mitotic	0.02766

GO:0019371	cyclooxygenase pathway	0.00026	GO:0006468	protein phosphorylation	0.02857
GO:0055072	iron ion homeostasis	0.00035	GO:0009629	response to gravity	0.02897
GO:0051775	response to redox state	0.00038	GO:0030397	membrane disassembly	0.02914
GO:0009266	**response to temperature stimulus**	0.00043	GO:0051257	meiotic spindle midzone assembly	0.0301
GO:0009939	**positive regulation of gibberellic acid mediated signaling pathway**	0.00044	GO:0000713	meiotic heteroduplex formation	0.0301
GO:0006730	one-carbon metabolic process	0.00044	GO:0043060	meiotic metaphase I plate congression	0.0301
GO:0031525	menthol biosynthetic process	0.00045	GO:0036500	ATF6-mediated unfolded protein response	0.03176
GO:0097295	morphine biosynthetic process	0.00045	GO:2000378	**negative regulation of reactive oxygen species**	0.03283
GO:0010197	polar nucleus fusion	0.00046	GO:0010080	regulation of floral meristem growth	0.03392
GO:0043388	positive regulation of DNA binding	0.00049	GO:0019253	reductive pentose-phosphate cycle	0.03535
GO:0010188	response to microbial phytotoxin	0.0005	GO:0019752	carboxylic acid metabolic process	0.03584
GO:0043065	**positive regulation of apoptotic process**	0.00059	GO:0031247	actin rod assembly	0.03614
GO:0070536	protein K63-linked deubiquitination	0.00065	GO:0030007	cellular potassium ion homeostasis	0.03654
GO:0006915	**apoptotic process**	0.00077	GO:0009626	plant-type hypersensitive response	0.03669
GO:0009704	de-etiolation	0.00079	GO:0006012	galactose metabolic process	0.03727
GO:0042373	vitamin K metabolic process	0.00082	GO:1901141	regulation of lignin biosynthetic process	0.03727
GO:0052576	carbohydrate storage	0.00086	GO:0071465	cellular response to desiccation	0.03727
GO:0070585	protein localization to mitochondrion	0.00096	GO:0000495	box H/ACA snoRNA 3'-end processing	0.03993
GO:0017144	drug metabolic process	0.00108	GO:0090669	telomerase RNA stabilization	0.03993
GO:0043516	**regulation of DNA damage response, signal transduction by p53 class mediator**	0.00133	GO:0015986	ATP synthesis coupled proton transport	0.0403
GO:0006636	**unsaturated fatty acid biosynthetic process**	0.00145	GO:0071318	cellular response to ATP	0.04073
GO:0010325	raffinose family oligosaccharide biosynthesis	0.00158	GO:0051262	protein tetramerization	0.04089
GO:0007049	cell cycle	0.00164	GO:0032088	negative regulation of NF-kappaB transcription factor activity	0.04132
GO:0009873	**ethylene-activated signaling pathway**	0.00166	GO:0010310	**regulation of hydrogen peroxide metabolic process**	0.0436
GO:0018026	peptidyl-lysine monomethylation	0.00184	GO:0007005	mitochondrion organization	0.04466
GO:0009088	threonine biosynthetic process	0.0019	GO:0007422	peripheral nervous system development	0.04676
GO:0071588	**hydrogen peroxide mediated signaling pathway**	0.00196	GO:0019563	glycerol catabolic process	0.04738
GO:0015979	**photosynthesis**	0.00201	GO:0031408	oxylipin biosynthetic process	0.04828
GO:0043693	monoterpene biosynthetic process	0.00201	GO:0002520	immune system development	0.04979

Table AIII.4 *List of enriched GO-BPs (FRD < 0.05) from heat-primed Posidonia oceanica. GO-BPs relate to thermal stress response are in* ***bold****.*

GO ID	GO - Biological process	FDR	GO ID	GO - Biological process	FDR
GO:0080167	response to karrikin	1.60E-23	GO:0009939	**positive regulation of gibberellic acid process**	0.00098
GO:0009715	chalcone biosynthetic process	5.80E-19	GO:0006544	glycine metabolic process	0.001
GO:0009629	response to gravity	4.30E-17	GO:0009306	protein secretion	0.00102
GO:0031540	regulation of anthocyanin biosynthetic process	8.10E-16	GO:0021762	substantia nigra development	0.0011
GO:0051131	chaperone-mediated protein complex assembly	1.90E-15	GO:0006970	response to osmotic stress	0.00132
GO:0009408	**response to heat**	9.00E-14	GO:0006817	phosphate ion transport	0.00151
GO:0009753	**response to jasmonic acid**	2.10E-13	GO:0042214	terpene metabolic process	0.00156
GO:0046686	response to cadmium ion	2.30E-12	GO:0043201	response to leucine	0.00158
GO:1902358	sulfate transmembrane transport	2.80E-12	GO:0098771	inorganic ion homeostasis	0.0018
GO:0071786	endoplasmic reticulum tubular network organization	5.20E-12	GO:0005992	trehalose biosynthetic process	0.00236
GO:0015706	nitrate transport	8.70E-12	GO:0043516	**regulation of DNA damage response**	0.00246
GO:0009704	de-etiolation	1.00E-11	GO:0044036	cell wall macromolecule metabolic process	0.00254
GO:0070981	L-asparagine biosynthetic process	1.20E-11	GO:0010244	response to low fluence blue light stimulus by blue low-fluence system	0.00289
GO:0042542	**response to hydrogen peroxide**	1.60E-11	GO:0071555	cell wall organization	0.00308
GO:0048527	lateral root development	2.40E-11	GO:0006098	pentose-phosphate shunt	0.00311
GO:0030241	skeletal muscle myosin thick filament assembly	3.40E-11	GO:0009737	**response to abscisic acid**	0.00316
GO:0048769	sarcomerogenesis	3.40E-11	GO:0055080	cation homeostasis	0.00329
GO:0010167	response to nitrate	6.40E-11	GO:0006564	L-serine biosynthetic process	0.00331
GO:0010157	response to chlorate	1.60E-10	GO:0018026	peptidyl-lysine monomethylation	0.00338
GO:0046688	response to copper ion	2.70E-10	GO:0010081	regulation of inflorescence meristem growth	0.00355
GO:0043617	**cellular response to sucrose starvation**	4.50E-10	GO:1904851	positive regulation of establishment of protein localization to telomere	0.00402
GO:0030240	skeletal muscle thin filament assembly	5.30E-10	GO:0010037	response to carbon dioxide	0.0041
GO:0050900	leukocyte migration	7.70E-10	GO:0009749	response to glucose	0.00417
GO:0071249	cellular response to nitrate	7.70E-10	GO:0006801	superoxide metabolic process	0.00431
GO:0009651	response to salt stress	1.40E-09	GO:0009617	response to bacterium	0.00476
GO:0080160	selenate transport	1.60E-09	GO:0009448	gamma-aminobutyric acid metabolic process	0.00521
GO:0006597	spermine biosynthetic process	3.40E-09	GO:0006569	tryptophan catabolic process	0.00521
GO:0000302	**response to reactive oxygen species**	3.50E-09	GO:0006611	protein export from nucleus	0.00532
GO:0010286	**heat acclimation**	1.20E-08	GO:0009873	**ethylene-activated signaling pathway**	0.00548
GO:0051973	positive regulation of telomerase activity	1.50E-08	GO:0032981	mitochondrial respiratory chain complex I assembly	0.00552
GO:0009063	cellular amino acid catabolic process	2.30E-08	GO:0015986	ATP synthesis coupled proton transport	0.00556
GO:0097164	ammonium ion metabolic process	2.80E-08	GO:0009682	induced systemic resistance	0.00556
GO:0098656	anion transmembrane transport	3.30E-08	GO:0061077	**chaperone-mediated protein folding**	0.00614
GO:0009751	**response to salicylic acid**	3.50E-08	GO:0000467	exonucleolytic trimming to generate mature 3'-end of 5.8S rRNA from tricistronic rRNA transcript	0.00621
GO:0006542	glutamine biosynthetic process	1.10E-07	GO:0015692	lead ion transport	0.00682
GO:0006541	glutamine metabolic process	1.30E-07	GO:0006952	defense response	0.00719
GO:0009611	response to wounding	1.50E-07	GO:0043388	positive regulation of DNA binding	0.00774
GO:0046685	response to arsenic-containing substance	1.60E-07	GO:0090414	molybdate ion export from vacuole	0.00786

GO:0009733	**response to auxin**	1.90E-07	GO:0006879	cellular iron ion homeostasis	0.00795
GO:0042538	hyperosmotic salinity response	2.00E-07	GO:0006096	glycolytic process	0.00823
GO:0045471	response to ethanol	2.80E-07	GO:0080024	indolebutyric acid metabolic process	0.0086
GO:0009934	regulation of meristem structural organization	2.80E-07	GO:0019373	epoxygenase P450 pathway	0.00898
GO:0009908	flower development	3.30E-07	GO:0009416	response to light stimulus	0.00911
GO:0009970	cellular response to sulfate starvation	4.00E-07	GO:0019637	organophosphate metabolic process	0.00917
GO:0010187	negative regulation of seed germination	9.50E-07	GO:0006457	**protein folding**	0.00924
GO:0042938	dipeptide transport	1.10E-06	GO:0006120	mitochondrial electron transport, NADH to ubiquinone	0.00928
GO:0060416	**response to growth hormone**	1.40E-06	GO:0034340	response to type I interferon	0.01014
GO:0090332	stomatal closure	1.40E-06	GO:1904874	positive regulation of telomerase RNA localization to Cajal body	0.01034
GO:0009414	response to water deprivation	1.50E-06	GO:0009735	response to cytokinin	0.01065
GO:0045446	endothelial cell differentiation	1.80E-06	GO:0032212	positive regulation of telomere maintenance via telomerase	0.01115
GO:0071277	cellular response to calcium ion	2.40E-06	GO:0010480	microsporocyte differentiation	0.01156
GO:0042939	tripeptide transport	3.30E-06	GO:0006919	activation of cysteine-type endopeptidase activity involved in apoptotic process	0.0118
GO:0040024	dauer larval development	3.60E-06	GO:0009759	indole glucosinolate biosynthetic process	0.0123
GO:0072593	**reactive oxygen species metabolic process**	3.90E-06	GO:0071049	nuclear retention of pre-mRNA with aberrant 3'-ends at the site of transcription	0.01275
GO:0009646	response to absence of light	4.00E-06	GO:0010506	regulation of autophagy	0.01285
GO:0009926	**auxin polar transport**	4.70E-06	GO:0044206	UMP salvage	0.01413
GO:0009409	response to cold	6.40E-06	GO:0006563	L-serine metabolic process	0.01433
GO:0031120	snRNA pseudouridine synthesis	7.40E-06	GO:0071034	CUT catabolic process	0.01508
GO:0009399	nitrogen fixation	8.10E-06	GO:0010582	floral meristem determinacy	0.01548
GO:0080148	negative regulation of response to water deprivation	8.30E-06	GO:0010188	response to microbial phytotoxin	0.01557
GO:0008295	spermidine biosynthetic process	9.60E-06	GO:0006749	glutathione metabolic process	0.01597
GO:0055114	oxidation-reduction process	1.10E-05	GO:0048229	gametophyte development	0.01654
GO:0042026	**protein refolding**	1.40E-05	GO:0006826	iron ion transport	0.01693
GO:0000495	box H/ACA snoRNA 3'-end processing	1.70E-05	GO:0046653	tetrahydrofolate metabolic process	0.01836
GO:0090669	telomerase RNA stabilization	1.70E-05	GO:0040035	hermaphrodite genitalia development	0.0187
GO:0019371	cyclooxygenase pathway	1.70E-05	GO:0015773	raffinose transport	0.01878
GO:0080168	**abscisic acid transport**	2.30E-05	GO:0006954	inflammatory response	0.01891
GO:1901684	arsenate ion transmembrane transport	2.30E-05	GO:0009723	**response to ethylene**	0.01908
GO:0045037	protein import into chloroplast stroma	2.60E-05	GO:0019464	glycine decarboxylation via glycine cleavage system	0.01919
GO:0009915	phloem sucrose loading	3.20E-05	GO:2001243	**negative regulation of intrinsic apoptotic signaling pathway**	0.02042
GO:0031525	menthol biosynthetic process	3.60E-05	GO:0015979	**photosynthesis**	0.02081
GO:0097295	morphine biosynthetic process	3.60E-05	GO:0098712	L-glutamate import across plasma membrane	0.02081
GO:0042128	nitrate assimilation	4.60E-05	GO:1903861	positive regulation of dendrite extension	0.02187
GO:1990481	mRNA pseudouridine synthesis	4.70E-05	GO:0032596	protein transport into membrane raft	0.02187
GO:0009266	**response to temperature stimulus**	4.80E-05	GO:1903265	positive regulation of tumor necrosis factor-mediated signaling pathway	0.02187
GO:0009744	response to sucrose	4.90E-05	GO:0071484	cellular response to light intensity	0.02367
GO:0010224	response to UV-B	5.40E-05	GO:0006986	**response to unfolded protein**	0.02394
GO:0030224	monocyte differentiation	6.00E-05	GO:0000494	box C/D snoRNA 3'-end processing	0.02496
GO:0009816	defense response to bacterium	6.70E-05	GO:1990258	histone glutamine methylation	0.02496
GO:0050890	cognition	6.80E-05	GO:0010120	camalexin biosynthetic process	0.02594

GO:0042373	vitamin K metabolic process	8.00E-05	GO:0071051	polyadenylation-dependent snoRNA 3'-end processing	0.02792
GO:0006182	cGMP biosynthetic process	8.40E-05	GO:0045041	protein import into mitochondrial intermembrane space	0.02856
GO:0009992	cellular water homeostasis	9.30E-05	GO:0042744	**hydrogen peroxide catabolic process**	0.03106
GO:0090408	phloem nitrate loading	9.60E-05	GO:0019919	peptidyl-arginine methylation, to asymmetrical-dimethyl arginine	0.03114
GO:0010075	regulation of meristem growth	1.00E-04	GO:0035335	peptidyl-tyrosine dephosphorylation	0.03266
GO:0009699	phenylpropanoid biosynthetic process	0.0001	GO:0009738	**abscisic acid-activated signaling pathway**	0.03292
GO:0050821	**protein stabilization**	0.0001	GO:0071038	nuclear polyadenylation-dependent tRNA catabolic process	0.03517
GO:0017144	drug metabolic process	0.00012	GO:0006950	**response to stress**	0.03552
GO:0010259	multicellular organism aging	0.00015	GO:0046464	acylglycerol catabolic process	0.03586
GO:0019684	**photosynthesis, light reaction**	0.00016	GO:0001666	response to hypoxia	0.03597
GO:0006807	nitrogen compound metabolic process	0.00016	GO:0006915	**apoptotic process**	0.03685
GO:0046677	response to antibiotic	0.00017	GO:0071588	**hydrogen peroxide mediated signaling pathway**	0.03726
GO:0009750	response to fructose	0.00017	GO:0006546	glycine catabolic process	0.03758
GO:0010189	vitamin E biosynthetic process	0.0002	GO:0080027	response to herbivore	0.03868
GO:0080052	response to histidine	0.00022	GO:0009684	indoleacetic acid biosynthetic process	0.03939
GO:0080053	response to phenylalanine	0.00022	GO:0051775	response to redox state	0.03977
GO:0010186	positive regulation of cellular defense	0.00024	GO:0071035	nuclear polyadenylation-dependent rRNA catabolic process	0.04012
GO:0006979	**response to oxidative stress**	0.00034	GO:0034971	**histone H3-R17 methylation**	0.04024
GO:0009644	response to high light intensity	0.00036	GO:0034972	**histone H3-R26 methylation**	0.04024
GO:0009407	toxin catabolic process	0.00043	GO:0009615	response to virus	0.04045
GO:0048366	leaf development	0.00053	GO:1902074	response to salt	0.04103
GO:0019530	taurine metabolic process	0.00054	GO:0031175	neuron projection development	0.04273
GO:0070314	G1 to G0 transition	0.00054	GO:0006730	one-carbon metabolic process	0.04317
GO:0009311	oligosaccharide metabolic process	0.00055	GO:0010241	ent-kaurene oxidation to kaurenoic acid	0.04327
GO:0051211	anisotropic cell growth	0.00061	GO:0030010	establishment of cell polarity	0.04507
GO:0055072	iron ion homeostasis	0.00078	GO:0034475	U4 snRNA 3'-end processing	0.04531
GO:0050789	regulation of biological process	0.0008	GO:0016266	O-glycan processing	0.04628
GO:0010039	response to iron ion	0.00082	GO:0006054	N-acetylneuraminate metabolic process	0.04628
GO:0031118	rRNA pseudouridine synthesis	0.00085	GO:0009642	response to light intensity	0.04829
GO:0006636	**unsaturated fatty acid biosynthetic process**	0.00085	GO:0050913	sensory perception of bitter taste	0.04929
GO:0048315	conidium formation	0.00089	GO:0010080	regulation of floral meristem growth	0.04994

Table AIII.5 *List of enriched GO-BPs (FRD < 0.05) from non-primed Cymodocea nodosa. GO-BPs relate to thermal stress response are in* ***bold****.*

GO ID	GO - Biological process	FDR	GO ID	GO - Biological process	FDR
GO:0070981	L-asparagine biosynthetic process	4.30E-18	GO:0006541	glutamine metabolic process	0.08942
GO:0006979	**response to oxidative stress**	1.90E-10	GO:0006559	L-phenylalanine catabolic process	0.09039
GO:0042026	**protein refolding**	6.60E-07	GO:0010205	photoinhibition	0.10211
GO:0006730	one-carbon metabolic process	0.00017	GO:0010206	**photosystem II repair**	0.1128
GO:0009686	**gibberellin biosynthetic process**	0.00117	GO:0010584	pollen exine formation	0.12074
GO:0000913	preprophase band assembly	0.00173	GO:0009693	**ethylene biosynthetic process**	0.12686
GO:0080022	primary root development	0.00492	GO:0042255	ribosome assembly	0.1286
GO:0006885	regulation of pH	0.0052	GO:0006629	lipid metabolic process	0.1731
GO:0009765	**photosynthesis, light harvesting**	0.00572	GO:0009734	**auxin-activated signaling pathway**	0.18009
GO:0019752	carboxylic acid metabolic process	0.00837	GO:0050790	regulation of catalytic activity	0.18688
GO:0009306	protein secretion	0.00885	GO:0010027	thylakoid membrane organization	0.19068
GO:0030865	cortical cytoskeleton organization	0.01279	GO:0006412	translation	0.20299
GO:0006805	xenobiotic metabolic process	0.01484	GO:0006108	malate metabolic process	0.23243
GO:0018298	protein-chromophore linkage	0.01957	GO:0009737	**response to abscisic acid**	0.25805
GO:0050994	regulation of lipid catabolic process	0.01974	GO:0031047	**gene silencing by RNA**	0.26505
GO:0000272	polysaccharide catabolic process	0.02231	GO:0009826	unidimensional cell growth	0.27685
GO:0006813	potassium ion transport	0.02838	GO:0006457	**protein folding**	0.30844
GO:0006065	UDP-glucuronate biosynthetic process	0.0314	GO:0051258	protein polymerization	0.31516
GO:0042542	**response to hydrogen peroxide**	0.03374	GO:0009636	response to toxic substance	0.31721
GO:0006751	glutathione catabolic process	0.04196	GO:0045454	cell redox homeostasis	0.33334
GO:0009845	seed germination	0.04261	GO:0009116	nucleoside metabolic process	0.37104
GO:0009698	phenylpropanoid metabolic process	0.0454	GO:0007017	microtubule-based process	0.37654
GO:0010029	regulation of seed germination	0.04746	GO:0006334	nucleosome assembly	0.38229
GO:0005987	sucrose catabolic process	0.04863	GO:0072593	**reactive oxygen species metabolic process**	0.39636
GO:0048510	regulation of timing of transition from vegetative to reproductive phase	0.05619	GO:0006754	ATP biosynthetic process	0.44569
GO:0009800	cinnamic acid biosynthetic process	0.06275	GO:0010150	leaf senescence	0.44625
GO:0005986	sucrose biosynthetic process	0.06835	GO:0006099	tricarboxylic acid cycle	0.48278
GO:0016036	cellular response to phosphate starvation	0.08375	GO:0044237	cellular metabolic process	0.48504
GO:0000226	microtubule cytoskeleton organization	0.08822			

Table AIII.6 *List of enriched GO-BPs (FRD < 0.05) from heat-primed Cymodocea nodosa. GO-BPs relate to thermal stress response are in* ***bold****.*

GO ID	GO - Biological process	FDR
GO:0052865	1-deoxy-D-xylulose 5-phosphate biosynthesis	1.50E-16
GO:0009228	thiamine biosynthetic process	6.70E-13
GO:0009800	cinnamic acid biosynthetic process	9.10E-11
GO:0006559	L-phenylalanine catabolic process	6.30E-10
GO:0016114	terpenoid biosynthetic process	1.60E-08
GO:0006885	regulation of pH	1.40E-05
GO:0042026	**protein refolding**	4.80E-05
GO:0009698	phenylpropanoid metabolic process	0.00015
GO:0006813	potassium ion transport	0.0002
GO:0009094	L-phenylalanine biosynthetic process	0.00024
GO:0010584	pollen exine formation	0.00144
GO:0045893	**positive regulation of transcription, DNA**	0.00561
GO:0006730	one-carbon metabolic process	0.0063
GO:0006754	ATP biosynthetic process	0.02672
GO:0070483	detection of hypoxia	0.02801
GO:0010223	secondary shoot formation	0.03344
GO:0009641	shade avoidance	0.038

