

# Photo-physiology and morphology reveal divergent warming responses in northern and southern hemisphere seagrasses

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11 **Keywords:** seagrasses, ocean warming, *Posidonia oceanica*, *Cymodocea nodosa*, *Posidonia australis*, *Zostera*  
12 *muelleri*, climax, pioneer, hemisphere, oceanic variability, heatwave

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21 **Abstract**

22 A better understanding of species and population responses to thermal stress is critical to predict changes in their  
23 distribution under warming scenarios. Seagrasses are a unique group of marine plants that play fundamental roles in  
24 marine environments and provide vital ecosystem services. Nevertheless, previous studies on seagrass thermal  
25 tolerance have focused exclusively on a handful of species, with the majority of these remaining virtually  
26 unexplored. Moreover, to date, no study has compared the response to thermal stress between northern and southern  
27 hemisphere seagrasses. Here, we conducted comparative mesocosm experiments using four seagrass species from  
28 the northern (i.e. Mediterranean: *Posidonia oceanica*, *Cymodocea nodosa*) and southern (i.e. Australia: *Posidonia*  
29 *australis* and *Zostera muelleri*) hemisphere as representative of two different life strategies, i.e. climax (*P. oceanica*,  
30 *P. australis*) and pioneer (*C. nodosa*, *Z. muelleri*). Plants acclimatized to the mesocosm conditions at ambient  
31 temperature (i.e. 26°C) during a five-week period, were exposed to a simulated marine heatwave (i.e. 32°C) for two  
32 weeks. Measurements of plant responses, including photo-physiology, morphology, and pigment content, were  
33 performed at the end of the warming exposure. Results showed that warming had no significant effects on  
34 photosynthetic performances of northern hemisphere seagrasses while negatively impacted their southern  
35 hemisphere counterparts. Similarly, warming favored the growth of northern hemisphere plants, but strongly  
36 inhibited the development of southern hemisphere species. Furthermore, photo-physiological and pigment content  
37 results suggested pioneer seagrasses better dealt with warming than climax species. Our study provides more  
38 insights into the field of seagrass ecology and yields potential implication for future seagrass conservation and  
39 restoration activities.

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## 53 1. Introduction

54 Seagrasses are a unique group of marine angiosperms occupying thousands of square kilometers along the shorelines  
55 of all continents, except for Antarctica (Short et al. 2007). Being among the most important habitat-forming species  
56 in the marine environment, seagrasses provide high primary productivity and nursery habitats for fish and  
57 invertebrates (Unsworth et al. 2019). Seagrasses help to stabilize coastal sediments, thus preventing erosion (Orth et  
58 al. 2006), and reduce pathogenic threats for humans, fish, and invertebrates (Lamb et al. 2017). Given that a vast  
59 majority of the human population inhabits coastal areas, seagrasses directly or indirectly influence the livelihoods of  
60 billions of people worldwide (Bertelli and Unsworth 2014). Economically, seagrass meadows are ranked among the  
61 most valuable ecosystems on Earth, contributing nearly \$2 trillion annually in ecosystem services (Waycott et al.  
62 2009; Costanza et al. 2014). Moreover, seagrasses represent the most significant natural carbon sink on our planet  
63 (Fourqurean et al. 2012; Macreadie and Hardy 2018), which can potentially be a part of future ocean solutions  
64 helping us to mitigate the negative effect of greenhouse gas emissions (Gattuso et al. 2018).

65 Nonetheless, seagrasses have undergone a global decline mainly due to human-induced environmental changes  
66 (Orth et al. 2006; Waycott et al. 2009). Across the globe, human activities are wiping out over 100 km<sup>2</sup> of seagrass  
67 meadows per annum. As a result, nearly 29% of their areal extent has been lost since 1879 (Waycott et al. 2009). For  
68 instance, the Mediterranean endemic species, *Posidonia oceanica*, already lost approximately 13-50% of its total  
69 areal extent since the mid-nineteenth century (Telesca et al. 2015). The decline of *P. oceanica* meadows is likely to  
70 continue as this species' ecological functions have even been predicted to go extinct by the end of this century  
71 (Marbà and Duarte 2010; Chefaoui et al. 2018).

72 Among human-induced stressors to seagrasses, ocean warming appears to be a key player (Nguyen et al. 2021).  
73 While ocean warming is commonly known as the gradual increment in the mean of seawater temperature, it also  
74 affects species in the form of extreme climatic events (i.e. marine heatwaves). Marine heatwaves (MHWs) are  
75 defined as abnormal warming events that last for over 5 days, with temperatures exceeding the 90<sup>th</sup> percentile of a  
76 three-decade historical baseline database (Hobday et al. 2016). For seagrasses (but also for other benthic organisms),  
77 the impact of MHWs is generally more detrimental than increases in mean seawater temperatures because seagrasses  
78 are generally susceptible to sudden thermal changes (Smale et al. 2019). In fact, MHWs have produced devastating  
79 consequences for seagrasses and associated communities across the globe (Coma et al. 2009; Harley et al. 2012;  
80 Wernberg et al. 2016; Smale 2020). MHWs were the main cause of massive die-off events of seagrass species  
81 including *Zostera marina* (Jarvis et al. 2014) and *Amphibolis antarctica* (Arias-Ortiz et al. 2018; Strydom et al.  
82 2020). MHWs not only cause the decline of seagrass meadows and all their ecological services but also fostered the  
83 release of greenhouse gases into the atmosphere, consequently contributing to the on-going global warming (Arias-  
84 Ortiz et al. 2018; Macreadie and Hardy 2018; Salinas et al. 2020).

85 With insights from previous studies, we now know that the capacity to cope with warming (or especially MHWs)  
86 varies among different seagrass species (Marín-Guirao et al. 2016; Collier et al. 2017; Nguyen et al. 2020b), but also

87 among populations of the same species from contrasting thermal environments (e.g. Bergmann et al. 2010; Winters  
88 et al. 2011; Marín-Guirao et al. 2018; Marín-Guirao et al. 2019). However, to date, the tolerance to anomalous  
89 thermal events of the majority of seagrasses (especially in the region of southeast Asia and northern Australia, a  
90 hotspot of seagrass diversity) are yet to be investigated (see Nguyen et al. 2021 for a complete review). Indeed, to  
91 the best of our knowledge, no study has compared the responses of northern versus southern hemisphere seagrasses  
92 to warming.