Chapter IV – Divergent warming responses among different seagrass species

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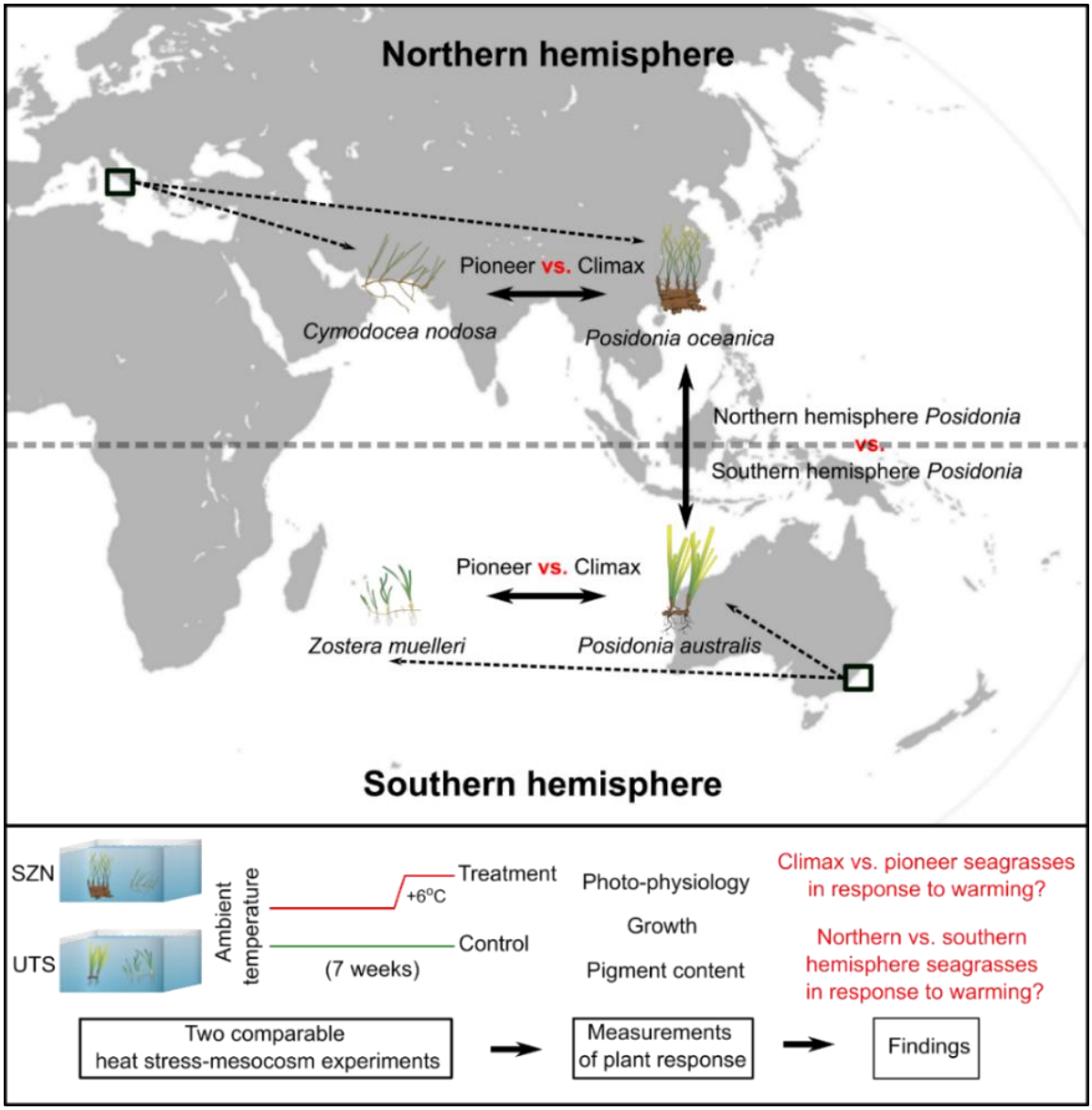


Figure 4.1 *Conceptual diagram illustrating the experiment presented in this chapter. Photo-physiology and morphology reveal divergent warming responses in northern and southern hemisphere seagrasses. Symbols were taken from the IAN symbol libraries, available at https://ian.umces.edu/media-library/. Maps were taken from SimpleMappr, available at https://www.simplemappr.net/.*

4.1 Introduction

4.1.1 Inter- and intra-specific differences in response to warming in plants

Previous studies have shown that the capacity to cope with warming varies among different seagrass species (Marín-Guirao *et al.*, 2016; Collier *et al.*, 2017), but also among populations of the same species from contrasting thermal environments (e.g. see Bergmann *et al.*, 2010; Winters *et al.*, 2011; Marín-Guirao *et al.*, 2018). However, to date, the tolerance to anomalous thermal events of the majority of seagrasses (especially in the region of southeast Asia and northern Australia, a hotspot of seagrass diversity) is yet to be investigated (see section 6.2.1). Indeed, to the best of my knowledge, no study has compared the responses of northern versus southern hemisphere seagrasses to warming.

Along the ecological succession, plants can be divided into pioneer species (i.e. fast-growing, often with small body size and annual) and climax species (i.e. slow-growing, long-lived, often with large body size and perennial), with different biological characteristics and ecological roles (Glenn-Lewin, Peet and Veblen, 1992). Likewise, some seagrass species can be classified as climax (e.g. *Posidonia oceanica*, *P. australis*, *Zostera marina*, *Thalassia testudinum*) while others as pioneer (e.g. *Cymodocea nodosa*, *Z. muelleri*). The contrasting characteristics between the two groups underpin large variations in the number and type of ecosystem services they provide. In seagrasses, most of their ecological services (e.g. sediment stabilization, nursery habitat, and blue carbon burial, etc.) depend upon their physical structure and primary productivity and, hence, climax seagrasses are considered more ecologically valuable than pioneer ones.

Studies from terrestrial plants have documented dissimilarities in response to environmental stressors between climax versus pioneer plants. For instance, studies from the Brazilian Atlantic Forest showed that pioneer trees were more tolerant against oxidative stress than climax plants (Favaretto *et al.*, 2011; Brandão *et al.*, 2017; Esposito *et al.*, 2018). In line with these studies from the southern hemisphere, a study from the Mediterranean region experimentally tested the responses to carbon assimilation under summer stress conditions (water deficits, high light, and temperature) in four Mediterranean trees, including climax and pioneer species (Faria *et al.*, 1998). This study indicated that, although both groups of trees suffered a decline in their photosynthetic capacities, the climax plants exhibited the lowest photosynthetic rates and the highest proportion of carotenoids to chlorophyll (i.e. an indicator of photo-protective mechanism activated under stressful conditions) while pioneer species maintained higher photosynthetic rates (Faria *et al.*, 1998). Hence, environmental stressors can

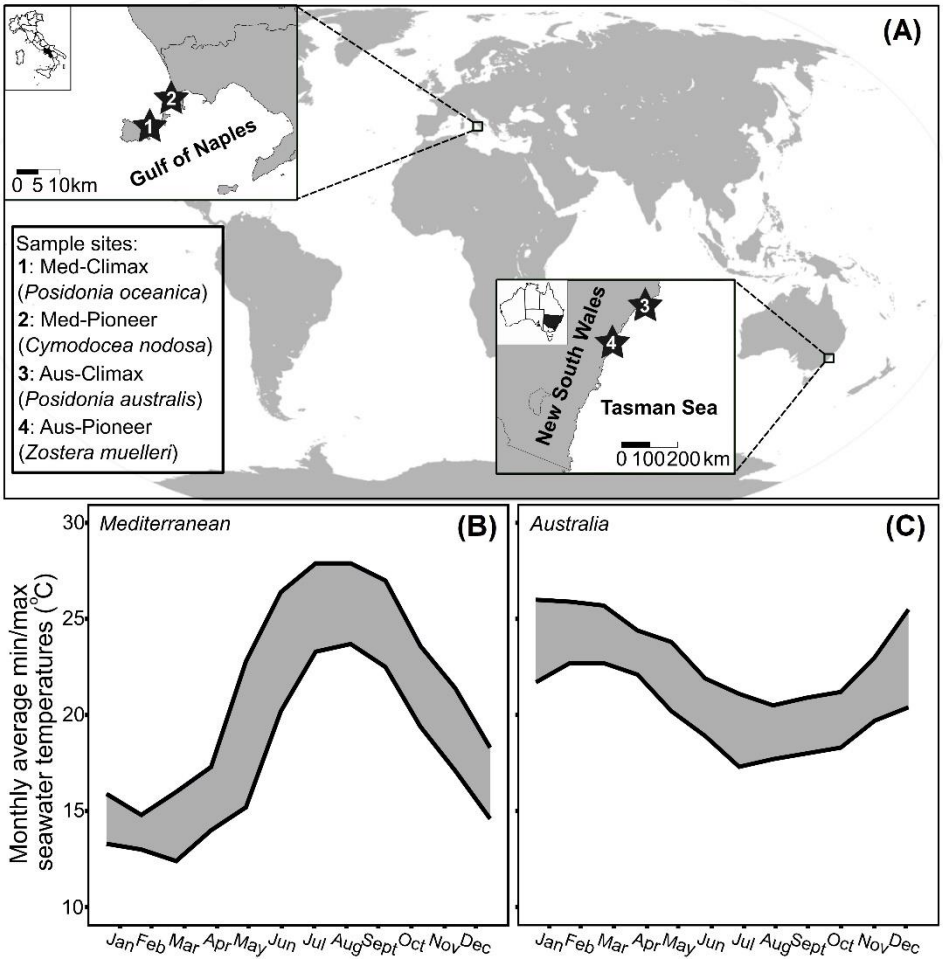
impact more strongly climax species, favoring the persistence of less complex and stable ecosystems, and providing less valuable ecosystem services. This is true also for seagrasses (Johnson *et al.*, 2003; Hyndes *et al.*, 2016; Shields, Parrish and Moore, 2019), where it appears essential to assess the response to stress of both climax and pioneer species to support timely and effective conservation and/or restoration actions. Few studies have experimentally compared the response of climax and pioneer seagrass species to warming (but see Masini and Manning, 1997; Seddon and Cheshire, 2001; Campbell, McKenzie and Kerville, 2006; Collier and Waycott, 2014; Marín-Guirao *et al.*, 2016, 2018; Collier *et al.*, 2017; Tutar *et al.*, 2017). Most of them suggested that pioneer species are more thermally tolerant than climax ones. These studies demonstrated that the fast-growing pioneer seagrasses exhibited a better ability to maintain unaltered plant carbon balances through improved photosynthetic thermal stability and performance as well as by inhibiting respiratory carbon consumption. Moreover, through a higher morphological plasticity, pioneer species can modify their plant architecture by increasing the above-ground (photosynthetic)/below-ground (non-photosynthetic) biomass ratio to deal with thermal stress (Collier *et al.*, 2017; Marín-Guirao *et al.*, 2018), and have also an increased ability to activate antioxidant defense mechanisms to protect themselves from heat-stress induced oxidative damages (Tutar *et al.*, 2017). Notwithstanding these evidences, the number of studies on this topic, especially on species with overlapping geographical distribution, remains scarce and deserves more effort.

4.1.2 The study

In this chapter, four seagrass species including *P. oceanica* and *C. nodosa* from the Mediterranean (northern hemisphere) and *P. australis* and *Z. muelleri* from South East Australia (southern hemisphere) were selected for a comparative study of their responses to warming. Plants were collected in the same seasonal conditions (i.e. late summer-early autumn: Mar-May in the southern hemisphere and Sept-Nov in the northern hemisphere) from both geographic areas and two mesocosm experiments were conducted following the same design. This study represents a unique opportunity to compare (1) two climax species of the genus *Posidonia* (*P. oceanica* and *P. australis*) with similar characteristics and ecological functions but distributed in the two hemispheres and (2) two couples of climax-pioneer species from both hemispheres (*P. oceanica* vs. *C. nodosa* and *P. australis* vs. *Z. muelleri*). On the first hand, it was hypothesized that the responses to warming of the two *Posidonia* species (i.e. *P. oceanica* and *P. australis*) would be different because sampled populations live under different thermal regimes (i.e. 13 – 28℃ for *P. oceanica*; **Fig. 4.2B** and 17 – 26℃ for *P. australis*; **Fig. 4.2C**)

and because their species thermal ranges are also different (i.e. 8 – 30℃ for *P. oceanica* and 12 – 28℃ for *P. australis*; **Fig. 1.10**). Additionally, it is noteworthy that the collection sites of the Australian seagrasses in this study did not fall into any Mediterranean-climate regions [see Cowling *et al.*, (1996) for a map of Mediterranean-climate regions and **Fig. 4.2A** for sample collection sites]. On the other hand, in both hemispheres, the climax seagrasses are expected to suffer more from thermal stress than their pioneer counterparts.

Figure 4.2 *Sample collection sites (**A**) and temperature conditions at collection sites (**B**, **C**).*



*(**B**) Monthly average sea surface temperature in Ischia, Italy (Mediterranean sites: **1** & **2**). (**C**) Monthly average sea temperature in Port Stephens, NSW, Australia (Australian sites: **3** & **4**). Data were taken from World sea temperature of 2020 (available at https://www.seatemperature.org/).*

4.2 Materials and Methods

4.2.1 Experiment 1: Northern hemisphere experiment

Plant collection

Plant fragments (i.e. ramets) of *P. oceanica* (40°44.020'N, 13°58.039'E at 5-6 m depth; **Fig. 4.2A-1**) and *C. nodosa* (40°47.021'N, 14°04.404'E at 8-10 m depth; **Fig. 4.2A-2**) were haphazardly collected by SCUBA diving in the Gulf of Naples (Italy) on the 18th September 2019. To reduce the likelihood of sampling the same genotype twice, plants were collected at a minimum distance of 10 m one from another. After collection, plants were kept in dark in a cooler filled with seawater at ambient temperature and transported to a benthic mesocosm facility at the Stazione Zoologica Anton Dohrn (SZN), Napoli, Italy [see Ruocco, Marín-Guirao and Procaccini (2019) for a detailed description of the experimental system]. Light intensity, salinity, and seawater temperature were measured at the time of plant sampling for setting up the experimental system. The two populations used in this study came from a similar thermal condition (i.e. 13-28℃, see **Fig. 4.2B**) which falls in the middle of the species thermal range (i.e.8-30℃ for both species, according to their species distribution ranges presented in **Fig. 1.10** and sea surface temperature data available at https://www.seatemperature.org/), therefore excluding the existence of a potential range-edge effect for the selected populations. Hereafter, I use Med-Climax for *P. oceanica* and Med-Pioneer for *C. nodosa*.

Experimental system

Once at the SZN experimental facility, twelve plant fragments (i.e. ramets) of each species composed of horizontal rhizomes of similar size and a similar number of interconnected vertical shoots (~ 10 shoots) were selected to standardize the experiment. Med-Climax ramets were transplanted in six plastic pots (i.e. two ramets per pot) filled with coarse carbonate sediments as described in Ruocco, Marín-Guirao and Procaccini (2019), while Med-Pioneer ramets were transplanted in twelve plastic pots (i.e. one ramet per pot) filled with natural sediments from the collection site as described in (Marín-Guirao *et al.*, 2018). After transplantation, pots of each species were randomly allocated into six 500L-aquaria with filtered and UV-treated natural seawater from a close area; each aquarium containing two ramets of the Med-Climax species and two ramets of the Med-Pioneer species. Transplant pots were distributed within aquaria to avoid crossed-species shading and their distance from the light source adjusted to reproduce similar light intensities to those measured at their collection sites (i.e. max noon irradiance: 300 and 200 μmol photons m^{-2} s^{-1} above the leaf canopy for

Med-Climax and Med-Pioneer, respectively). A 12h:12h light:dark photoperiod was applied, starting from 7:00., and progressively increasing to the maximum irradiance at 13:00 before a gradual reduction until dark at 19:00. Water temperature was measured automatically every 10 min using HOBO Pendant® Temperature/Light 64K Data Logger (Onset, USA) and manually checked twice a day with WTW Cond 3310 Set 1 (Xylem Analytics, Germany). Seawater salinity of 37.5±0.2 ppt was kept constant throughout the experiment through regular additions of purified water. Seawater quality was controlled via continuous mechanical filtration, weekly-UV sterilizations, and partial renewals.

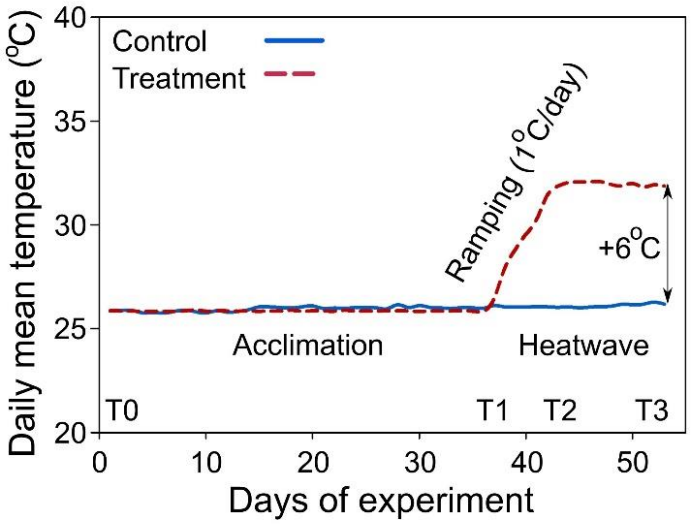


Figure 4.3 *Temperature profile during the two experiments.*

4.2.2 Experiment 2: Southern hemisphere experiment

Plant collection

Ramets of *P. australis* and *Z. muelleri* were collected, at distances > 25 m one from another to reduce the chance of sampling the same genotype twice. Plant fragments (i.e. ramets) were collected during low tides at ~70 cm depth at Port Stephens (PS), New South Wales (NSW), Australia (32°43'07.4"S 152°10'35.9"E; **Fig. 4.2A**) on the 19th of March 2019 and at Church Point (CP), NSW, Australia (33°38'46.8"S 151°17'11.9"E; **Fig. 4.2A**) on the 23rd of March 2019, respectively. Plant materials were brought to the seagrass mesocosm facility at the University of Technology Sydney (UTS) soon after collection. Light intensity, salinity, and seawater temperature were measured at the time of sample collection. Both studied populations experienced the highest temperature of 26℃ that is well below the upper limits of their species thermal ranges (i.e. 28℃ and 31℃ for *P. australis* and *Z. muelleri*, respectively, according to

their species distribution ranges presented in **Fig. 1.10** and sea surface temperature data available at https://www.seatemperature.org/). Therefore, I could preclude the potential existence of range-edge effects of the studied populations. However, it is worth noting that while the upper thermal limit of the *P. australis* population is 2℃ below its species' upper thermal limit (Fig. S1), the gap is 5℃ for the case of *Z. muelleri*. This may potentially result in different thermal tolerances between the two species. Hereafter, I use Aus-Climax for *P. australis* and Aus-Pioneer for *Z. muelleri*.

Experimental system

As soon as arrived at UTS, twelve ramets of each species with a similar number of shoots (i.e. 8-10 shoots) were selected and transplanted in individual plastic pots (i.e. one ramet per pot) filled with mini pebbles. Subsequently, pots were randomly distributed in tanks of the mesocosm facility: six 60-L aquaria for housing Aus-Climax pots and six 40-L aquaria for Aus-Pioneer pots (i.e. two ramets per aquarium). For both species, the irradiance level was set with a max. noon irradiance of 350 μmol photons m^{-2} s^{-1} at canopy height and a 12 h:12 h light:dark photo-period. Light cycle started from 7:30, with light levels progressively increasing to the maximum irradiance at 12:30. and kept for 2 hours, before a progressive reduction until dark at 7:30. Water temperature was measured automatically every 30 min using iButton data logger (iButtonLink, USA) and manually checked twice a day using a digital thermometer (FLUKE 52II, USA). During the experiment, purified water was added periodically to maintain constant seawater salinity (i.e. 34±0.2 ppt) similar to those in the fields. Approximately one third of seawater was renewed weekly in each aquarium to maintain water quality. See also **Figure AIV. 1** for a detailed explanation about the sampling regime and the experimental setup of both Experiment 1 and Experiment 2.

4.2.3 Experimental design

Both the northern and the southern hemisphere experiments shared the same experimental design. After transplantation and allocation within aquaria, plants of the four studied species were allowed to acclimate at 26℃, which is similar to the seawater temperatures recorded during plant collection at the four studied populations (i.e. in every case the difference was lower than 1℃). After a 5-week acclimation period, for each species. the temperature in half of the aquaria (i.e. three aquaria contained six plants) was progressively increased up to 32℃ at a heating rate of 1℃ day^{-1} to simulate a marine heatwave (MHW); whereas the temperature in the rest of the aquaria was maintained throughout the experiment (**Fig 4.3**). Therefore, for

each species, three tanks were randomly assigned to heat treatment (TM) and the other three remained as controls (CT). Seagrass responses were analyzed at the end of the MHW exposure, which lasted 12 and 10 days in the northern and southern hemisphere experiments, respectively. The aquarium is the true experimental unit for each seagrass species and variable so that measurements performed on plants of the same aquarium (i.e. '*pseudo* replicates') were averaged to obtain an independent replicated value. Therefore, the number of replicates used in statistical tests was $n = 3$.

4.2.4 Chlorophyll *a* fluorescence

Identical Diving-PAM fluorometers (Walz, Germany) were used to determine the photo-physiological responses of the four studied seagrass species (Med-Climax, Med-Pioneer, Aus-Climax, and Aus-Pioneer). Measurements included (*a*) maximum quantum yield of PSII (Fv/Fm), (*b*) Effective quantum yield of PSII (ΔF/Fm') and (c) non-photochemical quenching (NPQ) (a detailed explanation about the principle of PAM fluorometry as well as the information regarding the measurement of Fv/Fm, ΔF/Fm' and NPQ can be found in section 2.2.4). Fv/Fm was measured on night dark-adapted plants (around 6:00 - 7:00 before the light cycle started) while ΔF/Fm' was measured on light-adapted plants (around 12:30 - 13:30 while the irradiances were highest) and eventually NPQ was calculated by using the method described elsewhere (Maxwell and Johnson, 2000). To standardize the procedure, two chlorophyll *a* fluorescence measurements were conducted on the same middle portion of the second youngest leaf of each plant (Ruocco *et al.*, 2019). Selected leaves were marked to ensure the measurements of Fv/Fm and ΔF/Fm' were performed at the same place. Additionally, a dedicated underwater leaf clip was used to maintain a constant distance between the leaf and the fiber optic of the PAM fluorometer.

4.2.5 Plant growth

For both experiments, plant growth measurements were performed by adopting the leaf marking method (Zieman, 1974). Two plants from each aquarium and species were marked at the same position above the ligule at the end of the acclimation period and subsequently collected at the end of the heatwave to measure leaf elongation (mm). Then, the newly developed leaf segments were cleaned of epiphytes and dehydrated at 70°C for 24h before being weighted to assess biomass production (mg Dry weight).