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

102 Studies from terrestrial plants have documented dissimilarities in response to environmental stressors between  
103 climax versus pioneer plants. For instance, studies from the Brazilian Atlantic Forest showed that pioneer trees were  
104 more tolerant against oxidative stress than climax plants (Favaretto et al. 2011; Brandão et al. 2017; Esposito et al.  
105 2018). In line with these studies from the southern hemisphere, a study from the Mediterranean region  
106 experimentally tested the responses of carbon assimilation under summer stress conditions (water deficits, high  
107 light, and temperature) in four Mediterranean trees, including climax and pioneer species (Faria et al. 1998). This  
108 study indicated that, although both groups of trees suffered a decline in their photosynthetic capacities, the climax  
109 plants exhibited the lowest photosynthetic rates and the highest proportion of carotenoids to chlorophyll (i.e. an  
110 indicator of photo-protective mechanism activated under stressful conditions) while pioneer species maintained  
111 higher photosynthetic rates (Faria et al. 1998). Hence, environmental stressors can impact more strongly climax  
112 species, favoring the persistence of less complex and stable ecosystems, and providing less valuable ecosystem  
113 services. This is true also for seagrasses (Johnson et al. 2003; Hyndes et al. 2016; Shields et al. 2019), where it  
114 appears essential to assess the response to stress of both climax and pioneer species, in order to support timely and  
115 effective conservation and/or restoration actions. Few studies have experimentally compared the response to  
116 warming of climax and pioneer seagrass species (e.g. see Masini and Manning 1997; Seddon and Cheshire 2001;  
117 Campbell et al. 2006; Collier and Waycott 2014; Marín-Guirao et al. 2016, 2018; Collier et al. 2017; Tutar et al.  
118 2017). Most of them suggested that pioneer species are more thermal tolerant than climax ones. These studies  
119 demonstrated that the fast-growing pioneer seagrasses exhibited a better ability to maintain unaltered plant carbon  
120 balances through improved photosynthetic thermal stability and performance as well as by inhibiting respiratory  
121 carbon consumption. Moreover, through a higher morphological plasticity, pioneer species can modify their plant  
122 architecture by increasing the above-ground (photosynthetic)/below-ground (non-photosynthetic) biomass ratio to

123 deal with thermal stress (Collier et al. 2017; Marín-Guirao et al. 2018), and have also an increased ability to activate  
124 antioxidant defense mechanisms to protect themselves from heat-stress induced oxidative damage (Tutar et al.  
125 2017). Notwithstanding these evidences, the number of studies on this topic, especially on species with overlapping  
126 geographical distribution, remains scarce and deserves more effort.

127 In the present study, four seagrass species including *P. oceanica* and *C. nodosa* from the Mediterranean (northern  
128 hemisphere) and *P. australis* and *Z. muelleri* from South East Australia (southern hemisphere) were selected for a  
129 comparative study of their responses to warming. Plants were collected in the same seasonal conditions (i.e. late  
130 summer-early autumn: Mar-May in the southern hemisphere and Sept-Nov in the northern hemisphere) from both  
131 geographic areas and two mesocosm experiments were conducted following the same design. Our study represents a  
132 unique opportunity to compare (1) two climax species of the genus *Posidonia* (*P. oceanica* and *P. australis*) with  
133 similar characteristics and ecological functions but distributed in the two hemispheres and (2) two couples of  
134 climax-pioneer species from both hemispheres (*P. oceanica* vs. *C. nodosa* and *P. australis* vs. *Z. muelleri*). On the  
135 first hand, we hypothesized that the responses to warming of the two *Posidonia* species (i.e. *P. oceanica* and *P.*  
136 *australis*) would be different because sampled populations live under a different thermal regime (i.e. 13 – 28°C for  
137 *P. oceanica*; Fig. 1b and 17 – 26°C for *P. australis*; Fig. 1c) and because the species thermal ranges are also different  
138 (i.e. 8 – 30°C for *P. oceanica* and 12 – 28°C for *P. australis*; Fig. S1). Additionally, we note that the collection sites  
139 of the Australian seagrasses in this study did not fall into any Mediterranean-climate regions (see Cowling et al.  
140 1996 for a map of Mediterranean-climate regions and Fig. 1a for sample collection sites). On the other hand, in both  
141 hemispheres, the climax seagrasses are expected to suffer more from thermal stress than their pioneer counterparts.

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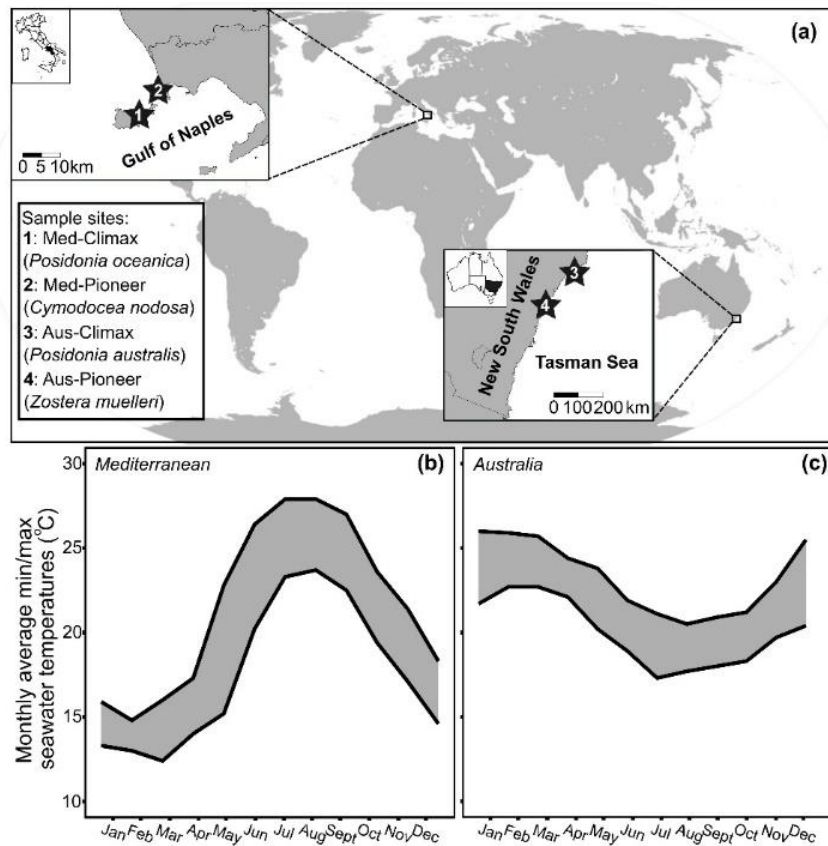
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149 Figure 1: Sample collection sites (a) and temperature conditions at collection sites (b, c). (b) Monthly average sea surface  
 150 temperature in Ischia, Italy (Mediterranean sites: 1 & 2). (c) Monthly average sea temperature in Port Stephens, NSW, Australia  
 151 (Australian sites: 3 & 4). Data were taken from World sea temperature of 2020 (<https://www.seatemperature.org/> data assessed  
 152 on 29<sup>th</sup> Nov 2020).