4.2.6 Pigment content

At the end of the experiments, two plants of each species and from each aquarium were collected for the analysis of leaf pigment content. Approximately 50 mm of leaf tissue from the middle portion of the second youngest leaf of climax species (Med-Climax and Aus-Climax) and the whole second youngest leaf of pioneer species (Med-Pioneer and Aus-Pioneer) was used for the analysis. Epiphytes were immediately removed from the collected material, which was then kept on ice in darkness until further processing. Pigment extractions were done on the same day of sample collection. After weight measurements, samples were homogenized in liquid nitrogen by using pestles and mortars before being transferred into 1.5 mL tubes filled with 1 mL of 100% methanol. Thenceforward, samples were kept in complete darkness at 4°C for 8 hours before centrifugation. 200 μL of the extracted solution was used to determine the absorbance at 4 different wavelengths (i.e. 470, 652, 665, and 750 nm) by the mean of microplate readers (TECAN Infinite® M1000PRO, Switzerland) to calculate chlorophyll *a*, chlorophyll *b*, chlorophyll *a*+*b*, chlorophyll *b*/*a* molar ratio and total carotenoids. Pigments were calculated using equations from Wellburn (1994) after converting microplate readings into 1cm cuvette readings following Warren (2008). Finally, results were normalized to milligrams of fresh weight.

4.2.7 Statistical analysis

Three-way analysis of variance (Three-way ANOVA), also known as three-factor ANOVA, is used to determine whether there is a three-way interaction among independent variables (i.e. factors) on an outcome (i.e. dependent variable) (Underwood *et al.*, 1997). In this study, I wanted to examine whether there was an interaction among the three factors (i.e. hemisphere, life-strategy and treatment) or which factor was the main player in determining the response of seagrasses to warming (i.e. dependent variable: photo-physiology, growth and pigment). Therefore, the response of seagrasses to experimental conditions was assessed using a three-way *ANOVA* (n = 3), including the following factors: Hemisphere (2 levels: northern and southern, fixed), Life-strategy (2 levels: climax and pioneer, fixed), and Treatment (2 levels: control and treatment, fixed). Cochran's C test was used to test homogeneity of variances and data were square-root transformed when necessary. Data were analyzed even when homogeneity of variances could not be achieved, as *ANOVA* is robust for this kind of assumption when the sizes of samples are equal (Underwood *et al.*, 1997). However, in this case, the significance was judged more conservatively ($p < 0.01$) when interpreting results to reduce the livelihood of Type I error (which is inflated by heterogeneous variances). For each

measurement, Student-Newman-Keuls (SNK) *post-hoc* tests were used to identify significant differences between (*1*) control and treatment plants of each Hemisphere, each Life-strategy, (*2*) northern and southern plants of each Life-strategy, each Treatment, and (*3*) climax and pioneer plants of each Hemisphere, each Treatment. All statistical analyses were conducted in R-studio v.1.2.5033 (R Core Team, 2018) using package *GAD* (Sandrini-Neto and Camargo, 2014).

Graphs were made with R-studio using package *ggplot2* (Wickham, 2009).

4.3 Results

4.3.1 Photo-physiological response

Northern versus southern hemisphere seagrasses

Warming had strong impacts on the southern hemisphere seagrasses while did not result in any significant changes for the northern hemisphere plants (ANOVA: H×T, F (1,16) = 12.030, p < 0.01) (**Fig. 4.4**). Warming significantly reduced Fv/Fm of the Aus-Climax plants (SNK test for 'H:L:T' among 'T' within 'H:L': p < 0.001; **Fig. 4.4A**, **Table AIV.1**), while slightly impacting the Med-Climax plants. Similarly, the Aus-Climax plants enhanced their NPQ with warming (SNK test for 'H:L:T' among 'T' within 'H:L': p < 0.001; **Fig. 4.4C**, **Table AIV.1**) while Med-Climax's NPQ remained relatively unchanged (**Fig. 4.4C**). Both Aus-species dramatically lowered their ΔF/Fm' as a result of thermal stress (**Fig. 4.4B**, **Table 1**), while the ΔF/Fm' values of Med-plants were not negatively affected but, rather, slightly increased under warming in the case of the Med-Pioneer species (**Fig. 4.4B**, **Table 1**). As a consequence, I detected a significant interaction in H×L×T for ΔF/Fm' measurements (ANOVA: F (1,16) = 14.267, p < 0.01). It is important to highlight that while the control plants exhibited a similar level of performance, heated Climax plants from the two hemispheres responded differently and significant differences were detected from all photo-physiological measurements (SNK test for 'H:L:T' among 'H' within 'L:T': p < 0.001; **Table AIV.1**).

Climax versus pioneer seagrasses

The simulated MHW strongly impacted the photosynthetic performances (both Fv/Fm and ΔF/Fm') of Aus-Climax plants, however, the level of warming impacts were much lower in the Aus-Pioneer plants (**Fig. 4.4A**). Climax-pioneer dissimilarities in response to warming were also found in the activation of NPQ machinery. While Aus-Climax plants significantly

activated their NPQ machinery (**Fig. 4.4C**) as mentioned above, on the other hand, Aus-Pioneer plants did not alter their NPQ even at the same warming condition (**Fig. 4.4C**). This is also evidenced from the SNK results for 'H:L:T' among 'L' within 'H:T' when no significant differences detected for Aus-control plants but Aus-treatment plants (SNK test: $p < 0.001$; **Table AIV.1**).

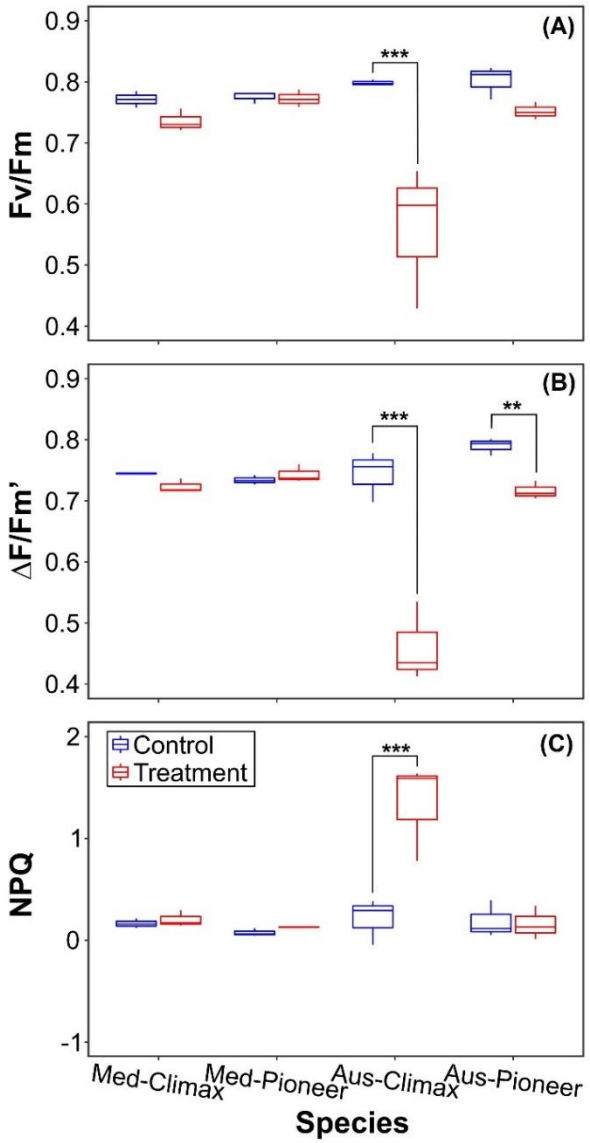


Figure 4.4 *Boxplots present photo-physiological results at the end of the experiment (n = 3). (**a**) Maximum quantum yield (Fv/Fm), (**b**) Effective quantum yield (ΔF/Fm'), and (**c**) Non-Photochemical quenching (NPQ). Asterisks indicate statistical differences between control and treatment within each species (Student-Newman-Keuls post-hoc test, ** p < 0.01, *** p < 0.001, more details can be found in Appendix IV, **Table AIV.1**).*

4.3.2 Plant growth response

Northern versus southern hemisphere seagrasses

There were differences in response to warming between northern versus southern hemisphere seagrasses in both biomass production and leaf elongation measurements (**Fig. 4**), as shown by the significant H×T interactions (ANOVA: F (1,16) = 14.532, $p < 0.01$ and F (1,16) = 10.151, $p < 0.01$, respectively). Among climax plants, warming significantly reduced biomass production (SNK test: $p < 0.05$; **Fig. 4.5A**) as well as leaf elongation (SNK test: $p < 0.01$; **Fig. 4.5B**) of the southern plants. On the other hand, warming favored the developments of the northern ones in terms of productivity (**Fig. 4.5**). Differently, warming increased the growth of northern pioneer plants (e.g. a significant difference between control versus treatment detected for biomass production, SNK test: $p < 0.05$; **Fig. 4.5A**). In contrast, the southern pioneer plants suffered a reduction in growth as a result of their exposure to a simulated MHW (**Fig. 4.5**).

Climax versus pioneer seagrasses

Even if no significant differences was detected between climax versus pioneer species within each hemisphere (ANOVA: L×T, $p > 0.05$ for both plant growth response measurements), it is interesting to note that there were significant interactions of H×L for both biomass production and leaf elongation (ANOVA: F (1,16) = 13.540, $p < 0.01$ and F (1,16) = 16.271, $p < 0.001$, respectively). For Med-seagrasses, even if warming generally enhanced the developments of both Med-Climax plants and Med-Pioneer plants, the levels of increments were significantly higher in Med-Pioneer plants in comparison with its climax counterpart (**Fig. 4.5**). Differently, Aus-Climax plants exhibited greater impact of warming when compared with their pioneer counterparts (**Fig. 4.5**) with significant differences between control versus treatment detected for both plant growth response measurements only for Aus-Climax plants (SNK test: $p < 0.05$ and $p < 0.01$; **Fig. 4.5**).

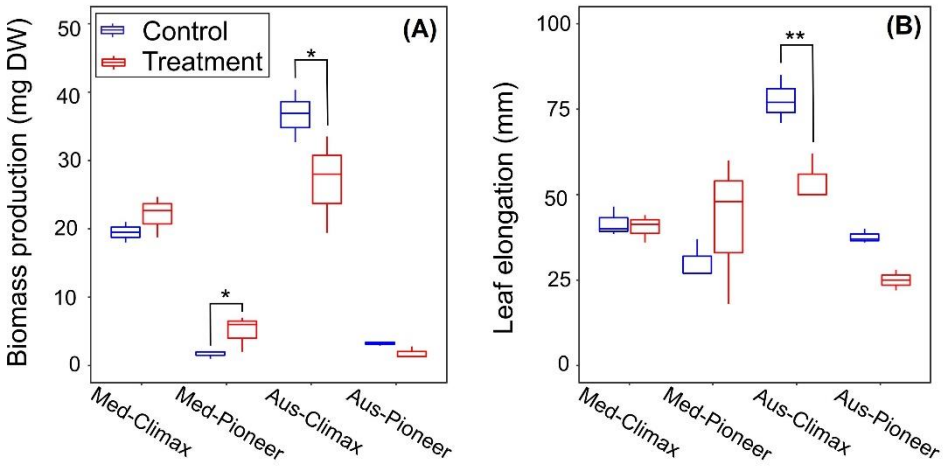


Figure 4.5 *Boxplots present plant growth response results at the end of the experiments (n = 3). Asterisks indicate statistical differences between control and treatment within each species (Student-Newman-Keuls post-hoc test, * p < 0.05, ** p < 0.01, more details can be found in Appendix IV,* ***Table AIV.1****).*

4.3.3 Pigment content response

Results from pigment content measurements showed complex interactions between northern versus southern as well as climax versus pioneer seagrasses in response to warming. Significant interactions were detected in H×L×T for all pigment measuring parameters (ANOVA, $p < 0.05$, **Table 4.1**). Details are presented below.

Northern versus southern hemisphere seagrasses

Warming significantly reduced all pigments content of Med-Climax plants such as Chl *a* (SNK test: $p < 0.01$, **Fig. 4.6A**), Chl *b* (SNK test: $p < 0.01$, **Fig. 4.6B**), Chl *a*+*b* (SNK test: $p < 0.01$, **Fig. 4.6C**), and total carotenoids (SNK test: $p < 0.01$, **Fig. 4.6D**) but did not result in any significant reduction in pigment content for Aus-Climax plants (except for the case of Chl *a*, although the level of reduction was greater in Med-Climax plants; **Fig. 4.6A,B,C,D**). Interestingly, while Med-Climax plants maintained their Chl *b/a* molar ratio during the simulated MHW, Aus-Climax plants significantly increased the ratio (SNK test: $p < 0.01$, **Fig. 4.6E**). It is worth mentioning that while Med-Pioneer plants accumulated more pigment content under the increased temperature, Aus-Pioneer plants reduced the accumulation of these pigments (see **Fig. 4.6A,B,C,D**). Likewise, Aus-Pioneer plants increased their Chl *b/a* molar ratio as a result of warming, while Med-Pioneer plants exposed to warming showed values similar to control plants (**Fig. 4.6E**).

Climax versus pioneer seagrasses

Warming greatly impacted the Med-Climax plants in terms of pigment contents including Chl *a*, Chl *b*, Chl *a*+*b* as well as total carotenoids with significant differences detected between control versus heated plants across all these measurements (**Fig. 4.6A,B,C,D**). On the contrary, warmed Med-Pioneer plants significantly improved pigment contents as a result of warming (**Fig. 4.6A,B,C,D**). Furthermore, a statistical difference was assessed between heated Med-Climax plants versus heated Med-Pioneer plants in terms of total carotenoids' response (SNK test for 'H:L:T' among 'L' within 'H:T': $p < 0.001$; **Table AIV.1**). For the southern hemisphere plants, warming negatively affected both climax and pioneer plants in terms of pigments (**Fig. 4.6**).

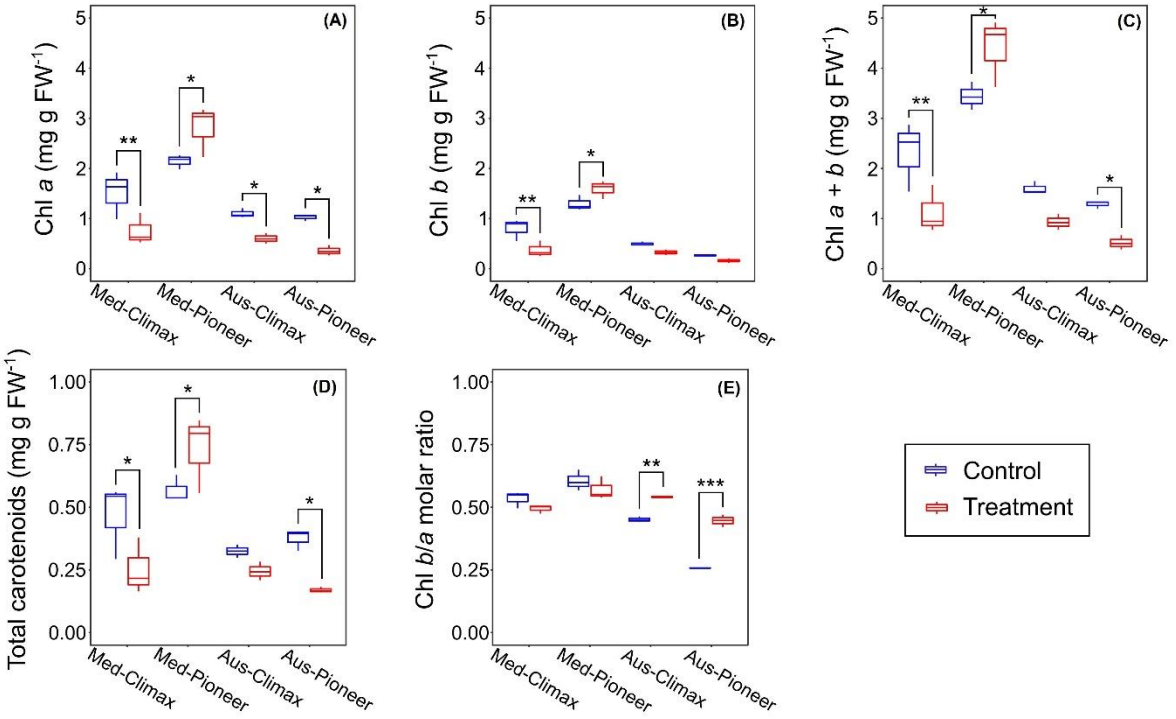


Figure 4.6 *Boxplots of pigment results at the end HW exposure (n = 3). Asterisks indicate statistical differences between control and treatment within each species (Student-Newman-Keuls post-hoc test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, more details can be found in Appendix IV,* ***Table AIV.1****).*

Table 4.1 *Results of three-way ANOVA analyses. Significant codes: *** p < 0.001; ** p < 0.01; * p < 0.05; ns p > 0.05; ϕ means 0.01 < p < 0.05 but not interpreted as significant because of variance heterogeneity in Cochran's C test; Sqrt: Square root.*

	Fv/Fm				**ΔF/Fm'**				**NPQ**				**Biomass production**				**Leaf elongation**			
Source of variation	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*
Hemisphere (H)	1	0.008	3.902	ns	1	0.021	24.429	***	1	0.340	15.795	**	1	1.183	7.162	*	1	596.670	7.381	ϕ
Life-strategy (L)	1	0.021	10.778	**	1	0.036	42.631	***	1	0.497	23.113	***	1	70.854	429.089	***	1	2327.230	28.790	***
Treatment (T)	1	0.040	20.650	***	1	0.051	60.076	***	1	0.180	8.377	*	1	0.015	0.091	ns	1	251.340	3.109	ns
H×L	1	0.009	4.629	ϕ	1	0.032	37.824	***	1	0.185	8.620	**	1	2.236	13.540	**	1	1315.230	16.271	***
H×T	1	0.023	12.030	**	1	0.045	52.699	***	1	0.068	3.174	ns	1	2.398	14.523	**	1	820.560	10.151	**
L×T	1	0.018	9.423	**	1	0.022	25.552	***	1	0.139	6.442	*	1	0.395	2.394	ns	1	214.000	2.647	ns
H×L×T	1	0.009	4.669	ϕ	1	0.012	14.267	**	1	0.186	8.642	**	1	0.017	0.103	ns	1	1.340	0.017	ns
Residual	16	0.002			16	0.001			16	0.022			16	0.165			16	80.830		
Transformation		None				None				*Sqrt*				*Sqrt*				None		
Cochran's C test		$p < 0.001$				$p < 0.01$				$p > 0.05$				$p > 0.05$				$p < 0.001$		

	Chlorophyll *a*				**Chlorophyll *b***				**Chlorophyll *a+b***				**Total carotenoids**				**Chlorophyll *b/a* molar ratio**			
Source of variation	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*	df	MS	*F*	*p*
Hemisphere (H)	1	6.422	80.732	***	1	2.983	176.301	***	1	18.158	109.855	***	1	0.308	37.524	***	1	0.097	131.972	***
Life-strategy (L)	1	2.086	26.225	***	1	0.648	38.290	***	1	5.059	30.606	***	1	0.116	14.151	**	1	0.007	9.783	**
Treatment (T)	1	0.585	7.360	*	1	0.061	3.633	ns	1	1.026	6.209	*	1	0.041	5.054	*	1	0.016	21.394	***
H×L	1	3.395	42.674	***	1	1.680	99.275	***	1	9.850	59.591	***	1	0.138	16.805	***	1	0.072	97.368	***
H×T	1	0.432	5.424	*	1	0.008	0.464	ns	1	0.556	3.362	ns	1	0.021	2.595	ns	1	0.046	62.871	***
L×T	1	0.596	7.491	*	1	0.230	13.619	**	1	1.567	9.483	**	1	0.024	2.907	ns	1	0.004	5.737	*
H×L×T	1	0.945	11.884	**	1	0.157	9.305	**	1	1.874	11.339	**	1	0.095	11.544	**	1	0.003	4.561	*
Residual	16	0.080			16	0.017			16	0.165			16	0.008			16	0.001		
Transformation		None				None				None				None				None		
Cochran's C test		$p > 0.05$				$p > 0.05$				$p > 0.05$				$p > 0.05$				$p > 0.05$		

4.4 Discussion

4.4.1 Difference between northern versus southern hemisphere seagrasses in response to warming

When two sister species of the genus *Posidonia* were exposed to a similar simulated marine heatwave (i.e. 32℃), their photo-physiological and plant growth responses clearly demonstrated that the southern hemisphere species *P. australis* (i.e. Aus-Climax) is more sensitive to anomalous thermal events than the northern hemisphere species *P. oceanica* (i.e. Med-Climax). Warming dramatically affected the photosynthetic performance of *P. australis*, while the photosynthetic functioning of *P. oceanica* was unaffected. The impairment of the photosynthetic apparatus, reflected as a reduction in the maximum (i.e. Fv/Fm) and effective photochemical efficiency (i.e. ΔF/Fm'), is a common response in seagrasses subjected to thermal stress (e.g. see Winters *et al.*, 2011; Marín-Guirao *et al.*, 2016; Ruocco, Marín-Guirao and Procaccini, 2019; Nguyen, Yadav, *et al.*, 2020) for some recent studies] and evidenced a higher photosynthetic thermal sensitivity in *P. australis* with regard to *P. oceanica*. This was further supported by the fact that only *P. australis* activated the NPQ machinery, a well-known photo-protective mechanism in plants (including seagrasses) that mitigates the damaging effects of a heat-induced photosynthetic malfunction by dissipating excess energy as heat [e.g. see Ashraf and Harris (2013) for a review in terrestrial plants and Marín-Guirao *et al.*, 2016; Ontoria *et al.*, 2019 for some recent studies in seagrasses]. Moreover, only *P. australis* experienced a significant growth inhibition during the warming exposure. Reduction in plant growth is a major consequence of growing under stress conditions and is commonly associated with photosynthetic constrains under high temperatures and with the diversion of resources from growth to sustain a heat-stress response and to repair heat-induced damage (Wahid *et al.*, 2007; Bita and Gerats, 2013; York *et al.*, 2013; Collier *et al.*, 2017; Marín-Guirao *et al.*, 2018). Interestingly, while warming reduced the overall pigment content (i.e. Chl *a*, Chl *b* and carotenoids content) of *P. oceanica* plants, the same level of warming only reduced Chl *a* content in the southern hemisphere plants. This resulted in a significant Chl *b/a* molar ratio increment (i.e. a proxy of PSII antenna size), suggesting that *P. australis* attempted to counterbalance their heat-impaired photosynthetic performance by enhancing their light-harvesting efficiency.

Both *P. oceanica* and *P. australis* together with 7 other species including *P. sinuosa*, *P. angustifolia*, *P. coriacea*, *P. denhartogii*, *P. kirkmanii*, *P. ostenfeldii*, and *P. robertsoniae* belong to the genus *Posidonia* which is among the most primitive marine angiosperm genus

(den Hartog, 1970; Kuo and Cambridge, 1984). Interestingly, while *P. oceanica* is endemic to the Mediterranean, the other 8 species (including *P. australis*) occur exclusively in the subtropical and temperate Australian seas (Kuo and Cambridge, 1984). It is still unclear when the single Mediterranean species and the Australian congeneric counterparts diverged. (Phillips and Menez, 1988) suggested it could have happened during the late Eocene, about 40 million years ago (Mya), while (Les *et al.*, 2003) estimated a more recent separation of 16.7 ±12.3 Mya. A more recent study from (Aires *et al.*, 2011) predicted this divergence would have taken place much earlier in the ancient Tethys Sea (i.e. over 60 Mya). In any case, the disconnection of Mediterranean *Posidonia* with the Australian ones has allowed the two groups to evolve in two contrasting environmental and evolutionary conditions (i.e. Mediterranean Sea versus Australian Seas). Compared to the Australian Seas, the Mediterranean has undergone massive changes during its history (Bianchi, Carlo and Morri, 2017). Especially, due to anthropogenic climate change, the Mediterranean Sea waters have warmed up at a faster pace (Bianchi, Carlo and Morri, 2017; Ozer *et al.*, 2017; Nguyen, Yadav, *et al.*, 2020), become saltier (Borghini *et al.*, 2014), and exhibited more frequent and intense extreme oceanic events [e.g. MHWs, see Darmaraki, Somot, Sevault, Nabat, *et al.*, (2019)]. In addition, not only the species but also the studied *P. oceanica* population thrives in a broader thermal regime (i.e. 13 – 28℃, **Fig. 4.2B**) than *P. australis* (i.e. 17 – 26℃, **Fig. 4.2C**); and this, together with the evolutionary differences among both *Posidonia* species stated above, may explain why the northern hemisphere *Posidonia* was less affected by warming than its southern hemisphere counterpart.

Regarding the pioneer seagrass species, this study also pinpoints some dissimilarities in the response to warming between *C. nodosa* (i.e. Med-Pioneer) and *Z. muelleri* (i.e. Aus-Pioneer). For example, warming significantly impacted the photosynthetic functioning of *Z. muelleri* (i.e. reduced ΔF/Fm' values) while no significant changes were detected for *C. nodosa*. Likewise, warming favored the growth and biomass production of *C. nodosa* but not for *Z. muelleri*, and similar divergences were also found in their responses at the level of photosynthetic pigments. These divergent responses to seawater warming manifested that the *C. nodosa* species, which is indeed benefited by increased temperatures, is more tolerant to anomalous heat events than the *Z. muelleri*. This finding suggests that the differences in response to warming among northern and southern hemisphere seagrasses may not be limited to the genus *Posidonia*, but extended to other seagrass species across hemispheres. However, since both pioneer species belong to a different family with contrasting origins and estimated ages [*Cymodoceaceae*: 67 Mya vs. *Zosteraceae*: 47Mya (Janssen and Bremer, 2004; Waycott *et al.*, 2007)], the

comparison is not as direct as in the two studied *Posidonia* species. Hence, further studies to compare the responses to warming of other seagrass species across the hemisphere are warranted.

4.4.2 Difference between climax versus pioneer seagrasses in response to warming

The northern hemisphere climax and pioneer species reacted almost in the same way to warming in terms of photo-physiology (i.e. no significant changes along with warming) and growth (i.e. greatly enhancements along with warming), whereas their responses differed in regard to pigment content modifications. The climax plants reduced all pigments (i.e. Chl *a*, Chl *b* and total carotenoids) during the warming exposure, while on the contrary, the pioneer plants increased the overall pigment content as a result of warming. These results indicated that, although both species came from the same thermal regime (both population and species), the climax seagrass was slightly impacted by the simulated marine heatwave while the pioneer species even benefited from the warming exposure. The differences in response to warming between climax and pioneer seagrasses became more obvious with photo-physiological results from the southern hemisphere experiment. *P. australis* plants experienced greater reductions in both Fv/Fm and ΔF/Fm' values compared with *Z. muelleri* plants (**Fig. 4.4A,B**). In addition, only the climax plants significantly increased their NPQ as a result of thermal stress. As explained in the previous section, these results are in line with findings from the northern hemisphere experiment on demonstrating that climax seagrasses are more prone to be adversely affected by warming than pioneer species. While results strongly support that the differences in response to warming between the two seagrass species in each hemisphere are due to the difference in their life strategies (i.e. climax vs. pioneer), I do acknowledge that there are some other factors potentially contributing to this outcome. First, at the species level, *P. australis* exhibits not only a narrower species thermal range (12-28℃) but also a lower upper thermal limit in respect to *Z. muelleri* (9-31℃) (see **Fig. 1.11**). Second, while *P. australis* is a completely subtidal species (Seddon and Cheshire, 2001), *Z. muelleri* survives in both subtidal and intertidal environments (Waycott *et al.*, 2004). Hence, *Z. muelleri* plants can be periodically exposed to air and extreme temperatures during low tides, which ultimately can increase the thermal tolerant level of the species. It has been shown that even the intertidal individuals of this species exhibit some characteristics that differ from the subtidal ones. They have a special leaf-pigment (i.e. dark red-brown) that is capable of enduring high light exposure (Waycott *et al.*, 2004) and smaller epidermal cells and air lacunae than do the subtidal individuals (Kuo, Ridge and Lewis, 1990). Another study on the seagrass *Zostera marina* also

demonstrated that intertidal plants were more photosynthetically active than subtidal ones, in [e.g. intertidal plants had higher carotenoid concentrations than subtidal plants to avoid photo damage during low-tide exposures or they had significantly higher NPQ than that of the subtidal ones (Park *et al.*, 2016)]. In this study, even if plants from both species were collected at the same depth during low tides (i.e 70 cm), I must aknowledge that *Z. muelleri* plants at the sampling site (i.e. Church Point, NSW, Australia) did experience lower tides and in some parts of the meadow, plants were even exposed to air (*My personal observation*). These factors can interact with the difference in life strategies to result in the different response to warming between *P. australis* and *Z. muelleri* observed in this study.

Differences between the response of climax versus pioneer species to environmental stressors have been previously documented in other seagrasses. For instance, Masini and Manning (1997) showed the pioneer seagrasses (i.e. *Amphibolis griffithii* and *A. antarctica*) were more resilient to changes in light and temperature when compared to two other climax seagrasses (i.e. *P. sinuosa* and *P. australis*) inhabiting in the same region of Western Australia. Similarly, the Mediterranean pioneer *C. nodosa* was also shown to be more thermally tolerant than the Mediterranean climax *P. oceanica* (Marín-Guirao *et al.*, 2018), but also to other abiotic stress factors including light (Olesen *et al.*, 2002) and salinity (Sandoval-Gil *et al.*, 2014), which seems to be related to their different levels of phenotypic plasticity (Pazzaglia, Reusch, *et al.*, 2021). Seddon and Cheshire (2001) also suggested that the climax *P. australis* is more vulnerable to desiccation in high-temperature conditions than the pioneer *A. antarctica*. All these evidences imply that warming can reshape the seagrass landscape by reducing the presence of climax species while enhancing the distribution of pioneer seagrasses. For instance, in Mission Bay, San Diego Bay, and Chesapeake Bay (USA), the climax seagrass *Z. marina* was replaced by the pioneer *Ruppia maritima* following extreme climatic events (Johnson *et al.*, 2003; Shields, Parrish and Moore, 2019). The same phenomenon is predicted to occur also in the Mediterranean, where ocean warming is expected to cause a decline of *P. oceanica* (Marbà and Duarte, 2010; Chefaoui, Duarte and Serrão, 2018) while favoring the expansion of some pioneer species [e.g. *C. nodosa, Halophila stipulacea* (Savva *et al.*, 2018; Winters *et al.*, 2020)]. Changes in seagrass meadow composition at the landscape scale would ultimately reduce their ecological value (Orth *et al.*, 2006; Lamb *et al.*, 2017; Unsworth, Nordlund and Cullen-Unsworth, 2019) and, hence, potentially affect the livelihoods of billions of people living in coastal areas (Bertelli and Unsworth, 2014). The replacement of climax seagrass

species, generally characterized by high biomass and productivity, by pioneer species will also decrease the capacity of seagrass meadows to mitigate the effects of carbon emissions (Gattuso *et al.*, 2018). Under some warming scenarios, the seagrass ecosystem may even switch metabolism from autotrophic to heterotrophic (Burkholz, Duarte and Garcias-Bonet, 2019), and enhance CO_2 and methane fluxes from the meadows into the atmosphere (Burkholz, Garcias-Bonet and Duarte, 2020), thus contributing to global warming.

Appendix IV

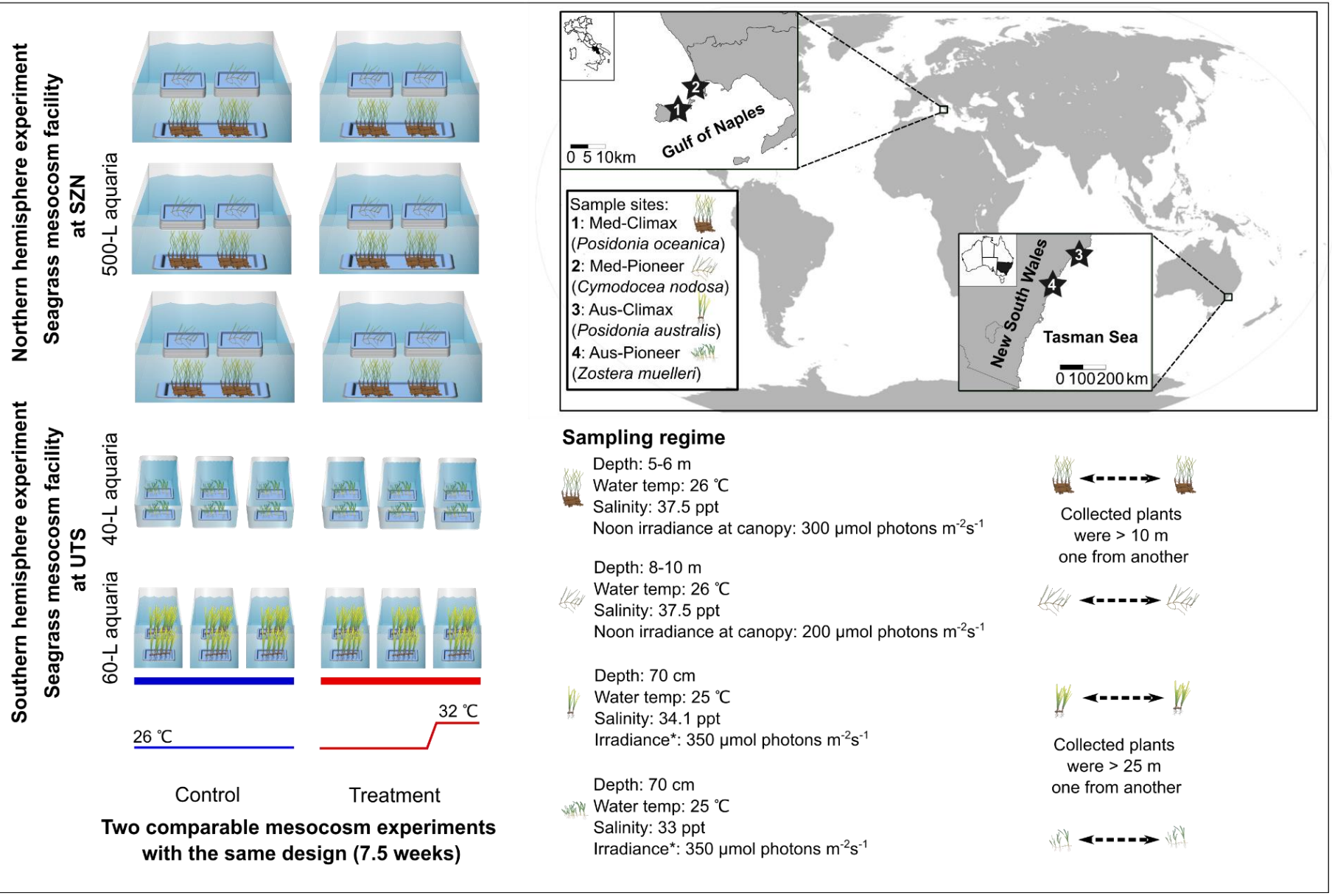

Northern hemisphere experiment
Seagrass mesocosm facility
at SZN
500-L aquaria
Southern hemisphere experiment
Seagrass mesocosm facility
at UTS
40-L aquaria
60-L aquaria
26 ℃
32 ℃
Control
Treatment
Two comparable mesocosm experiments
with the same design (7.5 weeks)
0 5 10km
Gulf of Naples
Sample sites:
1: Med-Climax
(Posidonia oceanica)
2: Med-Pioneer
(Cymodocea nodosa)
3: Aus-Climax
(Posidonia australis)
4: Aus-Pioneer
(Zostera muelleri)
New South Wales
Tasman Sea
0 100200 km
Sampling regime
Depth: 5-6 m
Water temp: 26 ℃
Salinity: 37.5 ppt
Noon irradiance at canopy: 300 µmol photons m-2s-1
Depth: 8-10 m
Water temp: 26 ℃
Salinity: 37.5 ppt
Noon irradiance at canopy: 200 µmol photons m-2s-1
Depth: 70 cm
Water temp: 25 ℃
Salinity: 34.1 ppt
Irradiance*: 350 µmol photons m-2s-1
Depth: 70 cm
Water temp: 25 ℃
Salinity: 33 ppt
Irradiance*: 350 µmol photons m-2s-1
Collected plants
were > 10 m
one from another
Collected plants
were > 25 m
one from another

Figure AIV.1 *Sampling regime and experimental setup of the northern hemisphere experiment (SZN) and the southern hemisphere experiment (UTS). Irradiance*: Saturating irradiance levels of plants in the field determined by rapid light curves using a PAM fluorometer. Experimental condition of the SZN experiment: Salinity: 37.5 ppt; 12h:12h light:dark period; Max. noon irradiance at canopy: 300 & 200 µmol photons* $m^{-2}s^{-1}$ *for P. oceanica and C. nodosa, respectively. Experimental condition of the UTS experiment: Salinity: 34 ppt; 12h:12h light:dark period; Max. noon irradiance at canopy: 350 µmol photons* $m^{-2}s^{-1}$ *for both P. australis and Z. muelleri.*

Table AIV.1 *Results of Student-Newman-Keuls (SNK) post-hoc tests. Significant codes: *** p < 0.001; ** p < 0.01; * p < 0.05; ns p > 0.05; Hemisphere (H), Life-trait (L) and Treatment (T).*

	Fv/Fm		**ΔF/Fm'**		**NPQ**		**Biomass production**		**Leaf elongation**	
SNK post-hoc tests										
for 'H:L:T' among 'T' within 'H:L'		Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer
	Med	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
	Aus	***	ns	***	**	***	ns	*	ns	**	ns
for 'H:L:T' among 'H' within 'L:T'		Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer
	Control	ns	ns	ns	*	ns	ns	***	ns	***	ns
	Treatment	***	ns	***	ns	***	ns	ns	*	ns	*
for 'H:L:T' among 'L' within 'H:T'		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
	Med	ns	ns	ns	ns	ns	ns	***	***	ns	ns
	Aus	ns	***	ns	***	ns	***	***	***	***	**

	Chlorophyll *a*		**Chlorophyll *b***		**Chlorophyll *a+b***		**Total carotenoids**		**Chlorophyll *b/a* molar ratio**	
SNK post-hoc tests										
for 'H:L:T' among 'T' within 'H:L'		Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer
	Med	**	*	**	*	**	*	*	*	ns	ns
	Aus	*	*	ns	ns	ns	*	ns	*	**	***
for 'H:L:T' among 'H' within 'L:T'		Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer	Climax	Pioneer
	Control	ns	***	*	***	*	***	ns	*	**	***
	Treatment	ns	***	ns	***	ns	***	ns	***	ns	***
for 'H:L:T' among 'L' within 'H:T'		Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment	Control	Treatment
	Med	*	***	***	***	**	***	ns	***	**	**
	Aus	ns	ns	*	ns	ns	ns	ns	ns	***	***

Chapter V – Signatures of local adaptation and micro-evolution in the seagrass *Posidonia oceanica*

The work presented in this chapter has been included in the following manuscript:

Hung Manh Nguyen, Miriam Ruocco, Emanuela Dattolo, Agostino Tomasello, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Signature of local adaptation to extreme environmental conditions in the Mediterranean seagrass *Posidonia oceanica*. *In preparation* targeting to Frontiers in Ecology and Evolution.

Special thanks

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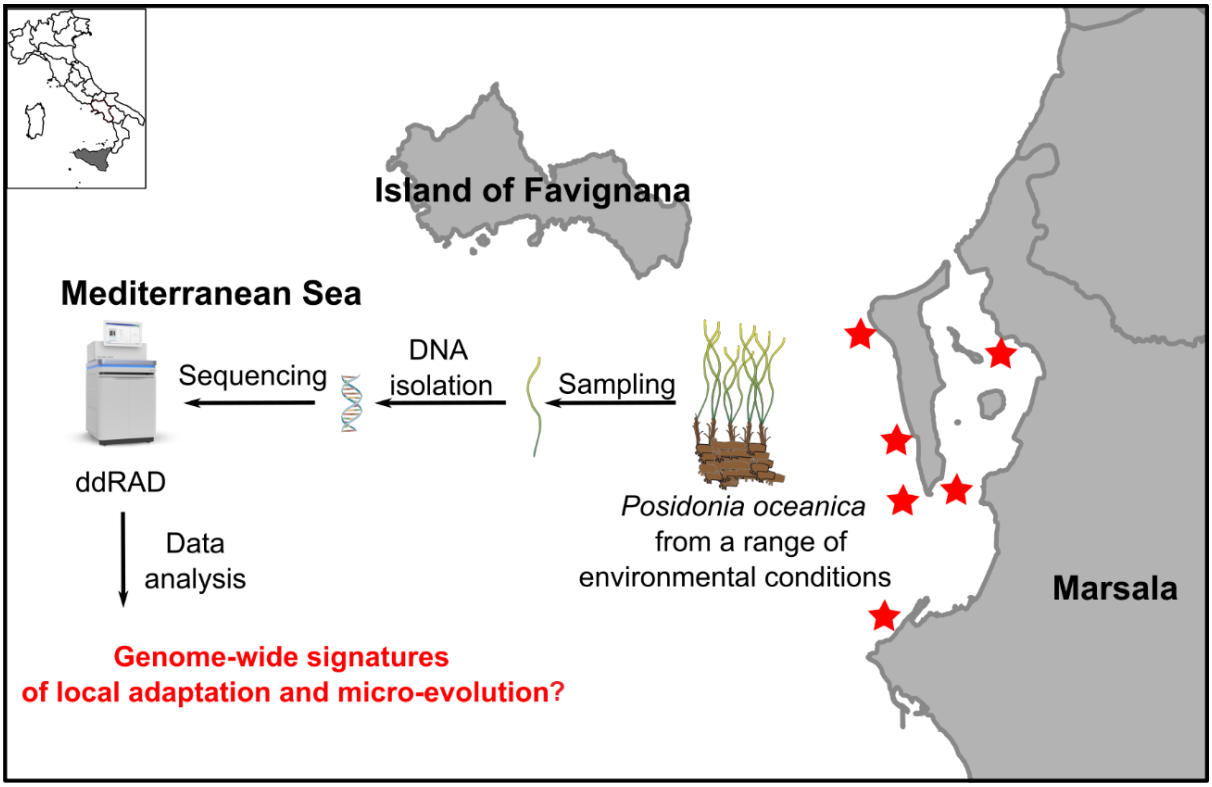

Figure 5.1 *Conceptual diagram illustrating the experiment presented in this chapter. Thermal-driven micro-evolution in seagrasses. Symbols were taken from the IAN symbol libraries, available at https://ian.umces.edu/media-library/. Maps were taken from SimpleMappr, available at https://www.simplemappr.net/.*

5.1 Introduction

5.1.1 Local adaptation in seagrasses

Local populations, if locally adapted, tend to exhibit traits that provide an advantage under local environmental conditions, therefore, having on average a higher relative fitness than populations originating from other habitats (Kawecki and Ebert, 2004). This phenomenon is commonly seen in a wide range of species across terrestrial (Jackrel and Wootton, 2014; Lascoux, Glémin and Savolainen, 2016; van Boheemen, Atwater and Hodgins, 2019) and marine environments (Barth *et al.*, 2017; van Oppen *et al.*, 2018; Cayuela *et al.*, 2020), including seagrasses (Hämmerli and Reusch, 2002; Blok, Olesen and Krause-Jensen, 2018; King *et al.*, 2018).

Several factors contribute to the establishment and the maintenance of local adaptation such as local selection, gene flow, mutation, genetic drift, migration, with the two formers appearing to be the main driving factors (Kawecki and Ebert, 2004; Lascoux, Glémin and Savolainen, 2016). In the past, it was commonly believed that local selection induces local adaptation while gene flow tends to erase it (Lascoux, Glémin and Savolainen, 2016). Recently, a growing body of evidence has demonstrated that gene flow instead can promote local adaptation and local adaptation can be maintained despite high gene flow (Tigano and Friesen, 2016).

In seagrasses, evidence of adaptation to local environmental conditions have accumulated in several species concerning several abiotic factors [e.g. light (Dattolo *et al.*, 2017), water quality (Maxwell *et al.*, 2014), nutrients (Pazzaglia *et al.*, 2020), salinity (Tomasello *et al.*, 2009), warming (Marín-Guirao *et al.*, 2018), among others] over a range of spatial scales [e.g. between sites of the same region (Maxwell *et al.*, 2014), between regions (Tuya *et al.*, 2019), along with depth gradients (Dattolo *et al.*, 2017), along latitudinal gradients (Jahnke *et al.*, 2019), and between seas (Nguyen, Yadav, *et al.*, 2020)]. Seagrass populations thriving in environmentally fluctuating locations are generally more capable to endure stress than those living in more stable environments (Hämmerli and Reusch, 2002; Blok, Olesen and Krause-Jensen, 2018; King *et al.*, 2018; Pazzaglia, Reusch, *et al.*, 2021). These populations could, therefore, provide material for assisting the evolution of natural populations and for improving seagrass restoration activities in order to secure a sustainable future for seagrasses and their associated ecosystem services (Pazzaglia, Nguyen, *et al.*, 2021). Despite the vital role of local adaptation on seagrass biology, intraspecific variation between populations is often ignored or under-estimated when assessing the relationship between seagrass species with their surrounding

environment, as well as, when predicting potential changes in their future distribution (Hu *et al.*, 2021; Pazzaglia, Reusch, *et al.*, 2021). On top of that, knowledge on the genetic basis underling local adaptation in seagrasses remains poorly understood.