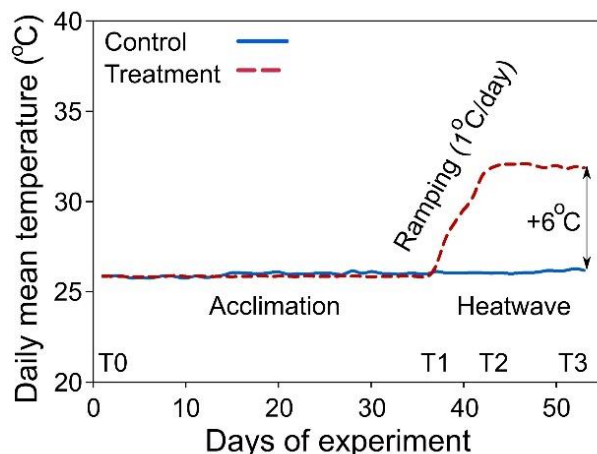
## 153 2. Materials and methods

### 154 2.1. Experiment 1: Northern hemisphere experiment

#### 155 2.1.1. Targeted species and Plant collection

156 *Posidonia oceanica* is endemic to the Mediterranean Sea (see Fig. S1 for the species distribution) and forms large  
 157 and dense monospecific meadows on rocks and sandy seabed ranging from shallow water (less than 1 m) down to 45  
 158 meter's depth (Procaccini et al. 2003). It ranks as one of the slowest-growing plants and among the longest-living  
 159 plants on Earth with single clones extending over kilometers and living for hundreds to thousands of years (Arnaud-  
 160 Haond et al. 2012). *Cymodocea nodosa* distributes throughout the Mediterranean Sea and extends also in nearby  
 161 subtropical Atlantic areas (see Fig. S1 for *C. nodosa* distribution). *C. nodosa* is a relative fast-growing species,  
 162 commonly found in shallow waters in both sandy and mud substrates where it forms both monospecific and mixed  
 163 meadows with other seagrass species (den Hartog 1970; Guidetti et al. 1998). Hereafter, we use Med-Climax for *P.*  
 164 *oceanica* and Med-Pioneer for *C. nodosa*.

165 Plant fragments (i.e. ramets) of Med-Climax (40°44.020'N, 13°58.039'E at 5-6 m depth; Fig. 1a-1) and Med-Pioneer  
 166 (40°47.021'N, 14°04.404'E at 8-10 m depth; Fig. 1a-2) were haphazardly collected by SCUBA diving in the Gulf of  
 167 Naples (Italy) on the 18<sup>th</sup> September 2019. To reduce the likelihood of sampling the same genotype twice, plants  
 168 were collected at a minimum distance of 10 m from each other. Both *P. oceanica* and *C. nodosa* experience a wide  
 169 species thermal range from 8°C in the winter (especially in the northern Adriatic Sea) to 30°C in the summer  
 170 (especially in the eastern Mediterranean Sea), see Fig S1 for more details. The two populations used in this study  
 171 came from a similar thermal condition (i.e. 13-28°C, see Fig. 1b) which falls in the middle of the species thermal  
 172 range, therefore excluding the existence of a potential range-edge effects for the selected populations. After  
 173 collection, plants were kept in dark in a cooler filled with seawater at ambient temperature and transported to a  
 174 benthic mesocosm facility at the Stazione Zoologica Anton Dohrn (SZN), Napoli, Italy (see Ruocco et al. 2019b for  
 175 a detailed description of the experimental system). Light intensity, salinity, and seawater temperature were measured  
 176 at the time of plant sampling for setting up the experimental system.



177 Figure 2: Temperature profile during the two experiments.

### 178 2.1.2. Experimental system

179 Once at the SZN experimental facility, twelve plant fragments (i.e. ramets) of each species composed by horizontal  
 180 rhizomes of similar size and a similar number of interconnected vertical shoots (~ 10 shoots) were selected to  
 181 standardize the experiment. Med-Climax ramets were transplanted in six plastic pots (i.e. two ramets per pot) filled  
 182 with coarse carbonate sediments as described in Ruocco et al. (2019b), while Med-Pioneer ramets were transplanted  
 183 in twelve plastic pots (i.e. one ramet per pot) filled with natural sediments from the collection site as described in  
 184 Marín-Guirao et al. (2018). After transplantation, pots of each species were randomly allocated into six 500L-  
 185 aquaria with filtered and UV-treated natural seawater from a close area; each aquarium containing two ramets of the  
 186 Med-Climax species and two ramets of the Med-Pioneer species. Transplant pots were distributed within aquaria to  
 187 avoid crossed-species shading and their distance from the light source adjusted to reproduce similar light intensities  
 188 to those measured at their collection sites (i.e. max noon irradiance: 300 and 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  above the  
 189 leaf canopy for Med-Climax and Med-Pioneer, respectively). A 12h:12h light:dark photoperiod was applied, starting  
 190 from 7:00 a.m., and progressively increasing to the maximum irradiance at 13:00 before a gradual reduction until

191 dark at 19:00. Water temperature was measured automatically every 10 min using HOBO Pendant®  
192 Temperature/Light 64K Data Logger (Onset, USA) and manually checked twice a day with WTW Cond 3310 Set 1  
193 (Xylem Analytics, Germany). Seawater salinity of  $37.5 \pm 0.2$  was kept constant throughout the experiment through  
194 regular additions of purified water. Seawater quality was controlled via continuous mechanical filtration, weekly-  
195 UV sterilizations, and partial renewals. An introductory video was made for this experiment and could be found on  
196 the website of Dr. Gabriele Procaccini's Laboratory (<https://gpgroupsxn.wixsite.com/website>; video: EpicSea2019).

## 197 **2.2. Experiment 2: Southern hemisphere experiment**

### 198 **2.2.1. Targeted species and Plant collection**

199 *Posidonia australis* is a slow-growing species found on sandy sediment between 1 to 15 m (Trautman and  
200 Borowitzka 1999). This species is distributed along the southern half of Australia, from Shark Bay in Western  
201 Australia to Port Macquarie in New South Wales, and along the northern coast of Tasmania (Fig. S1). With this  
202 distribution, *P. australis* exhibits the narrowest species thermal range (i.e. 12 – 28°C) of the four seagrass species  
203 included in this study. *Zostera muelleri* is a fast-growing species, commonly found in shallow water (< 4 m depth)  
204 on different sediments including fine sand, mud and others (Larkum et al. 2018). *Z. muelleri* is distributed along the  
205 eastern coast of Australia, Tasmania Kangaroo Island, Lord Howe Island, and New Zealand (Fig. S1; Waycott et al.  
206 2004). The species' thermal range varies between 9°C in the winter season (especially in the southern Tasmania  
207 Island and New Zealand) and 31°C during summer (see Fig. S1). Hereafter, we use Aus-Climax for *P. australis* and  
208 Aus-Pioneer for *Z. muelleri*.

209 Ramets of Aus-Climax and Aus-Pioneer were collected, at distances > 25 m one from another to reduce the chance  
210 of sampling the same genotype twice. Plant fragments (i.e. ramets) were collected during low tides at ~70 cm depth  
211 at Port Stephens (PS), New South Wales (NSW), Australia (32°43'07.4"S 152°10'35.9"E; Fig. 1a) on the 19<sup>th</sup> of  
212 March 2019 and at Church Point (CP), NSW, Australia (33°38'46.8"S 151°17'11.9"E; Fig. 1a) on the 23<sup>rd</sup> of March  
213 2019, respectively. Temperature ranges at both sampling locations between 17°C in winter and 26°C in summer. The  
214 maximum summer temperature is below the upper limits of species thermal ranges (i.e. 28°C and 31°C for *P.*  
215 *australis* and *Z. muelleri*, respectively; Fig. S1). Neither one of the two populations analyzed were at the edge of the  
216 species distribution range, but we acknowledged, in the interpretation of results, the difference in the upper limit of  
217 species thermal ranges. Light intensity and salinity were also measured at the time of sample collection. Plant  
218 materials were brought to the seagrass mesocosm facility at the University of Technology Sydney (UTS) soon after  
219 collection. A detailed description of the experimental system can be found in Nguyen et al. (2020b).