In 2006, a study was conducted on *Posidonia oceanica* populations in the area of Stagnone di Marsala, a semi-enclosed coastal lagoon along the western coasts of Sicily (Tomasello *et al.*, 2009). This particular lagoon represents a unique case where *P. oceanica* occurs under temperature and salinity conditions that exceed the theoretical thresholds of the species' tolerance (Tomasello *et al.*, 2009). By using 13 microsatellite markers for genotype screening together with lepidochronological analysis, Tomasello *et al.*, (2009) showed that *P. oceanica* populations in the inner-most area of the lagoon were genetically isolated in comparison with the meadows outside the lagoon. This study also suggested a possible selection of genotypes adapted to the persistent stressful conditions inside the lagoon. Nonetheless, to date, no information about the genetic mechanisms (e.g. the loci responsible for this local adaptation proving potentially adaptive advantages) are available.

Recently, the rapid development of next-generation sequencing (NGS) technologies together with the application of restriction enzyme digestion of genomic DNA have massively facilitated population genetic studies, especially in the fields of ecology and evolutionary ecology (Davey *et al.*, 2011; Ekblom and Galindo, 2011). These new approaches allow to produce thousands to millions of single nucleotide polymorphisms (SNPs) that can be used for subsequent analyses of population genetic structure at a very high resolution (Davey *et al.*, 2011). Double digest restriction-site-associated DNA sequencing (ddRAD) is a recently-developed methodology in which DNA is double-digested with restriction enzymes and the resulting fragments sequenced via NGS (Peterson *et al.*, 2012). The resulting NGS reads are computed across individuals for the detection of SNPs. ddRAD sequencing approach provides a relatively low-cost procedure with minimal starting material and especially requires no prior genomic knowledge (Peterson *et al.*, 2012) making this approach applicable to address a diversity of biological questions in a wide range of organisms, especially for those species whose genomes are not yet sequenced, as is the case of most seagrass species [except for *Zostera marina* (Olsen *et al.*, 2016) and *Z. muelleri* (Lee *et al.*, 2016)].

5.1.2 The study

In an era of rapid ocean warming, the populations of *P. oceanica* from the area of Stagnone di Marsala represent a natural experimental model system to study what might happen to

seagrasses in the future. Re-investigating the seagrass populations in the area after 15 years of being exposed to extreme environmental conditions using knowledge from a previous study (Tomasello *et al.*, 2009) and the application of a *state-of-the-art* approach in genetic research represents a very promising opportunity to better understand the genetic basis of local adaptation and micro-evolution that is possibly happening in the area. In Chapter V, I took this opportunity to broaden our understanding of the genetic basis of local adaptation in seagrasses. To this end, samples of *P. oceanica* were collected from two sites inside the lagoon and four surrounding sites outside the lagoon [that well corresponded with sampling localities in Tomasello *et al.*, (2009)]. Measurements included sea surface temperature, plant morphology and, especially, genome-wide screening using ddRAD approach for SNP identification and outlier detection. The selection of outliers could lead to the identification of selected traits in response to local environmental conditions.

5.2 Materials and Methods

5.2.1 The studied area

The Stagnone di Marsala lagoon is a shallow area with an average depth of 1.5 m and a surface area of about 2000 ha (Vizzini *et al.*, 2002). The lagoon can be sub-divided into a northern and a southern basin with different geomorphological and environmental characteristics. The northern basin has an average depth of 1.1 m and it is connected with the open sea through a channel of 400 m wide and 20–30 cm deep northwards (Sarà, Leonardi and Mazzola, 1999). This basin exhibits distinct lagoon features, such as limited water exchange and slow turnover, and has the highest annual variation in temperature and salinity among sites where the presence of *P. oceanica* has been reported [i.e. 10-30℃ and 33–48‰, respectively (Tomasello *et al.*, 2009)]. The northern channel opening the lagoon to the sea has been gradually blocked in recent years due to anthropogenic activities resulting in even higher variation in environmental conditions in the inner lagoon (Tomasello A., *personal communication*). In this basin, *P. oceanica* forms atoll-like structures (Tomasello *et al.*, 2009), a rare feature of *P. oceanica* meadows happening also in a few other localities along the Tunisian, Turkish and Corsican coasts [see Tomasello *et al.*, (2009) for related references]. For its part, the southern basin is slightly deeper (i.e. 2 m depth) and is connected with the surrounding open sea through a 3000 m wide opening (Tomasello *et al.*, 2009). In this area, annual water temperature ranges from 11.2 to 29.1℃ while salinity variates between 33 and 48‰ (Vizzini *et al.*, 2002). In this southern basin, *P. oceanica* forms a reef platform structure (Tomasello *et al.*, 2009). Lastly, the surrounding open sea is environmentally more stable with a year-round temperature ranging

from a minimum of 14.1℃ during winter to a maximum of 26.4℃ during summertime and a stable salinity level of 37‰ (Sarà, Leonardi and Mazzola, 1999). In this area, *P. oceanica* develops continuous and extensive meadows (Tomasello *et al.*, 2009).

5.2.2 Sample collection

On the 7th of September 2020, *P. oceanica* orthotropic shoots connected to their horizontal axis were haphazardly collected from six different sites (i.e. 20−30 from each site) including (***i***) two sites inside the Stagnone di Marsala lagoon [***Site 1*** (corresponds with Atolls site in Tomasello *et al.*, 2009), in the northern basin of the lagoon: samples were collected from 5 different atolls with an average of 4−6 samples per atoll (*atoll 1*: 37°52'54"N, 12°28'29"E; *atoll 2*: 37°52'49"N, 12°28'22"E; *atoll 3*: 37°52'54"N, 12°28'21"E; *atoll 4*: 37°52'55"N, 12°28'21"E; and *atoll 5*: 37°52'56"N, 12°28'19"E) & ***Site 2*** (corresponds with Récif site in Tomasello *et al.*, 2009), in the southern basin of the lagoon (37°50'35"N, 12°27'29"E)] and (***ii***) four sites outside the lagoon [***Site 3*** (corresponds with Plateau site in Tomasello *et al.*, 2009: 37°50'26"N, 12°26'45"E), ***Site 4*** (37°48'48"N, 12°25'53"E), ***Site 5*** (37°51'27"N, 12°26'35"E), and ***Site 6*** (37°53'18"N, 12°25'42"E)] (**Fig. 5.2**). Soon after collection, 96 leaf sub-samples (∼10 cm; 16 samples per site) were obtained for DNA extraction. Samples were gently cleaned out of epiphytes before being dried and stored with silica gel until further analysis. The rest of the collected material was kept in a cooler container filled with seawater and transported shortly to a laboratory for morphological measurements.

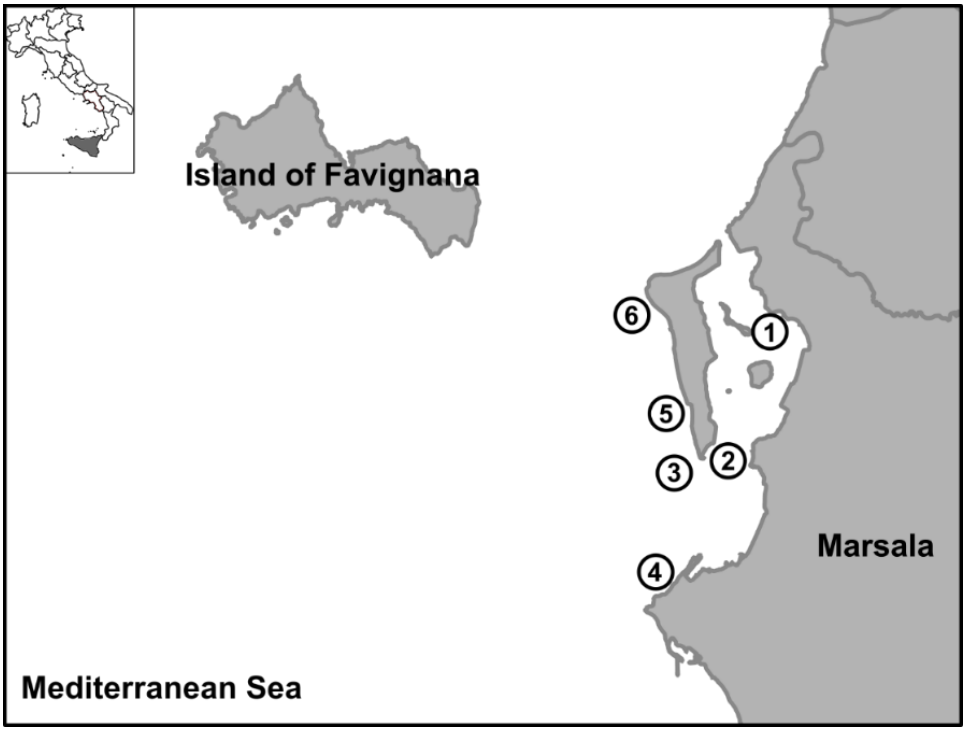

Figure 5.2 *Sample collection sites in this study.*

5.2.3 Sea surface temperature

SST data were obtained through image analysis based on satellite remote sensing data from the Sea and Land Surface Temperature Radiometer sensors installed on the Sentinel-3 mission satellites with a spatial resolution of 250 m (https://apps.sentinel-hub.com/). Data were collected from May to September for the years 2017 to 2020. Then, the data from the year 2017 was chosen because it contained the highest number of images. Selected images were analyzed using QGIS software (http://qgis.osgeo.org/) to obtain average and maximum temperatures during the May-September period for each studied site.

5.2.4 Morphological measurement

Morphological measurements were carried out on leaf bundles (Girard, 1977) with various categories defined by Giraud (1979). Measurements included leaf number per shoot, leaf length and shoot surface.

5.2.5 Statistical analysis

Prior to analysis, homogeneity of variance of the response variables was tested by Levene's test and Shapiro–Wilk test was used to validate data normality. As a result, data from shoot morphological measurements were normally distributed, however, with unequal variances. Therefore, One-way ANOVA was not appropriate for this case. Thus, I decided to use

Tamhane's T2 test [that is an all-pairs pairwise-t-test suitable for unequal variances (Tamhane, 1979)] to check for significant differences among sampling sites for shoot morphological measurements. Tamhane's T2 test was conducted in R-studio v.1.2.5033 (R Core Team, 2018).

5.2.6 DNA extractions, RAD-seq library preparation and sequencing

Total genomic DNA (gDNA) was isolated from about 30 mg of dried tissue using NucleoSpin® Plant II kit (Macherey-Nagel) by following the manufacturer's instructions. Total gDNA integrity was checked through 1% agarose gel electrophoresis and total gDNA purity was determined spectrophotometrically by examining 260/230 and 260/280 nm absorbance ratios using a NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific). Finally, DNA concentration was accurately measured by the Qubit dsDNA BR assay kit with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific).

Ninety-five ddRAD-seq library construction and sequencing were conducted at IGATech (Udine, Italy) using an IGATech custom protocol, with minor modifications with respect to Peterson's double digest restriction-site associated DNA preparation (Peterson *et al.*, 2012). In short, gDNA was double digested with both *Sph*I and *Mbo*I endonucleases (New England BioLabs). Fragmented DNA was purified with AMPureXP beads (Agencourt) and subsequently ligated with T4 DNA ligase (New England BioLabs). Samples were pooled on multiplexing batches and bead purified as before, and then they were size-selected and undergone several purification steps. RNA-seq libraries were sequenced with 150 cycles in paired end mode on NovaSeq 6000 instrument following the manufacturer's instructions (Illumina, San Diego, CA).

5.2.7 Single nucleotide polymorphism calling

Single nucleotide polymorphism (SNP) calling was performed using Stacks software package v2.53 (Catchen *et al.*, 2013). First, raw Illumina reads were demultiplexed using the *process_radtags* utility (Catchen *et al.*, 2013). The short-reads of each sample were assembled into exactly matching stacks using the *ustacks* utility (Catchen *et al.*, 2013). The creation of the loci catalog (i.e. a set of consensus loci from all the analyzed samples) was done using *cstacks* and matching each sample against the catalog using *sstacks* and *tsv2bam* utilities (Catchen *et al.*, 2013). *gstacks* ultility (Catchen et al. 2013) was used to pull in paired-end reads (if available), assemble the paired-end contigs and merge it with the single-end locus, align reads to the locus, and ultimately call SNPs. Finally, detected loci were filtered using the *populations* program included in Stacks v2.53 (Catchen et al. 2013) with option –R=0.75 in order to retain

only loci that are represented in at least the 75% of the whole metapopulation and with cutoff --*max-obs-het*=0.8 in order to process a nucleotide site at a locus with an observed heterozygosity at maximum of 80%.

5.2.8 Genetic variation analysis and clonality assessment

Individual genetic variation and population differentiation was assessed by a Principal Component Analysis (PCA) using the R packages, *gdsfmt* and *SNPRelate* (Zheng *et al.*, 2012) and by using ADMIXTURE 1.3.0 (Alexander and Lange, 2011). To choose the best estimate of the number of clusters (K), the ADMIXTURE's cross-validation procedure was used with default settings. The hypothetical number of K was set from 1 to 15 then the K value with the lowest cross-validation error was chosen to use for ADMIXTURE analysis. Pairwise genetic distances (Fst) across studied sites were calculated following a standard ANOVA as in Weir and Cockerham (1984) by using VCFtools (Danecek *et al.*, 2011). Number of distinct multilocus genotypes (MLGs) for each site was determined using the R package *poppr* (Kamvar, Brooks and Grünwald, 2015). Genetic distance limit for setting delimitation of clones was determined based on genetic distance detected between technical replicates (i.e. real "clones"). Inbreeding coefficient (F_{is}) was calculated using the R package *genepop* (Rousset, 2008) following standard ANOVA as in Weir and Cockerham (1984).

5.2.9 Outlier SNPs identification and functional annotation

Three genome scan methods were used to identify outlier SNPs. The first method was based on Fst values and implemented in the program *BAYESCAN* v.2.1 (Foll and Gaggiotti, 2008; Foll, 2012). Bayescan estimates Fst for each SNP locus to perform a genomic scan for outlier Fst values through a Bayesian method. It was used with prior odds set to 100 and using a threshold of q=0.5. The second method was also based on Fst values and implemented in the R package *OutFLANK* (Whitlock and Lotterhos, 2015). *OutFLANK* analysis was performed using default settings and SNPs with a p-value less than 0.01 were considered as 'suggestive' outliers [as done in a previous study (Andrew *et al.*, 2018)]. The last method based on multivariate analysis and implemented the R package *pcadapt* (Luu, Bazin and Blum, 2017) were used with default settings [that computed a test statistic based on Mahalanobis distance which is a multi-dimensional approach that measures how distant is a point from the mean (Luu, Bazin and Blum, 2017)]. To define the correct number of principle components (PCs) to use in *pcadapt* analysis, I started with K = 20 PCs then K = 3 was selected as the most appropriate value for the analysis based on an inspection of a scree plot (Luu, Bazin and Blum,

2017). In the last step, any SNP with a *p*-value less than 0.01 with Bonferroni correction was considered as an outlier SNP.

To reduce the likelihood of detecting false positives, a Venn diagram (available at http://bioinformatics.psb.ugent.be/webtools/Venn/) was used to identify shared and unique outliers detected from different methods. Only SNPs that were identified as outliers by at least two methods were considered as 'true' outliers and were used for subsequent analysis. Other SNPs (either detected as outliers by only one of the three methods or not detected as outliers by neither of the methods) were classified as neutral SNPs. Allelic frequencies of true outliers were determined using the R package *genepop* (Rousset, 2008) using default settings.

To determine whether an outlier SNP may represent any potential coding sequences, chromosomes regions of the true outlier SNPs were mapped against *P. oceanica* transcriptome data from MAGI (Marine Angiosperms Genome Initiative) project (JGI, USA, unpublished data) by using the BLASTn algorithm (Camacho *et al.*, 2009). Positive hits were identified if a homologous sequence around a SNP position with a high scoring stretches of sequence similarity of at least 70 bp with a percentage of identity greater than 85%. Subsequently, sequence similarity search was carried out between *P. oceanica* contigs (i.e. corresponding to the positive hits) against NCBI protein database using the BLASTx software (Camacho *et al.*, 2009) with an e-value cutoff of $1e^{-3}$ to identify potential protein functions corresponding to outlier SNPs.

5.3 Results

5.3.1 Sea surface temperature

Seawater temperature inside the lagoon was higher in comparison with the outside lagoon area (**Fig. 5.3**). In particular, average SST of Site 1 and Site 2 were 8℃ and 4℃ higher, respectively, than outside lagoon sites (**Fig. 5.3**). Similarly, while and maximum SST of Site 1 was 31.1℃ and Site 2 was 28.7℃, the maximum SST of the outside lagoon sites varied from 23.9−26.1℃. In addition, while temperature variation among the four outside lagoon sites was less than 2℃ (e.g. the average SSTs varied from 20.7−22.3℃ and the maximum SSTs varied from 23.9−26.1℃; **Fig. 5.3**), both average SST and maximum SST of Site 1 were 4.5℃ higher than those of Site 2 (**Fig. 5.3**).

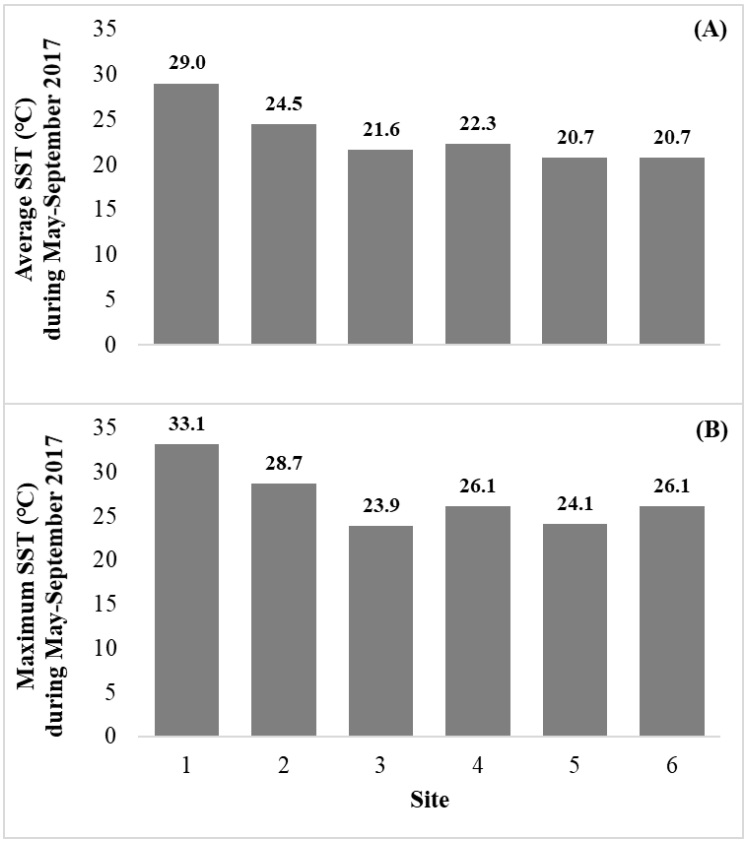

Figure 5.3 *Average and maximum SSTs at the studied sites during the period May-September 2017.*

5.3.2 Morphological results

There were significant differences among the studied sites for all morphological measurements (Tamhane's T2, $p < 0.05$**, Fig. 5.4**), being plants from Site 1 clearly different from plants from the rest of studied sites. In detail, the plants from Site 1 had on average three leaves per shoot, being significantly lower than the average number of leaves of plants from the other sites (i.e. ~ 5 leaves per shoot; **Fig. 5.4**). Similarly, plants from Site 1 had shooter leaves when compared with plants from the other sampling sites (Tamhane's T2 test, $p < 0.05$; **Fig. 5.4**) and in consequence, the shoot surface area at Site 1 was significantly lower than the surface area of plants from all other sites (**Fig. 5.4**). In particular, the shoot size of plants from Site 1 was 51% lower than the size of plants from inner Site 2 and 58-67% than plants from outside the lagoon (**Fig. 5.4**).

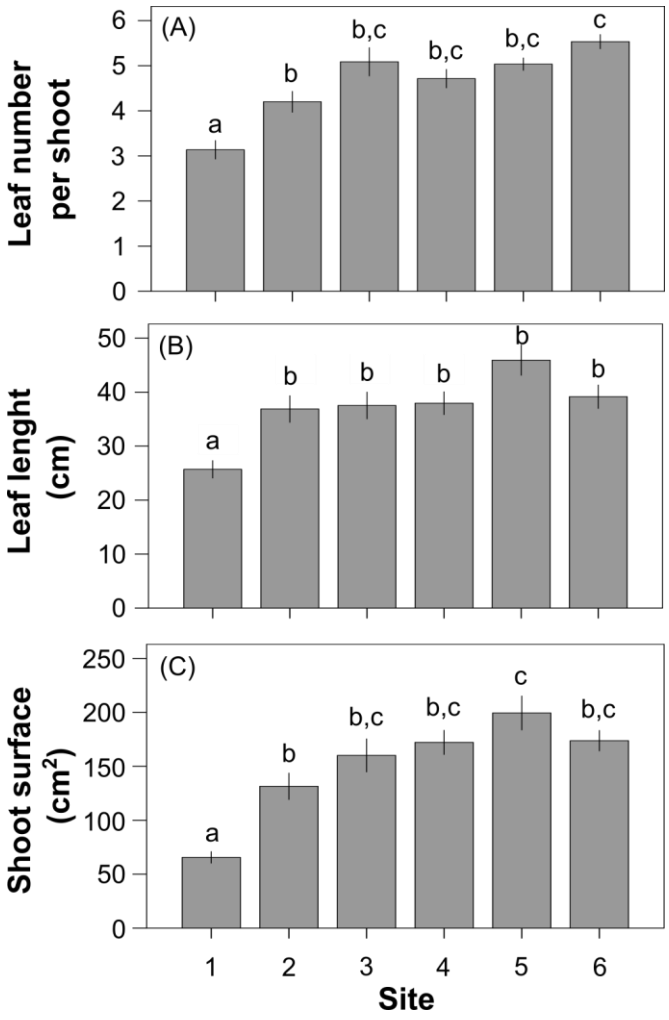

Figure 5.4 *Leaf morphological results. Data are mean ±SE. Letters over the bars indicate results of Tamhane's T2 test (p <0.05).*

5.3.3 Seagrass genetic differentiation among sites

The sequencing of RAD-seq libraries produced a total of 442,837,278 reads (i.e. ~4.7 million reads per sample). Subsequently, a total of 51,329 SNPs were identified across 95 *P. oceanica* samples.