### 220 **2.2.2. Experimental system**

221 As soon as arrived at UTS, twelve ramets of each species with a similar number of shoots (i.e. 8-10 shoots) were  
222 selected and transplanted in individual plastic pots (i.e. one ramet per pot) filled with mini pebbles. Subsequently,  
223 pots were randomly distributed in tanks of the mesocosm facility: six 60-L aquaria for housing Aus-Climax pots and  
224 six 40-L aquaria for Aus-Pioneer pots (i.e. two ramets per aquarium). For both species, the irradiance level was set  
225 with a max. noon irradiance of  $350 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  at canopy height and a 12 h:12 h light:dark photo-period.



226 Light cycle started from 7:30 a.m., with light levels progressively increasing to the maximum irradiance at 12:30  
227 p.m. and kept for 2 hours, before a progressive reduction until dark at 7:30 p.m. Water temperature was measured  
228 automatically every 30 min using iButton data logger (iButtonLink, USA) and manually checked twice a day using a  
229 digital thermometer (FLUKE 52II, USA). During the experiment, purified water was added periodically to maintain  
230 constant seawater salinity of  $34 \pm 0.2$  similar to those in the fields. Approximately one third of seawater was renewed  
231 weekly in each aquarium to maintain water quality.

### 232 **2.3. Experimental design**

233 Both the northern and the southern hemisphere experiments shared the same experimental design. After  
234 transplantation and allocation within aquaria, plants of the four studied species were allowed to acclimate at 26°C,  
235 which is similar to the seawater temperatures recorded during plant collection at the four studied populations (i.e. in  
236 every case the difference was lower than 1°C). After a 5-week acclimation period, temperature in half of the aquaria  
237 containing each seagrass species (i.e. 3 aquaria) was progressively increased up to 32°C at a heating rate of 1°C day<sup>-1</sup>  
238 to simulate a marine heatwave (MHW); whereas the temperature in the rest of aquaria was maintained throughout  
239 the experiment (Fig 2). Therefore, for each species, three tanks were randomly assigned to heat treatment (TM) and  
240 other three remained as controls (CT). Seagrass responses were analyzed at the end of the MHW exposure, which  
241 lasted 12 and 10 days in the northern and southern hemisphere experiments, respectively. The aquarium is the true  
242 experimental unit for each seagrass species and variable, so that measurements performed on plants of the same  
243 aquarium (i.e. 'pseudo replicates') were averaged to obtain an independent replicated value. Therefore, the number  
244 of replicates used in statistical tests was  $n = 3$ .

### 245 **2.4. Chlorophyll *a* fluorescence**

246 Identical Diving-PAM fluorometers (WALZ, Germany) were used to determine the photo-physiological responses  
247 of the four studied seagrass species (Med-Climax, Med-Pioneer, Aus-Climax, and Aus-Pioneer) following the  
248 methodology described in Marín-Guirao et al. (2013). To standardize the procedure, two chlorophyll *a* fluorescence  
249 measurements were conducted on the same middle portion of the second youngest leaf of each plant (Ruocco et al.,  
250 2019a). Measurements included (a) maximum quantum yield ( $F_v/F_m$ ) of photosystem II (PSII) measured on night  
251 dark-adapted plants (around 6:00 - 7:00 am before the light cycle started), (b) Effective quantum yield ( $\Delta F/F_m'$ )  
252 measured on light-adapted plants (around 12:30 - 13:30 while the irradiances were highest) and (c) Non-  
253 photochemical quenching ( $NPQ$ ) calculated by using the method described elsewhere (Maxwell and Johnson 2000)  
254 to estimate the amount of photosynthetic energy lost as heat (i.e. the photo-protective mechanisms associated to the  
255 xanthophyll-cycle pigments; Marín-Guirao et al. 2013).

### 256 **2.5. Plant growth**

257 For both experiments, plant growth measurements were performed by adopting the leaf marking method (Zieman  
258 1974). Two plants from each aquarium and species were marked at the same position above the ligule at the end of  
259 the acclimation period and subsequently collected at the end of the heatwave to measure leaf elongation (mm). Then,

260 the newly developed leaf segments were cleaned of epiphytes and dehydrated at 70°C for 24h before being weighted  
261 to assess biomass production (mg Dry weight).

## 262 **2.6. Pigment content**

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

## 276 **2.7. Statistical analyses**

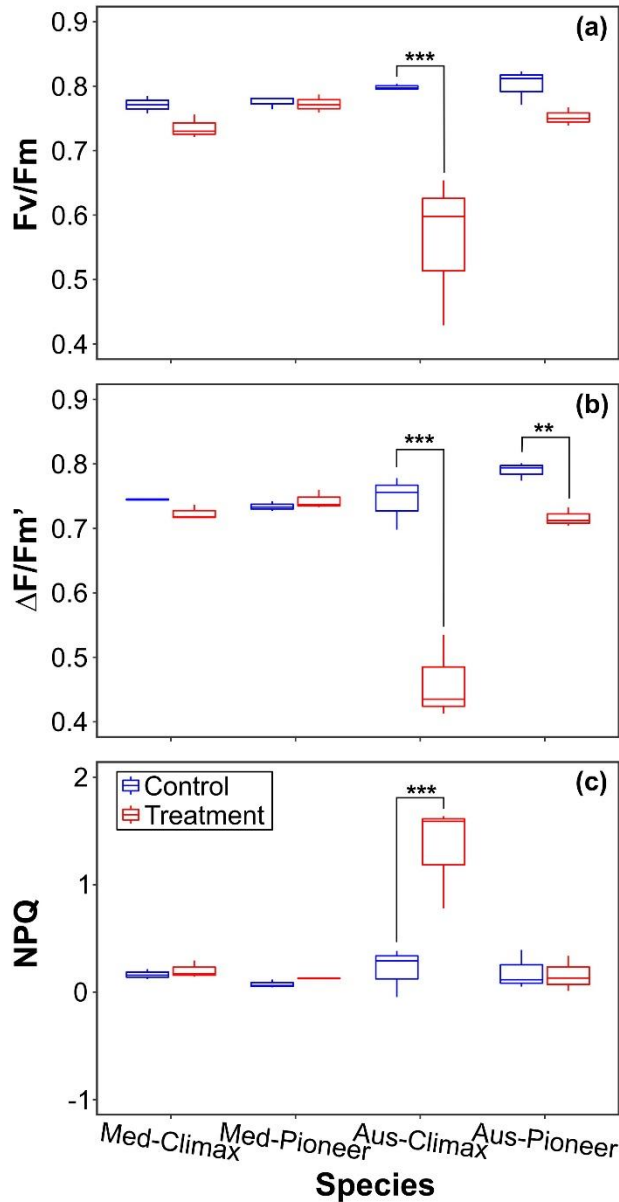
277 The response of seagrasses to experimental conditions was assessed using a three-way *ANOVA* ( $n = 3$ ), including the  
278 following factors: Hemisphere (2 levels: northern and southern, fixed), Life-strategy (2 levels: climax and pioneer,  
279 fixed), and Treatment (2 levels: control and treatment, fixed). Cochran's C test was used to test homogeneity of  
280 variances and data were square root transformed when necessary. Data were analyzed even when homogeneity of  
281 variances could not be achieved, as *ANOVA* is robust for this kind of assumption when the sizes of samples are equal  
282 (Underwood et al. 1997). However, in this case, the significance was judged more conservatively ( $p < 0.01$ ) when  
283 interpreting results to reduce the livelihood of Type I error (which is inflated by heterogeneous variances). For each  
284 measurement, Student-Newman-Keuls (SNK) *post-hoc* tests were used to identify significant differences between  
285 (1) control versus treatment plants of each Hemisphere, each Life-strategy, (2) northern versus southern plants of  
286 each Life-strategy, each Treatment, and (3) climax versus pioneer plants of each Hemisphere, each Treatment. All  
287 statistical analyses were conducted in R-studio v.1.2.5033 (R Core Team 2018) using package *GAD* (Sandrini-Neto  
288 and Camargo 2014).