PCA results showed a strong genetic differentiation of *P. oceanica* between (***i***) the two inside-lagoon sites (Site 1 & Site 2; **Fig. 5.5**) versus the four outside-lagoon sites (Site 3−6; **Fig. 5.5**) and (***ii***) between those from the inside lagoon (Site 1 versus Site 2). In detail, samples from Site 1 clearly separated from all samples of the other sites along the PC1 that explains 11.1% of the total variance of the data set. Moreover, most samples of Site 1 were grouped very compactly (**Fig. 5.5**). Interestingly, samples of Site 2 were divided into two distinct groups,

one group differentiated with all other samples along the PC2 (that accounts for 9% of the total variance) while the other group clustered with samples from Site 5, Site 4 and Site 6 (**Fig. 5.5**). Interestingly, even Site 1, Site 2 and Site 3 were geographically neighbouring sampling sites, PCA results showed no overlapping among plants from the three sites (**Fig. 5.5**).

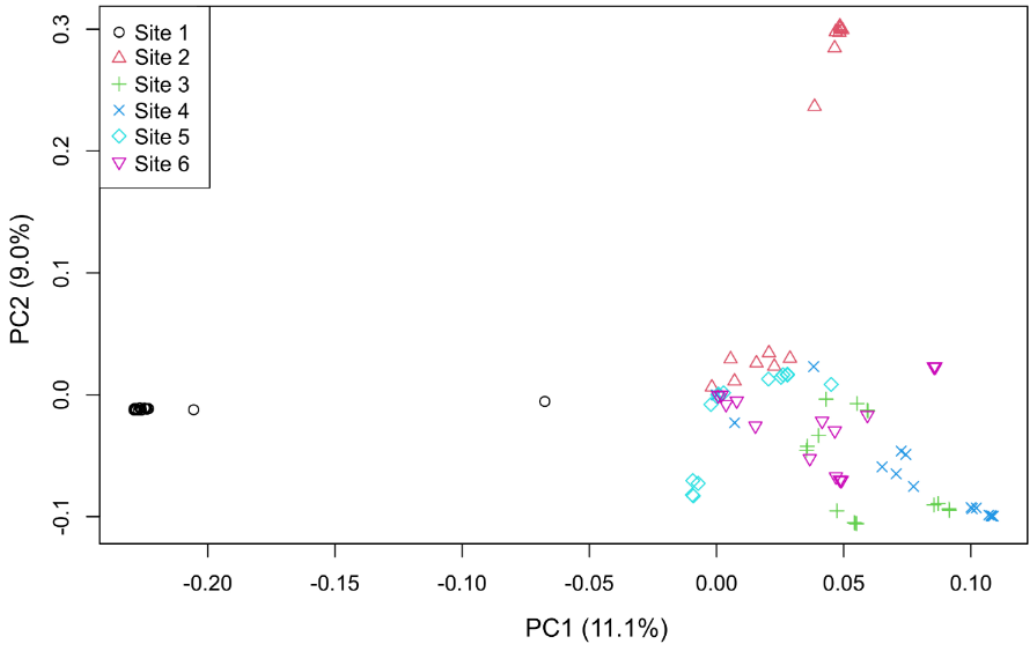

Figure 5.5 *PCA results based on all SNPs.*

Genetic partitioning among sites was further confirmed by results from ADMIXTURE analysis (**Fig. 5.6**). First, K=9 was identified as an 'optimal K' as it had the lowest cross-validation error of 0.177 among other K values (**Table AV.1**). Then, with the assumption of nine genetic clusters (K = 9), the clustering analysis implemented in ADMIXTURE showed clear divergences in genetic structures among sites (**Fig. 5.6**). No substructure was detected at Site 1 as this site was dominated by a single component (i.e. the red colour in **Fig. 5.6**). Especially, this structural component was also present, however in small proportion in all other sites (**Fig. 5.6**). On the other hand, all the other sites were characterized by diversified substructures (e.g. 8−9 components). It is important to note that the dominant substructure differed among these sites (**Fig. 5.6**).

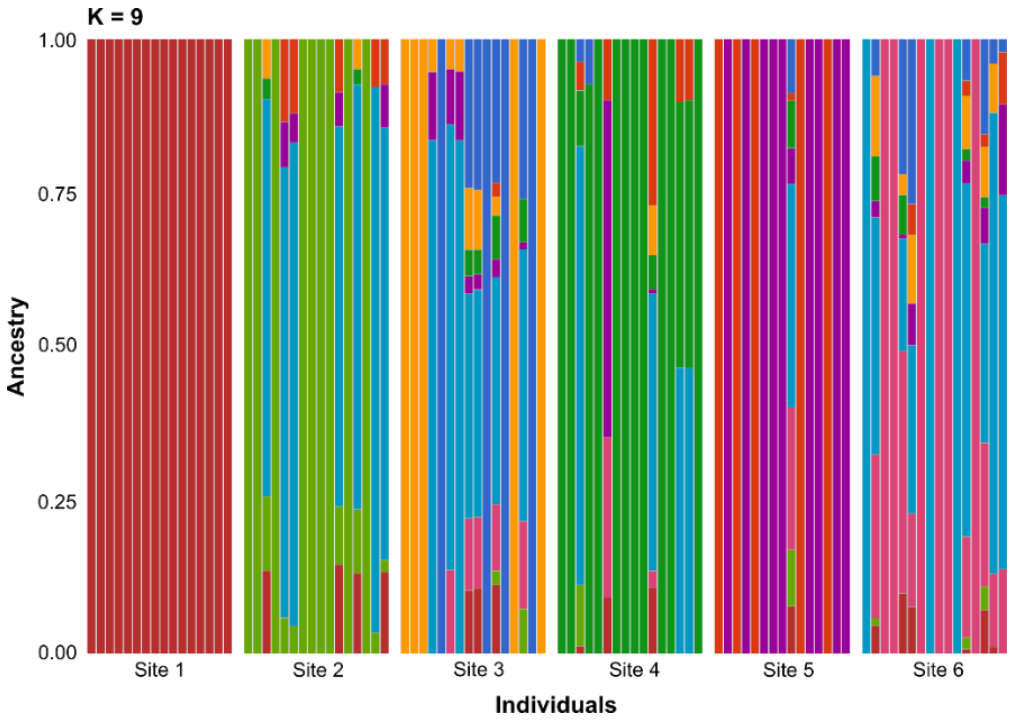

Figure 5.6 *ADMIXTURE results at K=9 with individuals on the x-axis (sorted by site) and assignment probability on the y-axis.*

Global pairwise Fst distances (i.e. genetic differentiation based on all SNPs) between Site 1 and any of the other sites were roughly double than the distance between two of these other sites (> 0.3; **Table 5.1**), suggesting a limited gene flow not only between Site 1 and the outside-lagoon sites but also between the two sites inside the lagoon. Similarly, when comparing Site 2 and the outside-lagoon sites, pairwise Fst values were greater than 0.2 also indicating a limited gene flow also between Site 2 and the outside-lagoon sites (**Table 5.1**). Among the four outside-lagoon sites, most pairwise Fst values between Site 4 and the other sites were among the highest values (mostly higher than 0.2; **Table 5.1**) pinpointing a strong genetic isolation of the southern-most site (Site 4) when compared with the other sites from outside the lagoon.

Table 5.1 *Global pairwise Fst distances among studied sites based on all SNPs. Darker colours indicate larger values.*

	Site 1	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**
Site 1	0.000					
Site 2	0.367	0.000				
Site 3	0.308	0.191	0.000			
Site 4	0.438	0.288	0.177	0.000		
Site 5	0.334	0.218	0.129	0.258	0.000	
Site 6	0.342	0.204	0.091	0.229	0.160	0.000

A total of 47 MLGs (multilocus genotypes) were detected among the six *P. oceanica* sites (**Fig. 5.7**), with only 3 MLGs found at Site 1. For the other sites, the number of MLGs varied from 8 to 10. MLG.3 was the dominant genotype at Site 1 and accounted for 87.5% of the total individuals at this site. MLGs detected at Site 1, Site 2 and Site 3 were unique for each of these sites while several MLGs were found in common among Site 4, Site 5 and Site 6. For instance, MLG.62 was found at Site 4, Site 5 and Site 6 and MLG.88 was found at Site 5 and Site 6.

The inbreeding coefficient (F_{is}) was negative in all studied sites with Site 1 having the lowest F_{is} of -0.993 and Site 3 having the highest F_{is} of -0.091 (**Table 5.2**).

Table 5.2 *Inbreeding coefficient (F_{is}) across sites.*

Site	F_{is}
1	-0.933
2	-0.275
3	-0.091
4	-0.335
5	-0.191
6	-0.132

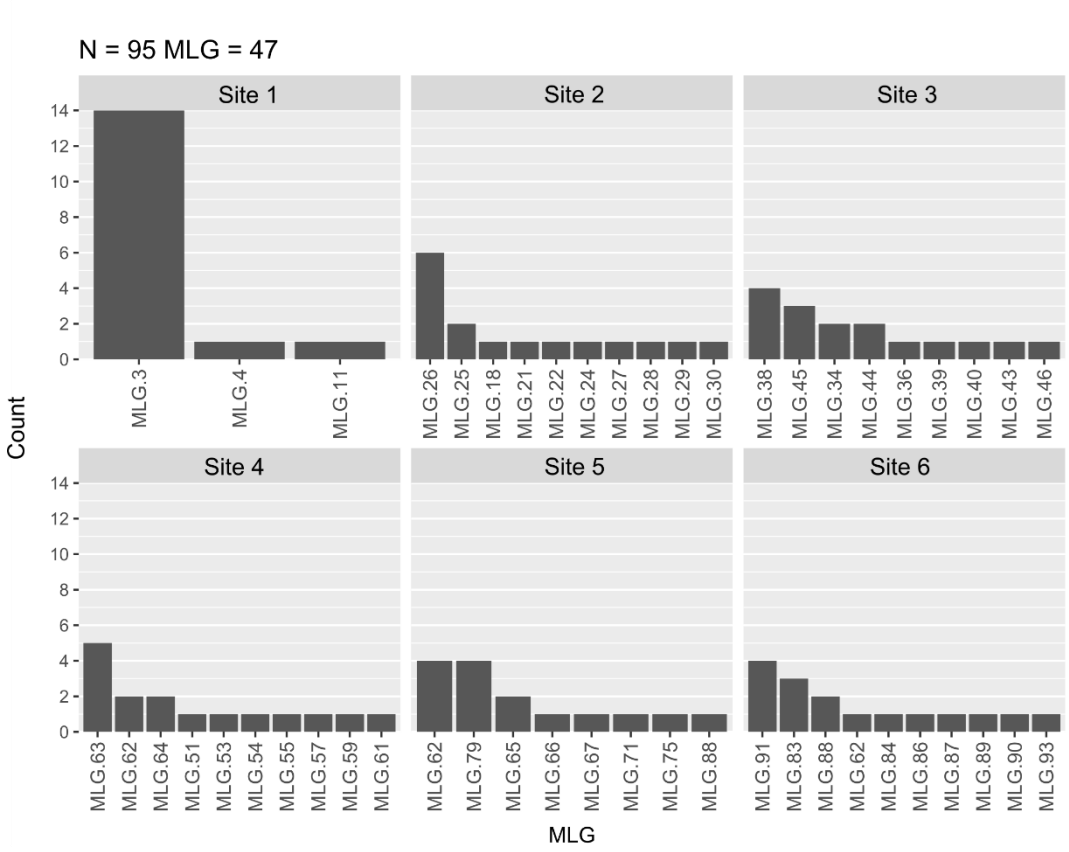

Figure 5.7 *Multilocus genotypes among sites: N=95: total number of individuals. MLG=47: total number of MLGs detected from all sites.*

5.3.4 Identification and annotation of outlier SNPs

The combination of three genome scan methods (*Bayescan*, *OutFLANK* and *pcadapt*) identified a total of forty-one 'true' outlier SNPs (**Fig. 5.8A,C**). After annotations, ten outlier SNPs were annotated with a total of eighteen different proteins (some outlier SNPs had more than one annotations; **Table 5.3**) including three proteins involved in stress responses, one related to epigenetics, one related to DNA replication, two were key regulators of plant-specific developmental events, one related to transporters, and the rest were either hypothetical protein or uncharacterised protein. *Mitogen-activated protein kinase kinase kinase* serves as a highly conserved central regulator in stress responses in eukaryotes (Nakagami, Pitzschke and Hirt, 2005) and mediates reactive oxygen species (ROS) homeostasis in *Arabidopsis* (Nakagami *et al.*, 2006). *Serine/threonine-protein phosphatase* involves directly to ROS mediated signaling pathways (Máthé *et al.*, 2019) and contributes critical functions in the regulation of adaptive stress responses to biotic and abiotic stresses [e.g. Water Deficit Stress, Cold Stress, Heat Stress, Mechanical Wounding, drought (Li *et al.*, 2008; País, Téllez-Iñón and Capiati, 2009)]. *Protein MAIN-LIKE* is required for meristem maintenance by sustaining genome integrity in stem cells and their descendants' cells (Wenig *et al.*, 2013), acts synergistically and redundantly with DNA methylation to silence transposable elements with demonstrated functions in several epigenetic pathways including DNA methylation and histone modifications (Nicolau *et al.*, 2020).

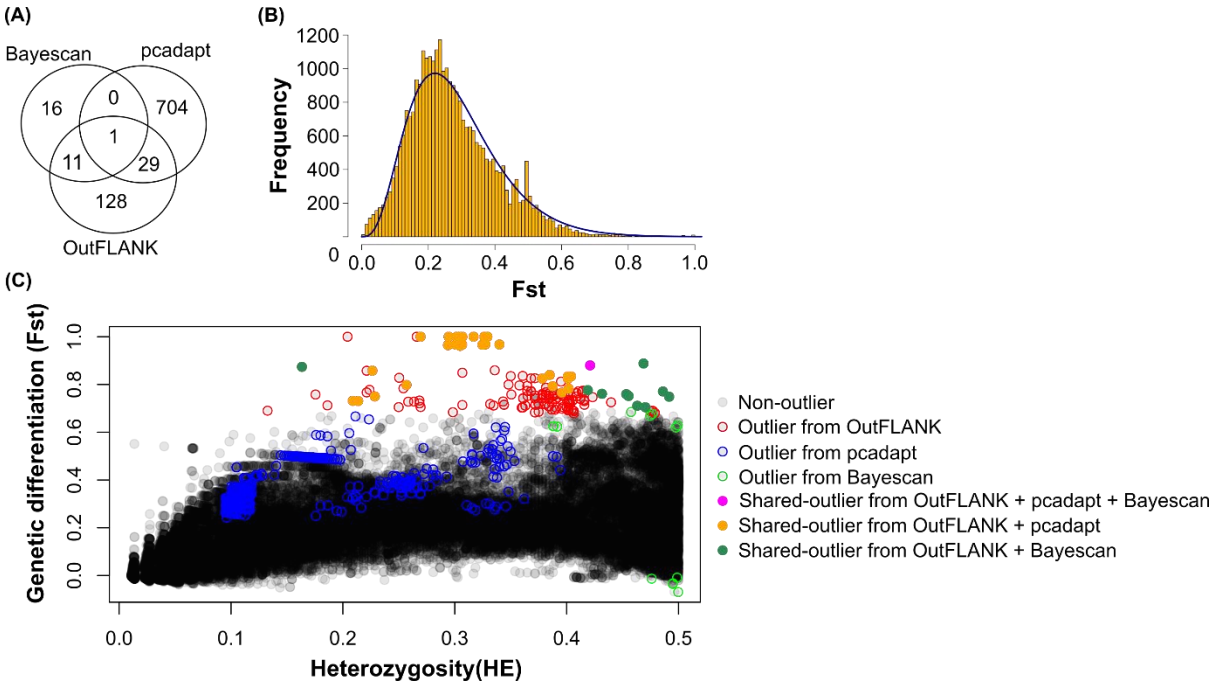

Figure 5.8 *Genetic differentiation and outlier SNPs. (A) Venn diagram showing outlier SNPs detected with the three selected methods. (B) Histogram presenting the genetic differentiation (Fst) frequency distribution for all SNPs from OutFLANK. (C) Genetic differentiation (Fst) versus expected heterozygosity (HE) and outlier SNPs detected from different methods.*

Allelic frequency of the outlier SNPs provided further hints about the genetic divergence between inside-lagoon populations and outside-lagoon populations. Firstly, 40/41 outlier SNPs were monoallelic at Site 1 and most of them were dominated by **Allele 2** (while **Allelle 1** was dominant in other sites; **Fig. 5.9**) suggesting a strong genetic differentiation in Site 1. Secondly, plants from Site 2 showed the highest number of biallelic SNPs (19/41), being **Allele 2**, the more abundant in this site (**Fig. 5.9**). This may indicate a 'transition phase' towards Site 1. Finally, it is worthy to mention that **Allelle 1** was the dominant allele in individuals from the outside-lagoon populations, 16/41 outlier SNPs were detected as biallelic at Site 3 while the number only ranged from 4 to 7 for Site 4, Site 5 and Site 6 (**Fig. 5.9**). This could be reflecting that the environmental conditions at Site 3 could also be under the influence of the inside-lagoon conditions.

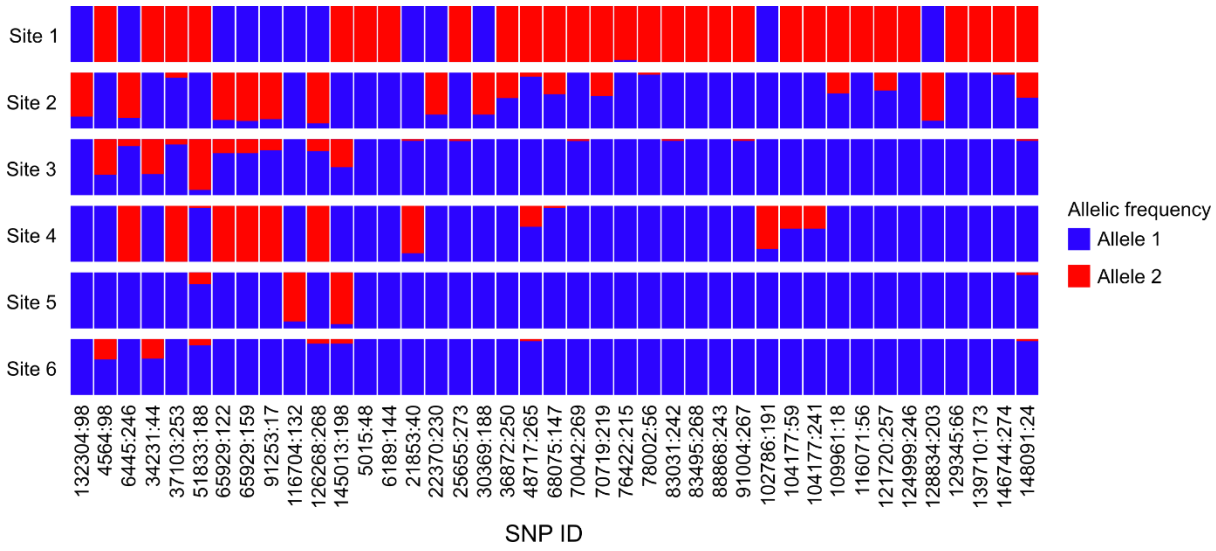

Figure 5.9 *Allelic frequencies of outlier SNPs.*

Table 5.3 *Reduced list of annotated outlier SNPs with known functions on NCBI protein database. Annotations associated with stress responses and epigenetics are in grey background.*

SNP ID	Contig name (MAGI database)	E-value	Bit score	Per. Ident (%)	Annotation of top BLASTx hit (NCBI database)	Function	Species	E-value	Bit score	Query Cover (%)	Per. Ident (%)	Accession number
21853:40	>TRINITY_DN1013_c0_g2_i1	3.0E-31	137	100	Putative WD-repeat protein	Regulators of plant-specific developmental events	*Zostera marina*	3.0E-63	229	21	77	KMZ59614.1
	>TRINITY_DN1013_c0_g2_i4	1.0E-70	268	100	WD repeat-containing protein WRAP73	Regulators of plant-specific developmental events	*Phoenix dactylifera*	0	769	57	82	XP_008798782.2
30369:188	>TRINITY_DN71066_c0_g1_i1	2.0E-41	170	91	Putative ribonuclease H protein	Mitochondrial DNA replication	*Ananas comosus*	1.0E-14	81	27	44	OAY74397.1
76422:215	>TRINITY_DN8381_c0_g1_i2	2.0E-28	127	90	Mitogen-activated protein kinase kinase kinase	Stress responses	*Posidonia oceanica*	3.0E-06	58	23	40	AEP40953.1
88868:243	>TRINITY_DN9430_c1_g1_i5	1.0E-30	135	90	Serine/threonine-protein phosphatase 7 long form	Stress responses	*Cinnamomum micranthum f. kanehirae*	5.0E-12	74	71	38	RWR83179.1
	>TRINITY_DN3903_c0_g3_i1	7.0E-38	159	91	Protein MAIN-LIKE 2	Epigenetics	*Rosa chinensis*	8.0E-18	100	25	35	XP_024180069.1
	>TRINITY_DN126_c4_g1_i1	4.0E-33	143	88	Serine/threonine-protein phosphatase	Stress responses	*Trifolium pratense*	4.0E-08	59	95	33	PNX70831.1
104177:241	>TRINITY_DN22961_c1_g2_i1	7.0E-35	149	89	Retrotransposon gag protein	Retrotransposon	*Asparagus officinalis*	3.0E-56	248	66	68	ABD63131.1
128834:203	>TRINITY_DN4295_c1_g1_i3	4.0E-61	236	97	Putative glycerol-3-phosphate transporter 1	Transporter	*Cinnamomum micranthum f. kanehirae*	0	726	59	76	RWR89814.1

5.4 Discussion

5.4.1 Differentiation in environmental condition, plant morphology and genetics among sites

This study highlights the differentiation in the summer environmental conditions (i.e. water temperature and salinity) between the lagoon waters versus the open sea surrounding it, as well as, between the northern and the southern basins of the lagoon. In fact, this phenomenon has been documented in previous studies (Vizzini *et al.*, 2002; Tomasello *et al.*, 2009) and can be explained by the limited water exchange together with the shallowness of the lagoon (La Loggia *et al.*, 2004). While Tomasello *et al.*, (2009) reported that the maximum temperature for the northern basin of the lagoon was 30℃ the maximum SST in this study was 33.1℃. Even if there could be some bias due to the different approaches used for measuring water temperature between Tomasello *et al.*, (2009) and this study, it is still clear that the water temperature in the northern basin has increased greatly over the last 15 years. This may be the result of two possible and non-exclusive factors including (*i*) the Mediterranean Sea is warming up [especially in the Eastern region (Nguyen, Yadav, *et al.*, 2020)] contributing to an overall increase in seawater temperature that also affects the lagoon and (*ii*) the 400-m wide channel in the north of the lagoon has been gradually blocked (Tomasello A., *personal communication*) contributing to further limit water exchange in the area. Also, a recent study has recorded a maximum value of 51.6‰ in the northern basin of the lagoon (Spinelli, 2018) pushing the upper limit of salinity tolerance known for *P. oceanica* to a new level [before it was known the upper limit of salinity tolerance for this species in the lagoon was 48‰ (Tomasello *et al.*, 2009)].

The significant reduction in plant size here observed in plants from the northern basin can be considered as signs of acclimation/adaptation for *P. oceanica* with the extreme condition in the area [both extreme temperature and extreme salinity (Fernández-Torquemada and Sánchez-Lizaso, 2005; Ruíz, Marín-Guirao and Sandoval-Gil, 2009; Marín-Guirao, Sandoval-Gil, *et al.*, 2013)]. This statement is supported by almost two decades of observations reporting undersized *P. oceanica* shoots growing at the northern basin of the Stagnone of Marsala lagoon (La Loggia *et al.*, 2004; Tomasello *et al.*, 2009; Spinelli, 2018). A similar shoot size reduction has been described in another *P. oceanica* population living under salinity levels above the normal threshold of tolerance of this stenohaline seagrass species (Marín-Guirao *et al.*, 2017). Authors proposed that this morphological adaptation serves as a stress-coping mechanism, helping plants from this population to inhabit survive under the continuous stress constraints imposed by an unfavorable environment. In terrestrial plants, in fact, a reduced size has been widely

described for plants subjected to long-term environmental stress as a morphological adaptation to cope with unfavourable environmental conditions (Lichtenthaler, 1996).

Results from this study concur with findings from Tomasello *et al.*, (2009) on demonstrating a high level of genetic isolation of the *P. oceanica* sites inside the Stagnone di Marsala lagoon in comparison with the open-sea sites. Principle component analysis shows a strong differentiation between *P. oceanica* populations from inside and outside the lagoon. This is further supported by clonality assessment analysis showing all genotypes found inside the lagoon are distinct from those from outside the lagoon.

Inside the lagoon, genetic overlapping between the northern- and southern-basin was no longer detectable in this study. While the difference in the number of studied individuals between this study (i.e. 16 samples per site) and Tomasello *et al.*, (2009) (i.e. 40 samples per site) may contribute to this observation, it is clear that the level of genetic differentiation between *P. oceanica* populations from the two basins was much greater compared with that in 2006. In Tomasello *et al.*, (2009), the pairwise Fst between the northern versus southern-basin site was lower than between the northern-basin site versus any of the outside-lagoon sites. In this study, the opposite result was obtained suggesting the separation between the northern and southern basin of the lagoon could have become stronger over the course of the last 15 years. It is worth mentioning that, Fst values among studied sites (except for the one from the northern basin) were always lower than 0.05 showing consistent levels of gene flow in the past (Tomasello *et al.*, 2009), while the Fst values among studied sites in this study varied from 0.09 to 0.28 hinting for a great reduction in the level of gene flow in the area. This should be noted that in Tomasello *et al.*, (2009), genetic markers were microsatellites and therefore resolutions of the analysis can also depend on different markers utilized.

In Tomasello *et al.*, (2009), six to eight individuals were sampled from each of six atolls and it was suggested that each atoll was composed of multiple genotypes. In this study, three to four individuals were sampled from each of five atolls and only three MLGs were detected in total. Taken into consideration also that the whole collected samples from this site were mostly dominated by one MLG supporting the so-called ‘natural selection of adaptive genotype’. This can help to explain why in the result of admixture analysis the only-dominated component at this site also appears in small fractions in all other sites. It could be understood that this component represents a set of genetic characteristics that might be suitable for the extreme environmental conditions existing in the northern basin. As a result, this component continues

to survive and gradually become dominant in the population while the other components are wiped out from the area. Since this area appears to be the most genetically isolated one, it would favour inbreeding in this particular inner-lagoon population. Interestingly, results from this study confirmed that the *P. oceanica* population in the northern basin has the lowest inbreeding coefficient among studied populations (Tomasello *et al.*, 2009) indicating a low level of inbreeding in this area. A possible explanation to this could be the fact that in the inner lagoon population, no flowering events were recorded during the last decades, whereas inflorescences were observed frequently in meadows outside the lagoon (Tomasello *et al.*, 2009).

In seagrasses, it has been demonstrated in several species that populations living in confined environments (such as coastal lagoons) have higher genetic isolation than those living in surrounding coastal areas [e.g. *Zostera marina* populations in San Quintin Bay, Mexico (Muñiz-Salazar *et al.*, 2006); *Halophila beccarii* populations in Cau Hai lagoon, Vietnam (Phan *et al.*, 2017) or recently *Halophila ovalis* populations in Dongsha Island, Taiwan (Liu and Hsu, 2021)]. Therefore, together with Tomasello *et al.*, (2009), this study confirms the existence of this phenomenon in *P. oceanica* populations in the Stagnone di Marsala lagoon.

The speed at which genetic isolation can occur depends on the level of physical isolation and the severity of environmental conditions (Hoffmann and Parsons, 1997). In the case of *P. oceanica* populations in the Stagnone di Marsala lagoon, together with a general effect of global warming, the gradual enclosure of the northern opening has disconnected the northern basin with the open sea and consequently limited water flow into and within the lagoon. As a result, the lagoon (especially the northern basin area) has become more isolated from surrounding areas and the environmental conditions have progressively become more extreme. This can help to explain a rapid genetic differentiation between populations from inside and outside the lagoon, as well as, a marked reduction in the number of clones from the northern basin during the last few decades.

Nonetheless, we should take into consideration that results in Tomasello *et al.*, (2009) were obtained through microsatellite genotyping, and that a difference in resolution between microsatellites and SNPs markers could explain some of the differences between the two studies.

5.4.2 Signatures of local adaptation to environmental conditions

This study identified some outlier SNPs associated with environmental stress responses and all of them are involved in the mediation of reactive oxygen species (ROS). In plants, environmental stressors including thermal stress and saline enhance the production of ROS, known as oxidative stress (Hasanuzzaman, Nahar and Fujita, 2013). In turn, plants produce ROS-scavengers (also known as antioxidants) to minimize the negative impacts of oxidative stress (Hasanuzzaman, Nahar and Fujita, 2013; Paridah *et al.*, 2016). In seagrasses, many previous studies have demonstrated the involvement of ROS-scavengers in response to warming (Reusch *et al.*, 2008; Winters *et al.*, 2011; Gu *et al.*, 2012; Liu *et al.*, 2016; Tutar *et al.*, 2017; Purnama *et al.*, 2019) and hypersaline (Marin-Guirao *et al.*, 2011; Capó *et al.*, 2020). Therefore, the genetic mechanisms underlying the mediation of ROS may play a critical role in promoting the local adaptation of *P. oceanica* to the environmental conditions at the Stagnone di Marsala lagoon. This is consistent with previous studies highlighting the role of ROS-managing mechanisms on the local adaption of organisms to different environmental conditions [e.g. the reef-building coral *Pocillopora damicornis* with temperature and light (van Oppen *et al.*, 2018)].

In addition, this study identified an outlier SNP related to epigenetic modifications (i.e. *Protein MAIN-LIKE*). In terrestrial plants, epigenetic modifications play crucial roles in response to several environmental stimuli (Chinnusamy and Zhu, 2009; Liu *et al.*, 2015). In seagrasses, results from previous studies (Ruocco *et al.*, 2019; Jueterbock *et al.*, 2020; Entrambasaguas *et al.*, 2021) together with results from this thesis (presented in Chapter II and III) agree on demonstrating the involvement of epigenetic modifications in seagrasses' response to environmental changes, including thermal stress. Thus, epigenetic modifications can contribute to the genetic mechanisms underlying the local adaptation of *P. oceanica* to the extreme environmental conditions at the Stagnone di Marsala lagoon.

In summary, my study suggests that the extreme environmental conditions (i.e. salinity and temperature) exiting within the Stagnone di Marsala coastal lagoon have promoted the local adaptation of the population living into this ecosystem. This local adaptation should have been promoted by the selection of genotypes genetically equipped to thrive under such adverse conditions. Together with this selection pressure, the limited gene flow exacerbated by the progressive isolation of the lagoon should have also participated in the local adaptation of the inner-lagoon population. Curiously, despite the isolation and low genetic diversity of the inner-

lagoon population, I do not find evidence of inbreeding, possibly due to the incapacity of these plants to flourish and hence, to reproduce sexually in such an extreme ecosystem as described in Tomasello *et al.*, (2009). Moreover, this population is dominated by a low number of "resistant" genotypes with exclusive mutations (outlier SNPs) on genes with stress-protective functions, as those involved in the antioxidant defense system or in orchestrating the plant stress response (i.e. epigenetic-related genes). These findings offer new clues in our attempt to assist the adaptation of seagrasses. The identification of putative heritable loci under selection for a given stressor (i.e. thermal stress) could then be combined with manipulative stress experiments to examine the resilience and the potential trade-offs of genotypes possessing such loci (Anderson *et al.*, 2015). This information could be crucial for improving seagrass restoration outcomes by facilitating an informed decision-making process about the provenance and genetic background of the transplant material. This information can also be useful for guiding the genetic modification of seagrasses to produce improved and more resilient individuals through genetic engineering (e.g. CRISPR/CAS9) and selective breeding approaches. These approaches, however, require a high level of human intervention, are more socially and ethically controversial and still far from be applied in seagrasses, although they are common in terrestrial plants and animals and have been proposed in certain cases of coral reef restoration (van Oppen *et al.*, 2015; Van Oppen *et al.*, 2017).

Appendix V

Table AV.1 *ADMIXTURE's cross-validation errors of different K values. The smallest number is in* ***bold****.*

K value	Cross-validation error
1	0.407
2	0.352
3	0.298
4	0.263
5	0.247
6	0.222
7	0.219
8	0.185
9	**0.177**
10	0.179
11	0.182
12	0.195
13	0.197
14	0.236
15	0.265

Chapter VI – Conclusions

The work presented in this chapter (6.2) has been published previously:

Hung Manh Nguyen, Peter J. Ralph, Lázaro Marín-Guirao, Mathieu Pernice and Gabriele Procaccini. Seagrasses in an era of ocean warming: a review (2021) *Biological Reviews* **96**(5): 2009−2030. https://doi.org/10.1111/brv.12736

6.1 Contribution of this thesis

Seagrasses are remarkable for their unique evolution [the only group of angiosperms that have re-inhabited the marine habitats (Les, Cleland and Waycott, 1997; Waycott *et al.*, 2004)] and also for their ecosystem services in the coastal environment [e.g. engineering species, global carbon cycle, etc. (Orth *et al.*, 2006; Fourqurean *et al.*, 2012)]. Nonetheless, the increasingly accumulated pressures due to human activities are challenging the existence of these marine plants as well as their ecosystem services (Orth *et al.*, 2006; Waycott *et al.*, 2009; Grech *et al.*, 2012). Studying the relationship between seagrasses and their surrounding environments helps us to better understand one of the most fascinating and critical groups of organisms living in our oceans. Research on seagrasses and their environment also has the potential to improve forecasting their future in order to plan and develop timely and effective actions (research, management and restoration) that can ultimately secure a sustainable future for these species and their ecosystem services. In this regard, this thesis provides a comprehensive contribution to our knowledge regarding the relationship between seagrasses and seawater warming with potential implications in future seagrass protection activities. In **Table 6.1**, I summarize the main scientific questions addressed and the main findings in each chapter.

Table 6.1 *Summary of the main scientific questions addressed in this thesis*

Chapter	Question	Main finding
I	What are the main common effects of warming on seagrasses?	Warming affects seagrasses across levels of biological organization. • At the molecular level, warming induces the production of ROS, causes protein unfolding and degradation, and ultimately damages DNA molecules. • At the biochemical/physiological level, warming decreases photosynthetic capacity while increasing respiration. Warming degrades photosynthetic pigments, enhances sulphide stress and damages cellular membrane fluidity. • At the morphological/population level, warming reduces plant growth and induces plant mortality which eventually results in population downsizing and even population die-off. • At the ecosystem/planetary level, warming switches seagrass ecosystems from autotrophic to heterotrophic thus contributing to climate change. Warming threatens the existence of seagrass habitats but also their neighbouring ones (such as corals).
	What are the main common responses of seagrasses with warming?	In turn, seagrasses develop several mechanisms for minimizing the negative impacts of warming. • At the molecular level, seagrasses express genes encoding heat shock proteins and ROS-scavengers. Other molecular mechanisms involved in proteolysis, ubiquitination, DNA repair or apoptosis are also activated. Moreover, epigenetic modifications seem to have vital roles in the response of seagrasses to warming.

		• At the biochemical/physiological level, seagrasses activate photo-protective mechanisms, modify their pigments content and re-allocate carbohydrates for fuelling the stress response as well as for optimizing energy resources. • At the morphological/population level, seagrasses modify the plant architecture (i.e. above-ground biomass) and anticipate sexual reproduction (flowering). Seagrass populations can acclimatize, adapt or even escape by the means of fragmentation/seed dispersal. • At the ecosystem/planetary level, temperate meadows may transform into tropical meadows. Even with less valuable in terms of ecosystem services as well as blue carbon storage, this represents in some cases the only game-saving option. • The response of seagrasses to warming varies amongst species (tropical species are generally more tolerant than temperate species), populations (populations living in fluctuating environmental conditions tend to have a higher capacity to endure warming than those living in stable conditions), and over depths (deeper plants are likely more susceptible to warming than their shallower counterparts).
II, III	Does thermal stress memory exist in seagrasses?	These chapters provide evidence indicating the positive effects of thermal stress memory in all four studied seagrass species. Preheated plants performed photosynthetically better than non-preheated ones as seen in the case of *P. australis*, *Z. muelleri* and *P. oceanica*. Preheated *Z. muelleri* better maintained their productivity in thermal stress conditions than non-preheated plants. Similarly, preheated *P. oceanica* were able to better adjust their growth to minimize the impact of warming than their non-preheated counterparts. The pigment content of preheated seagrasses (*P. australis*, *Z. muelleri*, and *C. nodosa*) was

		not affected or even enhanced by warming while that of non-preheated plants was negatively impacted. Lastly, gene expression results showed non-preheated plants exhibiting more signs of stress than preheated plants.
III	What are the molecular mechanisms underlying thermal stress memory in seagrasses?	This chapter outlines some molecular mechanisms potentially involved in seagrass thermal stress memory. • *A 'know-how' mechanism:* global gene expression analysis showed the strong transcriptomic activation of non-preheated plants, whereas gene expression downregulation was the dominant response of preheated plants (*P. oceanica* and *C. nodosa*). Gene functional and GO analysis demonstrated the response to warming of preheated and non-preheated plants differed in terms of genes and biological processes involved. In brief, preheated seagrasses better cope with warming by inducing a more energy-effective response through (*i*) minimizing the number of active genes required for the response and (*ii*) involving in the response more energy-efficient pathways. • *Epigenetic modifications:* my analysis identified several epigenetic-related genes and processes in the transcriptomic response to warming of both preheated and non-preheated *P. oceanica* plants. However, this response was more complete in preheated than in non-preheated plants. Hence, epigenetic modifications may contribute to the formation of thermal stress memory during the priming phase but may also play important roles in the activation of this memory during the triggering phase. • *Thermal stress memory-related genes*: gene expression analysis showed significant expression of some thermal stress memory-related genes (e.g. HSP18.2, HSP22 and FBA6) from both preheated and non-preheated plants suggesting their functions in both priming and memory phase.

IV	Are there any differences in response to warming between northern versus southern hemisphere seagrasses?	This chapter demonstrates the differences in response to warming between northern and southern hemisphere *Posidonia*. This phenomenon can also potentially occur among other seagrass species across the hemisphere. However, more species need to be studied before the differential thermal affinity among northern and southern hemisphere seagrasses can be generalized.
	Does differences in response to warming between climax versus pioneer seagrasses occur in both hemisphere?	This chapter provides evidence showing that pioneer species (*C. nodosa* and *Z. muelleri*) responded better to warming than their climax counterparts (*P. oceanica* and *P. australis*) and this difference seems consistent in both northern and southern hemispheres.
V	What are the genetic mechanisms underlying local adaptation to warming in seagrasses?	This chapter identifies several outlier loci that may potentially underlie the local adaptation of the *P. oceanica* to the extreme environmental conditions existing inside the Stagnone di Marsala lagoon. SNPs (i.e. outlier loci) were detected within genes involved in environmental stress responses (i.e. ROS in particular) and epigenetic modifications.

Starting with a comprehensive review in **Chapter I**, I identified potential commonalities in the effects of warming and the responses of seagrasses across different levels in the hierarchy of life ranging from molecular to planetary. In addition, in the published review (Nguyen *et al.*, 2021), I also summarized current knowledge concerning the combined effects of ocean warming with other environmental stressors on seagrasses, offering a more realistic view of the relationship between seagrasses and global warming. Together, this chapter provides a substantial contribution to seagrass research by serving as a starting/referencing material for future research about seagrasses and ocean warming.

In **Chapter II**, I demonstrated the existence of thermal stress memory for the first time in seagrasses. These findings have opened up an emerging topic in seagrass research with great potential applications in seagrass management and restoration activities. In fact, the positive effect of thermal stress memory may partly explain the surprisingly weak effects of repeated heat events on natural populations. For instance, after the abrupt decline of *P. oceanica* population following the 2006 MHW (Marbà and Duarte, 2010; Jordà, Marbà and Duarte, 2012), no further mass mortality event has been documented even after several subsequent more intense and longer-lasting MHWs in the Mediterranean [e.g., 2012, 2015, 2017; see Darmaraki, Somot, Sevault and Nabat (2019)]. On the other hand, thermal stress memory can also be a part of the so-called 'assisted evolution' approach (Bulleri *et al.*, 2018) to produce 'super seagrasses' that can survive future harsh environmental conditions and enhance the success of seagrass restoration activities. This emerging topic of stress memory, indeed, provides us with more ***optimism*** about the future of seagrasses.

In **Chapter III**, the molecular mechanisms underlying thermal stress memory in seagrasses were explored through a high-throughput gene-expression profiling approach (i.e. RNA-seq). Results suggest a '*know-how*' mechanism that heat-primed plants acquired during the priming event and that eventually equipped them with the ability to induce more energy-effective responses when the thermal stress recurred. This finding has broadened our understanding in the field of seagrass ecology (ecological epigenetics) but also in the field of terrestrial plant ecology where knowledge on this topic is also largely scarce (Bäurle, 2016). Additionally, molecular results from this chapter have added to the mounting evidence suggesting an important role of epigenetic modifications with thermal stress response in seagrasses (e.g. see Lazaro Marín-Guirao *et al.*, 2017; Duarte *et al.*, 2018; Marín-Guirao *et al.*, 2019). Especially, results from this chapter have identified the involvement of thermal stress memory-related

genes in such processes that can serve as a starting point for future molecular studies in this topic.

In **Chapter IV**, using a cross-hemisphere study, I investigated the response to warming of four seagrass species representing (*i*) two *Posidonia* species from the northern and southern hemisphere and (*ii*) one couple of climax-pioneer seagrass species from each hemisphere. This research provides new evidence that strengthen interspecific differences in the response of seagrasses to warming (e.g. see Marín-Guirao *et al.*, 2016; Collier *et al.*, 2017). More importantly, this study highlights a different behaviour to seawater warming between climax and pioneer seagrasses that would have potential consequences on the future distribution of these two groups of seagrasses and thus, on the tropicalization of temperate seagrass meadows (Hyndes *et al.*, 2016). One major conclusion I want to emphasize from this study is the need to protect and adequately manage (e.g. reinforce) climax seagrass populations due to their higher ecological and socioeconomic value and their higher susceptibility to warming with regard to pioneer seagrass populations.

In **Chapter V**, using *P. oceanica* populations from and around the Stagnone di Marsala as a natural experimental model system, I studied the molecular basis of local adaptation in seagrasses. Morphological results together with environmental data suggest *P. oceanica* has significantly reduced the shoots size as a stress-coping mechanism (i.e. morphological adaptation) to survive the extreme conditions (i.e. salinity and temperature) existing in the inner lagoon areas (the northern basin). Moreover, ddRADseq analysis reveals differences in genetic structure among sites and identifies several outlier loci that may be potentially responsible for the local adaptation of the inner lagoon populations. Some outlier loci are involved in environmental stress responses (i.e. ROS in particular) and epigenetic modifications. Overall, this study provides novel insights into the field of seagrass ecology and widens our understanding of how seagrasses might genetically evolve in the near future to survive a changing ocean (e.g. a warmer ocean).

6.2 Future directions for seagrass research

To ensure '***our children's children will still be able to see seagrasses not in documentaries or in the form of specimens in museums but thriving in the ocean***' as I stated at the beginning of this thesis, more efforts should be devoted to seagrass research, restoration and management activities. Here, I want to conclude this thesis by discussing some significant gaps in knowledge and recommend future directions for seagrass studies.

6.2.1 Enlarging the number of studied species and populations

Previous studies have shown that the effects of ocean warming are not the same for all seagrass species and populations. Therefore, it remains difficult to predict the future of seagrasses accurately. Most seagrass studies in the context of ocean warming come mainly from three regions: the Mediterranean Sea, the USA, and Australia (**Fig. 6.1**). Most of these studies have focused on only a few seagrass species: *Z. marina*, *P. oceanica* and *C. nodosa* (**Table AVI.2**). This highlights a significant gap in our understanding of how warming affects seagrasses; the vast majority of the world's seagrass species have not been studied in this context to date. Future studies should focus not only on additional species but also on more populations within each species to deliver a more comprehensive picture of how seagrasses will respond to a future-changing climate.

Since warming potentially interacts with many other stress factors acting at the local or regional levels, seagrass meadows are currently under multiple anthropogenic pressures. Populations that are chronically stressed (e.g. under eutrophic conditions) might have a different tolerance or ability to respond to warming compared to healthy populations. There is, therefore, an urgent need to explore how seagrass responses to warming differ between healthy and chronically stressed populations, as well as, how other biotic and abiotic factors interact with warming amplifying or alleviating the ability of seagrasses to withstand increased seawater temperatures. This knowledge will be critical for improving the management and protection of valuable seagrass ecosystems by managing local factors that directly affect seagrass health, potentially altering their resilience to warming.