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

## 290 **3. Results**

### 291 **3.1. Photo-physiological responses**

292



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294 Figure 3: Boxplot graphs present photo-physiological results at the end of the experiment ( $n = 3$ ). (a) Maximum quantum yield  
 295 ( $F_v/F_m$ ), (b) Effective quantum yield ( $\Delta F/F_m'$ ), and (c) Non-Photochemical quenching ( $NPQ$ ). Asterisks indicate statistical  
 296 differences between control and treatment within each species (Student-Newman-Keuls *post-hoc* test, \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ,  
 297 more details can be found in Supplementary data, Table S1).

298 3.1.1. Northern versus southern hemisphere seagrasses

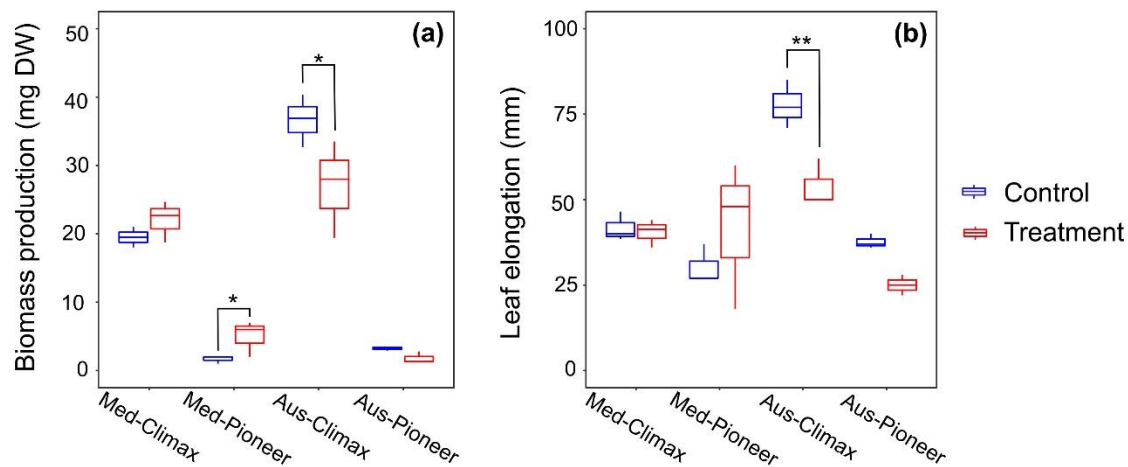
299 Warming had strong impacts on the southern hemisphere seagrasses while did not result in any significant changes  
 300 for the northern hemisphere plants (ANOVA:  $H \times T$ ,  $F(1,16) = 12.030$ ,  $p < 0.01$ ) (Fig. 3). Warming significantly  
 301 reduced  $F_v/F_m$  of the Aus-Climax plants (SNK test for 'H:L:T' among 'T' within 'H:L':  $p < 0.001$ ; Fig. 3, Table  
 302 S1), while slightly impacted the Med-Climax plants. Similarly, the Aus-Climax plants enhanced their  $NPQ$  with

303 warming (SNK test for 'H:L:T' among 'T' within 'H:L':  $p < 0.001$ ; Fig. 3, Table S1) while Med-Climax's  $NPQ$   
 304 remained relatively unchanged (Fig. 3). Both Aus-species dramatically lowered their  $\Delta F/Fm'$  as a result of thermal  
 305 stress (Fig. 3, Table 1), while the  $\Delta F/Fm'$  values of Med-plants were not negatively affected but, rather, slightly  
 306 increased under warming in the case of the Med-Pioneer species (Fig. 3, Table 1). As a consequence, we detected a  
 307 significant interaction in  $H \times L \times T$  for  $\Delta F/Fm'$  measurements (ANOVA:  $F(1,16) = 14.267$ ,  $p < 0.01$ ). It is important  
 308 to highlight that while the control plants exhibited a similar level of performance, heated Climax plants from the two  
 309 hemispheres responded differently and significant differences were detected from all photo-physiological  
 310 measurements (SNK test for 'H:L:T' among 'H' within 'L:T':  $p < 0.001$ ; Table S1).

### 311 3.1.2. Climax versus pioneer seagrasses

312 The simulated MHW strongly impacted the photosynthetic performances (both  $F_v/F_m$  and  $\Delta F/F_m'$ ) of Aus-Climax  
 313 plants, however, the level of warming impacts were much lower in the Aus-Pioneer plants (Fig. 3). Climax-pioneer  
 314 dissimilarities in response to warming were also found in the activation of NPQ machinery. While Aus-Climax  
 315 plants significantly activated their NPQ machinery (Fig. 3) as mentioned above, on the other hand, Aus-Pioneer  
 316 plants did not alter their NPQ even at the same warming condition (Fig. 3). This is also evidenced from the SNK  
 317 results for 'H:L:T' among 'L' within 'H:T' when no significant difference detected for Aus-control plants but Aus-  
 318 treatment plants (SNK test:  $p < 0.001$ ; Table S1).

### 319 3.2. Plant growth responses



320  
 321 Figure 4: Boxplot graphs present plant growth response results at the end of the experiments ( $n = 3$ ). Asterisks indicate statistical  
 322 differences between control and treatment within each species (Student-Newman-Keuls *post-hoc* test, \*  $p < 0.05$ , \*\*  $p < 0.01$ ,  
 323 more details can be found in Supplementary data, Table S1).

### 324 3.2.1. Northern versus southern hemisphere seagrasses

325 There were differences in response to warming between northern versus southern hemisphere seagrasses in both  
 326 biomass production and leaf elongation measurements (Fig. 4), as shown by the significant  $H \times T$  interactions  
 327 (ANOVA:  $F(1,16) = 14.532$ ,  $p < 0.01$  and  $F(1,16) = 10.151$ ,  $p < 0.01$ , respectively). Among climax plants,

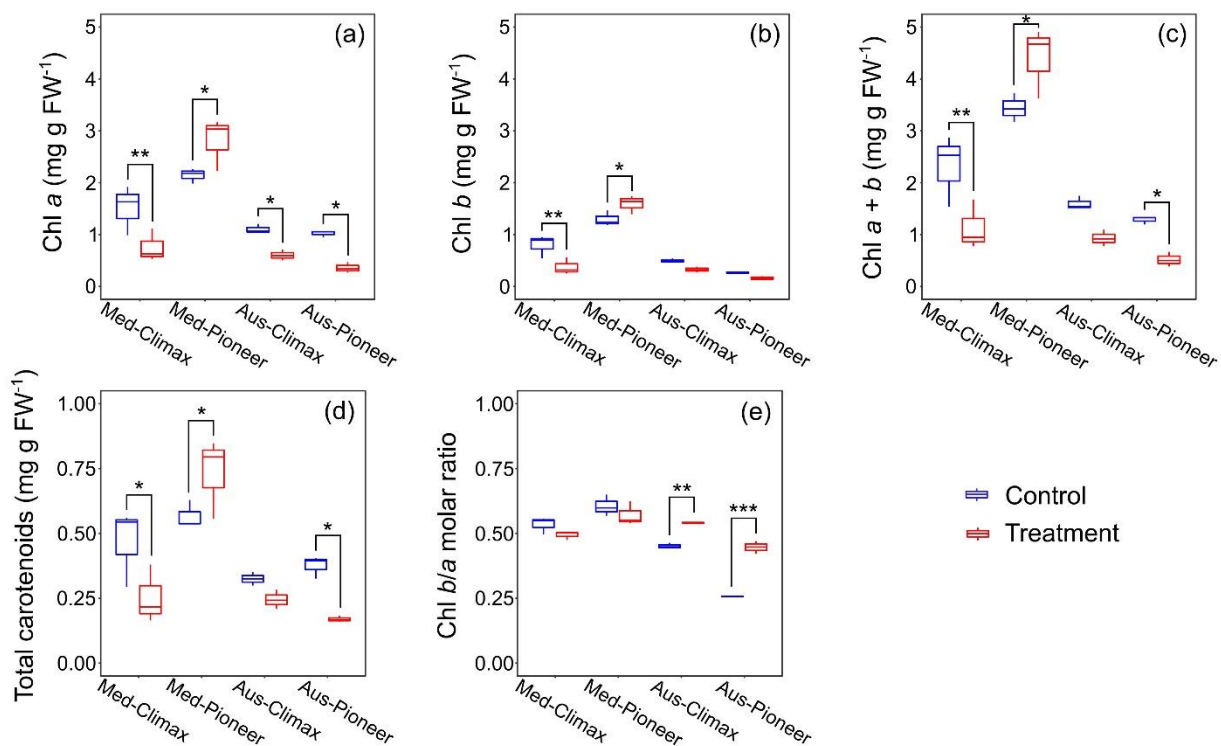
328 warming significantly reduced biomass production (SNK test:  $p < 0.05$ ; Fig. 4a) as well as leaf elongation (SNK  
 329 test:  $p < 0.01$ ; Fig. 4b) of the southern plants. On the other hand, warming favored the developments of the northern  
 330 ones in terms of productivity (Fig. 4a,b). Differently, warming increased the growth of northern pioneer plants (e.g.  
 331 a significant difference between control versus treatment detected for biomass production, SNK test:  $p < 0.05$ ; Fig.  
 332 4a). In contrast, the southern pioneer plants suffered a reduction in growth as a result of their exposure to a  
 333 simulated MHW (Fig. 4a,b).

### 334 3.2.2. Climax versus pioneer seagrasses

335 Even if we did not detect any significant difference between climax versus pioneer species within each hemisphere  
 336 (ANOVA:  $L \times T$ ,  $p > 0.05$  for both plant growth response measurements), it is interesting to note that there were  
 337 significant interactions of  $H \times L$  for both biomass production and leaf elongation (ANOVA:  $F(1,16) = 13.540$ ,  $p <$   
 338  $0.01$  and  $F(1,16) = 16.271$ ,  $p < 0.001$ , respectively). For Med-seagrasses, even if warming generally enhanced the  
 339 developments of both Med-Climax plants and Med-Pioneer plants, the levels of increments were significantly higher  
 340 in Med-Pioneer plants in comparison with its climax counterpart (Fig. 4a,b). Differently, Aus-Climax plants  
 341 exhibited greater impact of warming when compared with their pioneer counterparts (Fig. 4a,b) with significant  
 342 differences between control versus treatment detected for both plant growth response measurements only for Aus-  
 343 Climax plants (SNK test:  $p < 0.05$  and  $p < 0.01$ ; Fig. 4a, b).

### 344 3.3. Pigment content responses

345



346

347 Figure 5: Boxplot graphs of pigment results at the end HW exposure ( $n = 3$ ). Asterisks indicate statistical differences between  
348 control and treatment within each species (Student-Newman-Keuls *post-hoc* test, \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , more  
349 details can be found in Supplementary data, Table S1).

350 Results from pigment content measurements showed complex interactions between northern versus southern as well  
351 as climax versus pioneer seagrasses in response to warming. Significant interactions were detected in H×L×T for all  
352 pigment measuring parameters (ANOVA,  $p < 0.05$ , Table 1). Details are presented below.

### 353 3.3.1. Northern versus southern hemisphere seagrasses

354 Warming significantly reduced all pigments content of Med-Climax plants such as Chl *a* (SNK test:  $p < 0.01$ , Fig.  
355 5a), Chl *b* (SNK test:  $p < 0.01$ , Fig. 5b), Chl *a+b* (SNK test:  $p < 0.01$ , Fig. 5c), and total carotenoids (SNK test:  $p <$   
356 0.01, Fig. 5d) but did not result in any significant reduction in pigment content for Aus-Climax plants (except for the  
357 case of Chl *a*, although the level of reduction was greater in Med-Climax plants; Fig. 5a,b,c,d). Interestingly, while  
358 Med-Climax plants maintained their Chl *b/a* molar ratio during HW, Aus-Climax plants significantly increased the  
359 ratio (SNK test:  $p < 0.01$ , Fig. 5e). It is worth mentioning that while Med-Pioneer plants accumulated more pigment  
360 content under the increased temperature, Aus-Pioneer plants reduced the accumulation of these pigments (see Fig.  
361 5a,b,c,d). Likewise, Aus-Pioneer plants increased their Chl *b/a* molar ratio as a result of warming, while Med-  
362 Pioneer plants exposed to warming showed values similar to control plants (Fig. 5e).

### 363 3.3.2. Climax versus pioneer seagrasses

364 Warming greatly impacted the Med-Climax plants in terms of pigment contents including Chl *a*, Chl *b*, Chl *a+b* as  
365 well as total carotenoids with significant differences detected between control versus heated plants across all these  
366 measurements (Fig. 5a,b,c,d). On the contrary, warmed Med-Pioneer plants significantly improved pigment contents  
367 as a result of warming (Fig. 5a,b,c,d). Furthermore, we assessed a statistical difference between heated Med-Climax  
368 plants versus heated Med-Pioneer plants in terms of total carotenoids' response (SNK test for 'H:L:T' among 'L'  
369 within 'H:T':  $p < 0.001$ ; Table S1). For the southern hemisphere plants, warming negatively affected both climax  
370 and pioneer plants in terms of pigments (Fig. 5).