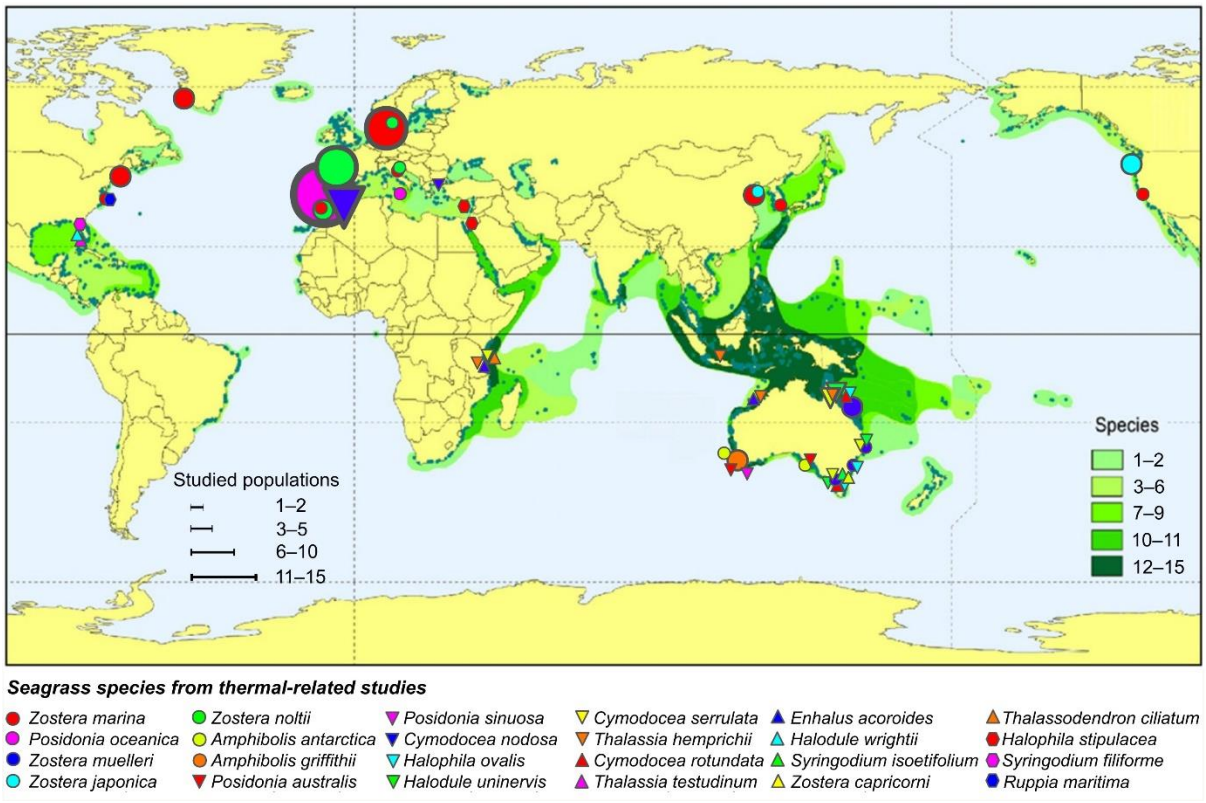

Figure 6.1 *World map of seagrass populations used for thermal-related studies from 1985 to 2020. Colored symbols indicate study populations, with symbols sizes scaled according to the key on the left. World distributions of species are shown by background green shading, with species numbers indicated according to the key on the right. Data were collected from Google Scholar using the keywords "seagrass thermal stress", "seagrass heat stress", "seagrass temperature" and "seagrass warming" together with my knowledge. Figure created by adapting Fig. 3 in Short et al., (2007). Details of locations and related publications are provided in Appendix VI (**Table AVI.1**).*

6.2.2 Developing more precise and detailed seagrass distribution maps

Some seagrass species are losing habitat at a rapid rate (Robblee *et al.*, 1991; Seddon, Connolly and Edyvane, 2000; Jordà, Marbà and Duarte, 2012; Marbà, Díaz-Almela and Duarte, 2014) or are being replaced by the rapid expansion of other species (Gambi, Barbieri and Bianchi, 2009; Scheibling, Patriquin and Filbee-Dexter, 2018; Nguyen, Yadav, *et al.*, 2020). Some studies (Short *et al.*, 2007; Jayathilake and Costello, 2018) have provided general information regarding seagrass distribution, but many regions remain unmapped (Assis *et al.*, 2020; McKenzie *et al.*, 2020). Building seagrass distribution maps can be challenging, especially for species that have a wide distribution range with different depths or inhabit mixed-species meadows. Effective methods have been developed to map seagrasses (see a review by

McKenzie, Finkbeiner and Kirkman, 2001) and more recently, a low-cost field-survey method using snorkeling and perpendicular transects (Winters *et al.*, 2017) was developed at a small regional scale. Advanced technologies [e.g. satellite remote sensing, Geographic Information System (GIS) technologies, camera-equipped drones] have also been employed in seagrass mapping research (Barrell *et al.*, 2015; Phinn *et al.*, 2018). Future work is encouraged both in developing advanced technologies and in building more detailed seagrass maps across different regions of the world.

6.2.3 Long-term monitoring programs

Many countries across the globe have implemented seagrass long-term monitoring networks appropriate to their local species and habitats. Since the end of the 20th century, many monitoring programs have been aggregating data to create regional and/or global monitoring networks to preserve seagrass meadows and to increase scientific knowledge and public awareness about these threatened and valuable ecosystems. At the global level, Seagrass-Watch (http://www.seagrasswatch.org/) and SeagrassNet (http://www.seagrassnet.org/) integrate hundreds of sites distributed along the coasts of dozens of countries for the long-term ecological monitoring of seagrasses. In the Mediterranean, the regional integration of existing networks is lacking, but initiatives are currently ongoing, for instance, the POSIMED project (http://posimed.org/).

Data from long-term seagrass monitoring programs are not only providing valuable information to unravel the status and trends of natural populations at the global, regional, and local scales (e.g. see Thomas, Unsworth and Rasheed, 2010; Rasheed and Unsworth, 2011; de los Santos *et al.*, 2019), but they are also helping in the development and execution of international environmental protection policies (e.g. the Marine Strategy European Directive). Seagrass monitoring programs are also recording environmental data, including water temperature, to correlate seagrass decline with warming trends or extreme warming events (Marbà and Duarte, 2010; Richardson, Lefcheck and Orth, 2018; Shields, Parrish and Moore, 2019). However, ecosystem modelling and forecasting activities for seagrasses are still needed. This could be facilitated by bridging global and local observations, and by linking long-term data series from seagrass monitoring programs to the continuous recording of coastal environmental conditions. Currently, platforms and sensor systems to measure physical, chemical, geological, and biological properties are increasingly being installed in coastal areas and oceanic regions worldwide [e.g. GOOS (https://www.goosocean.org/) and OOI

(https://oceanobservatories.org/)]. The production of high-throughput data from multidisciplinary studies is a promising advance towards improving all aspects of seagrass conservation, from dynamic model development to forecast validation. These are powerful holistic approaches to monitoring seagrass ecosystems and their evolution in a rapidly changing ocean, as well as contributing to their effective conservation and the management of human activities in coastal areas (Capotondi *et al.*, 2019). The integration of time-series data through multivariate statistics and/or machine-learning algorithms could also provide promising tools to monitor coastal ecosystems in a changing climate (Danovaro *et al.*, 2016; Crise *et al.*, 2018).

6.2.4 More realistic experiments in controlled conditions

Many past studies suffered from experimental constraints/limitations such as using unrealistic temperature levels, warming rates, experimental duration, small water volumes, or even single seagrass shoots that prevented clonal integration (e.g. see reviews by Bulthuis, 1987; Lee, Park and Kim, 2007). By contrast, recent experiments have become more realistic due to the development of sophisticated mesocosm systems for the culture of seagrasses in optimal conditions, and their use to conduct finely tuned and highly controlled experiments (e.g. see Bergmann *et al.*, 2010; Marín-Guirao *et al.*, 2011; Georgiou *et al.*, 2016; Cambridge *et al.*, 2017; Oscar, Barak and Winters, 2018; Ruocco, Marín-Guirao and Procaccini, 2019 and also mesocosm systems used in this thesis). These new systems have enabled more robust experiments to obtain not only a significant amount of knowledge in a short period but also novel results (Bulthuis, 1987; Lee, Park and Kim, 2007). In the near future, the application of more advanced technologies are expected to push the boundary of seagrass research even further by enabling in situ experiments (e.g. see Egea *et al.*, 2019) and near-natural simulated environment experiments (e.g. see Saha *et al.*, 2019).

6.2.5 The study of the holobiont

There is now increasing recognition of the fundamental interactions between symbiotic microorganisms (bacteria, fungi, and archaea) and their host organisms. From both an ecological and evolutionary point of view, we should perhaps consider the organisms and their symbiotic microorganisms not separately but together. The term ‘holobiont’ has been used to describe this combination of the host organism and its microbiome (see a review by Vandenkoornhuyse *et al.*, 2015). In terrestrial plants, the number of studies considering the holobiont has increased, uncovering important functions of the microbiome in plant nutrition, resistance to biotic and abiotic stresses, and evolution (Vandenkoornhuyse *et al.*, 2015). In

marine environments, interactions across holobionts are expected to be more flexible, with faster microbial community shifts, and greater phylogenetic diversity compared to terrestrial ecosystems (Dittami *et al.*, 2021). Compared with terrestrial plants, the importance of the holobionts in marine ecosystems is understudied. Some pivotal investigations of seagrass–bacteria interactions have suggested many important roles in providing nutrients, sustaining fitness, enhancing growth, and protecting seagrasses from toxic compounds and pathogens (see reviews by Ugarelli *et al.*, 2017; Tarquinio *et al.*, 2019; Conte *et al.*, 2021). In the face of ocean warming, the activity of the seagrass–bacterial community in relation to carbon remineralization is expected to increase, consequently reducing carbon accumulation rates in seagrass meadows (Trevathan-Tackett *et al.*, 2017). Future studies of the seagrass holobiont should focus on a better understanding of (1) the components of the seagrass microbiome, (2) seagrass–microbiome interactions in an ecological context, and especially (3) how the seagrass microbiome can help seagrasses to be more resilient to environmental changes. Such studies will not only broaden our understanding of this important aspect of seagrass ecology but will also be extremely useful for seagrass restoration activities, as symbiotic microorganisms could potentially be used to enhance the survival of transplanted seagrasses (both seedlings and adult plants).

6.2.6 Seagrasses as a solution to mitigate climate change

Adopting the concept of Gattuso *et al.*, (2018) that ocean solutions may allow us to address climate change, it is essential to restore and conserve healthy seagrass meadows worldwide in order to preserve the ecosystem services they provide in mitigating climate change (e.g. carbon sequestration) and its associated effects (e.g. coastal erosion by increasing sea storms). In the agriculture and food industries, the application of genetic engineering has significantly improved the productivity and quality of crops and commercial species (see a review by Janni *et al.*, 2020). To the best of my knowledge, such approaches (e.g. CRISPR/Cas9 which is short for clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9) have never been applied in seagrass research. These novel approaches provide a promising way to select, breed, or produce genotypes that can survive future harsh environmental conditions [i.e. assisted evolution (Bulleri *et al.*, 2018)]. Such potential "super-seagrasses" could help us to re-establish ecosystems in areas where seagrasses have been completely destroyed due to natural and/or human-induced catastrophic events or to reinforce natural populations endangered by the ongoing climate change. Additionally, as seagrasses growing in extreme environments (e.g. under anthropogenic pressures, highly fluctuant environments,

frequent MHWs, etc.) are expected to be more resilient to ocean warming, transplantations using plants from such populations could be highly useful in seagrass restoration (see a review by Tan *et al.*, 2020). Moreover, emerging knowledge in the field of thermal stress memory and epigenetic memory in seagrasses as presented in this thesis (see also Jueterbock *et al.*, 2020) could yield many potential applications in seagrass restoration. Together, the application of such new approaches could support attempts to reinforce natural populations or to restore degraded seagrass meadows effectively and sustainably at a global scale and consequently protect their ecosystem services (Reynolds *et al.*, 2016), thus ultimately mitigating the negative impacts of climate change.

Appendix VI

Table AVI.1 *List of thermal-related studies on seagrasses from 1985 to 2020. Data were collected from Google Scholar as indicated in the legend to* ***Fig. 6.1***.

Year	Author + Year	Species	Location
2020	(Nguyen, Yadav, *et al.*, 2020)	*Halophila stipulacea*	*34.705556, 33.123333 29.546375, 34.964293*
2020	(Kim *et al.*, 2020)	*Zostera marina*	*34.800000, 128.583333*
2019	(Ontoria *et al.*, 2019)	*Posidonia oceanica*	*42.106389, 3.171111*
2019	(Yaping *et al.*, 2019)	*Zostera marina*	*37.042222, 122.567778*
2019	(Purnama *et al.*, 2019)	*Thalassia hemprichii*	*-6.878000, 112.214028*
2019	(Marín-Guirao *et al.*, 2019)	*Posidonia oceanica*	*37.728333, -0.712500 42.106333, 3.171167*
2018	(Soissons *et al.*, 2018)	*Zostera noltii*	*36.500000, -6.166667; 40.133333, -8.833333; 43.416667, -3.800000; 43.350000, -1.766667; 44.700000, -1.133333; 44.716667, -1.150000; 48.600000, -2.350000; 51.433333, -4.083333; 51.566667, -3.983333; 54.900000, -8.316667*
2018	(Savva *et al.*, 2018)	*Cymodocea nodosa* and *Posidonia oceanica*	*39.532344, 2.588575; 39.533172, 2.587664*
2018	(Mota *et al.*, 2018)	macroalga *Fucus vesiculosus* and *Zostera marina*	*64.161111, -51.556111; 36.997500, -7.828056*
2018	(Marín-Guirao *et al.*, 2018)	*Posidonia oceanica* and *Cymodocea nodosa*	*42.106333, 3.171167; 37.728333, -0.712500;*
2018	(Collier *et al.*, 2018)	*Cymodocea serrulata, Halodule uninervis,* and *Zostera muelleri*	*-19.176867, 146.828950; -20.634981, 148.701806*
2018	(Traboni *et al.*, 2018)	*Posidonia oceanica*	*37.728333, -0.712500*
2018	(George *et al.*, 2018)	*Thalassia hemprichii, Cymodocea serrulata, Enhalus acoroides,* and *Thalassodendron ciliatum*	*-6.350000, 39.333333*
2018	(Egea *et al.*, 2018)	*Cymodocea nodosa*	*36.488833, -6.264750*
2018	(Beca-Carretero, Olesen, *et al.*, 2018)	*Zostera marina*	*64.150000, -51.550000; 64.466667, -50.216667; 56.383333, 16.550000*
2018	(Beca-Carretero, Guihéneuf, *et al.*, 2018)	*Posidonia oceanica* and *Cymodocea nodosa*	*42.106389, 3.171111; 37.572444, -1.207806*
2018	(Ruiz *et al.*, 2018)	*Posidonia oceanica*	*42.106389, 3.171111*

2017	(Tutar *et al.*, 2017)	*Posidonia oceanica* and *Cymodocea nodosa*	*37.716667, -0.700000*
2017	(Wilkinson *et al.*, 2017)	*Halophila ovalis*	*-19.181333, 146.843833*
2017	(Lazaro Marín-Guirao *et al.*, 2017)	*Posidonia oceanica*	*37.727778, -0.696667*
2017	(Collier *et al.*, 2017)	*Cymodocea serrulata, Halodule uninervis,* and *Zostera muelleri*	*-16.754833, 145.973000; -27.492167, 153.401500*
2017	(Zhang, Zhang and Yang, 2017)	*Zostera japonica*	*37.910000, 120.730000*
2017	(Malandrakis *et al.*, 2017)	*Cymodocea nodosa*	*40.933461, 24.418042*
2016	(Liu *et al.*, 2016)	*Zostera marina*	*36.054318, 120.368965*
2016	(Georgiou *et al.*, 2016)	*Halophila stipulacea*	*34.706111, 33.123889*
2016	(Jueterbock *et al.*, 2016)	*Zostera marina*	*56.717833, 8.474100; 43.966167, 12.764333; 43.064467, -70.872417; 41.554000, -70.510833*
2016	(Marín-Guirao *et al.*, 2016)	*Posidonia oceanica* and *Cymodocea nodosa*	*37.716667, -0.700000*
2016	(Pedersen *et al.*, 2016)	*Thalassia hemprichii* and *Enhalus acoroides*	*-16.402200, 123.139567*
2015	(Olsen and Duarte, 2015)	*Cymodocea nodosa*	*39.553238, 2.686711*
2015	(Kaldy, Shafer and Dale Magoun, 2015)	*Zostera japonica*	*44.627000, -124.013000*
2014	(Olsen, Coyer and Chesney, 2014)	*Posidonia oceanica*	*39.553238, 2.686711*
2014	(Kong *et al.*, 2014)	*Zostera marina*	*36.050000, 120.333333*
2014	(Collier and Waycott, 2014)	*Thalassia hemprichii, Cymodocea rotundata, Halodule uninervis,* and *Halophila ovalis*	*-18.682683, 146.512094*
2014	(Franssen *et al.*, 2014)	*Zostera marina* and *Nanozostera noltii*	*56.953404, 10.417567; 43.970295, 12.759893*
2013	(York *et al.*, 2013)	*Zostera muelleri*	*-33.125833, 151.613611*
2013	(Kaldy and Shafer, 2013)	*Zostera japonica*	*48.574700, -122.538600; 44.614167, -124.028417; 43.316181, -124.311290*
2013	(García *et al.*, 2013)	*Posidonia oceanica*	*39.966667, 3.133333; 39.150000, 2.950000; 38.980000, 1.430000; 38.420000, 1.260000*
2012	(Gu *et al.*, 2012)	*Zostera marina* and *Zostera noltii*	*56.953404, 10.417567; 43.970295, 12.759893*
2012	(Olsen *et al.*, 2012)	*Posidonia oceanica* and *Cymodocea nodosa*	*39.600000, 3.390000*

2011	(Franssen *et al.*, 2011)	*Zostera marina*	*56.717833, 8.474167; 43.966167, 12.764333*
2011	(Massa *et al.*, 2011)	*Zostera noltii*	*37.012262, -8.007767*
2011	(Winters *et al.*, 2011)	*Zostera marina*	*56.717833, 8.474100; 56.208317, 10.577450; 43.966117, 12.764317*
2011	(Collier, Uthicke and Waycott, 2011)	*Halodule uninervis* and *Zostera muelleri*	*-19.181333, 146.843833*
2010	(Marbà and Duarte, 2010)	*Posidonia oceanica*	*39.150000, 2.933333*
2010	(Bergmann *et al.*, 2010)	*Zostera marina*	*56.717833, 8.474100; 56.208317, 10.577450; 43.966117, 12.764317*
2009	(Tomasello *et al.*, 2009)	*Posidonia oceanica*	*37.865566, 12.454006*
2009	(Massa *et al.*, 2009)	*Zostera noltii*	*37.005000, -7.966944; 37.004167, -7.987778*
2008	(Ehlers, Worm and Reusch, 2008)	*Zostera marina*	*54.683333, 10.000000*
2008	(Reusch *et al.*, 2008)	*Zostera marina*	*54.683333, 10.000000*
2008	(Nejrup and Pedersen, 2008)	*Zostera marina*	*55.442505, 10.448000*
2007	(Koch *et al.*, 2007)	*Halodule wrightii* and *Thalassia testudinum*	*25.228056, -80.793611; 24.922222, -80.792500*
2006	(Ransbotyn and Reusch, 2006)	*Zostera marina*	*54.427500, 10.172778*
2006	(Campbell, McKenzie and Kerville, 2006)	*Halophila ovalis, Zostera capricorni, Syringodium isoetifolium, Cymodocea rotundata, Cymodocea serrulata, Halodule uninervis,* and *Thalassia hemprichii*	*-38.250000, 145.350000; -16.883333, 145.766667*
2001	(Seddon and Cheshire, 2001)	*Amphibolis antarctica* and *Posidonia australis*	*-33.516667, 137.891667*
1999	(Ralph, 1999)	*Halophila ovalis*	*-33.846119, 151.248425*
1998	(Ralph, 1998)	*Halophila ovalis*	*-33.846119, 151.248425*
1997	(Masini and Manning, 1997)	*Posidonia sinuosa, Posidonia australis, Amphibolis griffithii,* and *Amphibolis antarctica*	*-31.983333, 115.733333*
1995	(Walker and Cambridge, 1995)	*Amphibolis antarctica and Amphibolis griffithii*	*-29.300000, 114.883333*
1995	(Masini *et al.*, 1995)	*Posidonia sinuosa, Posidonia australis,* and *Amphibolis griffithii*	*-35.053333, 117.910000*
1992	(Romero and Perez, 1992)	*Cymodocea nodosa*	*40.633683, 0.805979*
1989	(Zimmerman, Smith and Alberte, 1989)	*Zostera marina*	*36.607364, -121.872065*
1986	(Evans, Webb and Penhale, 1986)	*Zostera marina* and *Ruppia maritima*	*37.266667, -76.350000*

1986	(Marsh, Dennison and Alberte, 1986)	*Zostera marina*	*41.525319, -70.677860*
1985	(Barber and Behrens, 1985)	*Thalassia testudinum* and *Syringodium filiforme*	*27.799059, -82.526777*
1985	(Kerr and Strother, 1985)	*Zostera muelleri*	*-38.233333, 144.666667*

Table AVI.2 *Number of studied populations from each seagrass species used for thermal-related studies from 1985 to 2020. Data were collected from Google Scholar as indicated in the legend to* ***Fig. 6.1****.*

Species	Number of studied populations
Zostera marina	19
Posidonia oceanica	14
Cymodocea nodosa	10
Zostera muelleri	5
Halophila ovalis	5
Halodule uninervis	5
Thalassia hemprichii	5
Zostera noltii	4
Cymodocea serrulata	4
Zostera japonica	3
Amphibolis antarctica	3
Amphibolis griffithii	3
Posidonia australis	3
Posidonia sinuosa	2
Cymodocea rotundata	2
Thalassia testudinum	2
Enhalus acoroides	2
Halophia stipulacea	2
Halodule wrightii	1
Syringodium isoetifolium	1
Zostera capricorni	1
Thalassodendron ciliatum	1
Syringodium filiforme	1
Ruppia maritima	1

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