371

372

Source of variation	Fv/Fm				$\Delta F/Fm'$				NPQ				Biomass production				Leaf elongation			
	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	$\phi$
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	$\phi$	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	$\phi$	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				<i>Sqrt</i>				<i>Sqrt</i>				None		
Cochran's C test		$p < 0.001$				$p < 0.01$				$p > 0.05$				$p > 0.05$				$p < 0.001$		

Source of variation	Chlorophyll a				Chlorophyll b				Chlorophyll a+b				Total carotenoids				Chlorophyll b/a molar ratio			
	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		$p > 0.05$				$p > 0.05$				$p > 0.05$				$p > 0.05$				$p > 0.05$		

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Table 1: Results of three-way ANOVA analyses. Significant codes: \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; ns  $p > 0.05$ ;  $\phi$  means  $0.01 < p < 0.05$  but not interpreted as significant because of variance heterogeneity in Cochran's C test; *Sqrt*: Square root.

## 377 4. Discussion

### 378 4.1. Difference between northern versus southern hemisphere seagrasses in response to warming

379 When two sister species of the genus *Posidonia* were exposed to a similar simulated MHW (i.e. 32°C), their photo-  
380 physiological and plant growth responses clearly demonstrated that the southern hemisphere species *P. australis* (i.e.  
381 Aus-Climax) is more sensitive to anomalous thermal events than the northern hemisphere species *P. oceanica* (i.e.  
382 Med-Climax). Warming dramatically affected the photosynthetic performance of *P. australis*, while the  
383 photosynthetic functioning of *P. oceanica* was unaffected. The impairment of the photosynthetic apparatus, reflected  
384 as a reduction in the maximum (i.e.  $F_v/F_m$ ) and effective photochemical efficiency (i.e.  $\Delta F/F_m$ ), is a common  
385 response in seagrasses subjected to thermal stress (e.g. see Winters et al. 2011; Marín-Guirao et al. 2016; Ruocco et  
386 al. 2019a; Nguyen et al. 2020a for some recent studies) and evidenced a higher photosynthetic thermal sensitivity in  
387 *P. australis* with regard to *P. oceanica*. This was further supported by the fact that only *P. australis* activated the  
388 NPQ machinery, a well-known photo-protective mechanism in plants (including seagrasses) that mitigates the  
389 damaging effects of a heat-induced photosynthetic malfunction by dissipating excess energy as heat (e.g. see Ashraf  
390 and Harris 2013 for a review in plants and Marín-Guirao et al. 2016; Ontoria et al. 2019 for some recent studies in  
391 seagrasses). Moreover, only *P. australis* experienced a significant growth inhibition during the warming exposure.  
392 Reduction in plant growth is a major consequence of growing under stress conditions and is commonly associated  
393 with photosynthetic constrains under high temperatures and with the diversion of resources from growth to sustain a  
394 heat-stress response and to repair heat-induced damage (e.g. Wahid et al. 2007; Bitá and Gerats 2013; York et al.  
395 2013; Collier et al. 2017; Marín-Guirao et al. 2018). Interestingly, while warming reduced the overall pigment  
396 content (i.e. Chl *a*, Chl *b* and carotenoids content) of *P. oceanica* plants, the same level of warming only reduced  
397 Chl *a* content in the southern hemisphere plants. This resulted in a significant Chl *b/a* molar ratio increment (i.e. a  
398 proxy of PSII antenna size), suggesting that *P. australis* attempted to counterbalance their heat-impaired  
399 photosynthetic performance by enhancing their light harvesting efficiency.

400 Both *P. oceanica* and *P. australis* together with 7 other species including *P. sinuosa*, *P. angustifolia*, *P. coriacea*, *P.*  
401 *denhartogii*, *P. kirkmanii*, *P. ostenfeldii*, and *P. robertsoniae* belong to the genus *Posidonia* which is among the  
402 most primitive marine angiosperm genus (den Hartog 1970; Kuo and Cambridge 1984). Interestingly, while *P.*  
403 *oceanica* is endemic to the Mediterranean, the other 8 species (including *P. australis*) occur exclusively in the  
404 subtropical and temperate Australian seas (Kuo and Cambridge 1984). It is still unclear when the single  
405 Mediterranean species and the Australian congeneric counterparts diverged. Phillips and Meinez (1988) suggested it  
406 could have happened during the late Eocene, about 40 million years ago (Mya), while Les et al. (2003) estimated a  
407 more recent separation of  $16.7 \pm 12.3$  Mya. A more recent study from Aires et al. (2011) predicted this divergence  
408 would have taken place much earlier in the ancient Tethys Sea (i.e. over 60 Mya). In any case, the disconnection of  
409 Mediterranean *Posidonia* with the Australian ones has allowed the two groups to evolve in two contrasting  
410 environmental and evolutionary conditions (i.e. Mediterranean Sea versus Australian Seas). Compared to the  
411 Australian Seas, the Mediterranean has undergone massive changes during its history (Bianchi, Carlo and Morri  
412 2017). Especially, due to anthropogenic climate change, the Mediterranean Sea waters have warmed up at a faster



413 pace (Bianchi, Carlo and Morri 2017; Ozer et al. 2017; Nguyen et al. 2020a), become saltier (Borghini et al. 2014),  
414 and exhibited more frequent and intense extreme oceanic events (e.g. MHWs, see Darmaraki et al. 2019). In  
415 addition, not only the species but also the studied *P. oceanica* population thrives in a broader thermal regime (i.e. 13  
416 – 28°C, Fig. 1B; see Fig. S1 for more details about species distribution and species thermal range) than *P. australis*  
417 (i.e. 17 – 26°C, Fig. 1c; see Fig. S1 for more details about species distribution and species thermal regime); and this,  
418 together with the evolutionary differences among both *Posidonia* species stated above, may explain why the  
419 northern hemisphere *Posidonia* was less affected by warming than its southern hemisphere counterpart.

420 Regarding the pioneer seagrass species, this study also pinpoints some dissimilarities in the response to warming  
421 between *C. nodosa* (i.e. Med-Pioneer) and *Z. muelleri* (i.e. Aus-Pioneer). For example, warming significantly  
422 impacted the photosynthetic functioning of *Z. muelleri* (i.e. reduced  $\Delta F/F_m'$  values) while no significant changes  
423 were detected for *C. nodosa*. Likewise, warming favored the growth and biomass production of *C. nodosa* but not  
424 for *Z. muelleri*, and similar divergences were also found in their responses at the level photosynthetic pigments.  
425 These divergent responses to seawater warming manifested that the *C. nodosa* species, which is indeed benefited by  
426 increased temperatures, is more tolerant to anomalous heat events than the *Z. muelleri*. This finding suggests that the  
427 differences in response to warming among northern and southern hemisphere seagrasses may not be limited to the  
428 genus *Posidonia*, but extended to other seagrass species across hemispheres. However, since both pioneer species  
429 belong to a different family with contrasting origins and estimated ages (*Cymodoceaceae*: 67 Mya vs. *Zosteraceae*:  
430 47Mya; Janssen and Bremer 2004; Waycott et al. 2007), the comparison is not as direct as in the two studied  
431 *Posidonia* species. Hence, further studies to compare the responses to warming of other seagrass species across  
432 hemisphere are warranted.

#### 433 **4.2. Difference between climax versus pioneer seagrasses in response to warming**

434 The northern hemisphere climax and pioneer species reacted almost in the same way to warming in terms of photo-  
435 physiology (i.e. no significant changes along with warming) and growth (i.e. greatly enhancements along with  
436 warming), whereas their responses differed in regard to pigment content modifications. The climax plants reduced  
437 all pigments (i.e. Chl *a*, Chl *b* and total carotenoids) during the warming exposure, while on the contrary, the pioneer  
438 plants increased the overall pigment content as a result of warming. These results indicated that, although both  
439 species came from the same thermal regime (both population and species), the climax seagrass was slightly  
440 impacted by the simulated MHW while the pioneer species even benefited from the warming exposure. The  
441 differences between the southern hemisphere species in response to warming was stronger for the photo-  
442 physiological parameters. *P. australis* plants experienced greater reductions in both  $F_v/F_m$  and  $\Delta F/F_m'$  values  
443 compared with *Z. muelleri* plants (Fig. 3a,b). In addition, only the climax plants significantly increased their NPQ as  
444 a result of thermal stress. Additionally, the Australian species did not show differences between climax and pioneer  
445 species in regard to pigment content response. These results are in line with findings from the northern hemisphere  
446 experiment on demonstrating that climax seagrasses are more prone to be adversely affected by warming than  
447 pioneer species. The fact that we observed the 'climax-pioneer' pattern in different traits for the two hemispheres  
448 suggests that species also differed in their mechanisms of response to warming.

449 In order to correctly interpret this result, we should take into consideration that the difference in the species thermal  
450 range can affect the species response to the temperature imposed during the experiment. *P. australis* was exposed to  
451 a warming treatment that goes above its theoretical thermal tolerance, possibly masking the effect due to its life  
452 strategy. Nevertheless, species temperature range was here assessed looking at species distribution, but maximum  
453 temperature tolerance limits can be higher. This is the case of *P. oceanica*, where the species occurs in a coastal  
454 enclosed lagoon (i.e. the Stagnone di Marsala, Italy) exhibiting temperature and salinity tolerant levels above its  
455 theoretical tolerance limit (Tomasello et al. 2009).

456 Differences between the response of climax versus pioneer species to environmental stressors have been previously  
457 documented in other seagrasses. For instance, Masini and Manning (1997) showed the pioneer seagrasses (i.e.  
458 *Amphibolis griffithii* and *A. antarctica*) were more resilient to changes in light and temperature when compared to  
459 two other climax seagrasses (i.e. *P. sinuosa* and *P. australis*) inhabiting in the same region of Western Australia.  
460 Similarly, the Mediterranean pioneer *C. nodosa* was also shown to be more thermal tolerant than the Mediterranean  
461 climax *P. oceanica* (Marín-Guirao et al. 2018), but also to other abiotic stress factors including light (Olesen et al.  
462 2002) and salinity (Sandoval-Gil et al. 2014), which seems to be related to their different levels of phenotypic  
463 plasticity (Pazzaglia et al. 2021). Seddon and Cheshire (2001) also suggested that the climax *P. australis* is more  
464 vulnerable to desiccation in high-temperature conditions than the pioneer *A. antarctica*. All these evidences imply  
465 that warming can reshape the seagrass landscape by reducing the presence of climax species while enhancing the  
466 distribution of pioneer seagrasses. For instance, in Mission Bay, San Diego Bay, and Chesapeake Bay (USA), the  
467 climax seagrass *Z. marina* was replaced by the pioneer *Ruppia maritima* following extreme climatic events (Johnson  
468 et al. 2003; Shields et al. 2019). The same phenomenon is predicted to occur also in the Mediterranean, where ocean  
469 warming is expected to cause a decline of *P. oceanica* (Marbà and Duarte 2010; Chefaoui et al. 2018) while  
470 favoring the expansion of some pioneer species (e.g. *C. nodosa*, *Halophila stipulacea*) (Savva et al. 2018; Winters et  
471 al. 2020). Changes in seagrass meadow composition at the landscape scale would ultimately reduce their ecological  
472 value (Orth et al. 2006; Lamb et al. 2017; Unsworth et al. 2019) and, hence, affect the livelihoods of billions of  
473 people living in coastal areas (Bertelli and Unsworth 2014). The replacement of climax seagrass species, generally  
474 characterized by high biomass and productivity, by pioneer species will also decrease the capacity of seagrass  
475 meadows to mitigate the effects of carbon emissions (Gattuso et al. 2018). Under some warming scenarios,  
476 seagrasses ecosystem may even switch metabolism from autotrophic to heterotrophic (Burkholz et al. 2019), and  
477 enhance CO<sub>2</sub> and methane fluxes from the meadows into the atmosphere (Burkholz et al. 2020).

### 478 **4.3. Future perspectives**

479 Our study brings evidence of differences in the response to warming between climax and pioneer seagrasses and  
480 suggests that these differences are consistent between the northern and southern hemispheres. Ocean warming is  
481 happening fast and accelerating the tropicalization of temperate seagrass meadows (see review by Hyndes et al.  
482 2016). Our results emphasize the need to protect climax seagrasses because they are more ecologically valuable but  
483 also more susceptible to warming than pioneer species. Optimistically, we can enhance the resilience of climax  
484 seagrasses to warming through genotype selection, synthetic biology, assisted evolution, and the use of microbiome

485 (Bulleri et al. 2018). Such approaches require, however, a deeper understanding of the relationship between  
486 seagrasses and the surrounding environments.

#### 487 **Compliance with ethical standards**

488 *Conflicts of interest:* The authors declare that there is no conflict of interest.

489 *Ethical approval:* This article does not contain any studies with animals performed by any of the authors.

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495 Procaccini; **Resources:** Mathieu Pernice, Gabriele Procaccini; **Supervision:** Lázaro Marín-Guirao, Mathieu Pernice,  
496 Gabriele Procaccini.

#### 497 **Acknowledgment**

498 HN was supported by an SZN Ph.D. fellowship via the Open University. This work was funded by EPIC-SEA  
499 Project, Extra-Eu Scientific Research and Cooperation Grant of the SZN, by the project Marine Hazard,  
500 PON03PE\_00203\_1, Italian Ministry of Education, University and Research (MIUR) and by the project Assemble  
501 Plus EU-FP7. The authors are grateful for precious support from Peter J. Ralph, Mikael Kim, Nasim Shah  
502 Mohammadi, Paul Brooks, Scott Allchin, Susan Fenech, and Kun Xiao (UTS) for the Aus-mesocosm experiment at  
503 UTS, Australia as well as Emanuela Dattolo, Alex Santillán-Sarmiento, Ludovica Pedicini and Jessica Pazzaglia  
504 (SZN) for the Med-mesocosm experiment at SZN, Italy. Moreover, the authors thank three anonymous reviewers for  
505 many constructive criticisms and suggestions.

506

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