



## Field thermo acclimation increases the resilience of *Posidonia oceanica* seedlings to marine heat waves

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### ABSTRACT

Acclimation is a response that results from chronic exposure of an individual to a new environment. This study aimed to investigate whether the thermal environment affects the early development of the seagrass *Posidonia oceanica*, and whether the effects of a field-simulated Marine Heat Wave (MHW) on seedlings change depending on acclimation. The experiment was done in the field using a crossed design of Acclimation (acclimated vs unacclimated) and MHW (present vs absent) factors. Acclimation has initially constrained the development of *P. oceanica* seedlings, but then it increased their resilience to the MHW, under both a morphological and biochemical (fatty acid saturation) level. This treatment could be considered in *P. oceanica* restoration projects in a climate change-impaired sea, by purposely inducing an increased resistance to heat before transplants.

### 1. Introduction

Extreme climate events (ECEs), highly associated to climate warming, have received a lot of attention lately due to their increasing frequency and their role as drivers of contemporary and future ecological dynamics (Smith, 2011). ECEs, such as heat waves, drought, rainfall with consequent floods, can have strong impacts on ecosystems also because they provide the organisms no time for adaptation to the new but temporary conditions (Babcock et al., 2019). Similar to heat waves in the atmosphere, marine heat waves (MHWs), prolonged discrete anomalously warm water events, occur when sea surface temperature (SST) exceeds for at least five days a climatological threshold (Hobday et al., 2016). They have increased in frequency and duration in the past decades (Ummenhofer and Meehl, 2017; Darmaraki et al., 2019a) and affect the status of several marine ecosystems in all major ocean (Frölicher and Laufkötter, 2018; Smale et al., 2019). According to some predicted future scenarios, these events will become even more frequent, prolonged and intense depending on increasing greenhouse gas forcing (Meehl and Tebaldi, 2004; Darmaraki et al., 2019b).

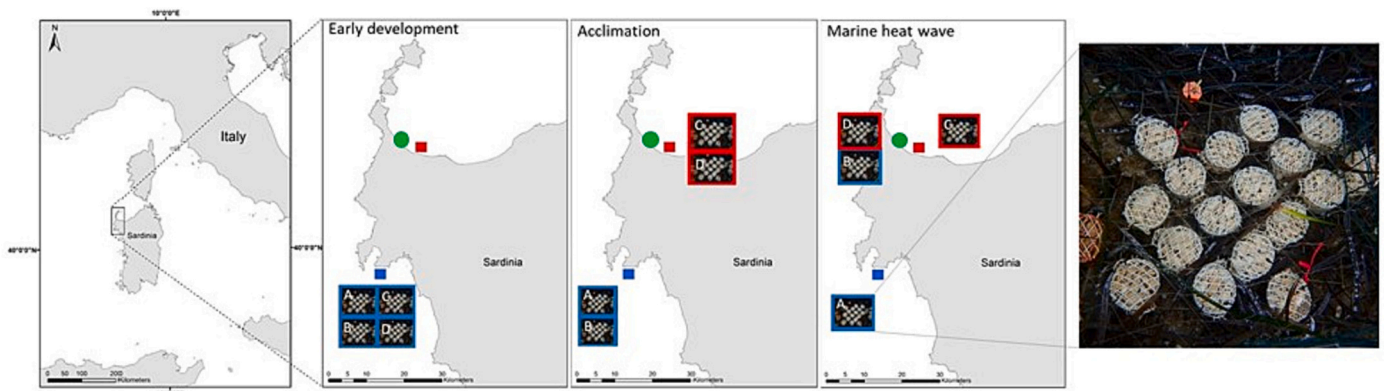
Sea warming is among the most important threats for the Mediterranean biodiversity (Coll et al., 2010; Chefaoui et al., 2018) where

temperature seems to increase faster than in other regions in the world (Jordà et al., 2012). The observed increases in SST affect species occurrence as revealed by some correlative evidence (Duffy, 2003; Yeruham et al., 2015) and this scenario is also complicated by the recurrence of MHWs which have triggered warming-induced severe mortality events of a large number of species from different taxa (Garabou et al., 2022). MHWs can cause serious effects on seagrass populations and distribution (Arias-Ortiz et al., 2018) and a relationship between increasing temperature during MHWs and shoot mortality rates in seagrasses has already been found (Marbà and Duarte, 2010; Strydom et al., 2020). As a result, understanding how organisms will cope with these events, has become an urgent issue for marine ecologists.

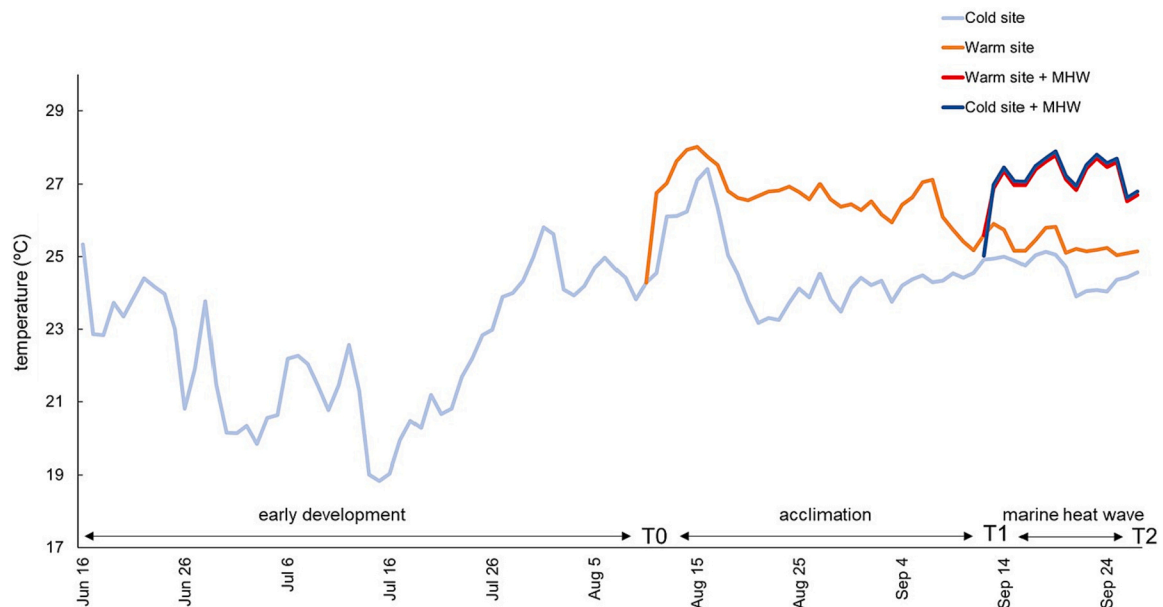
Acclimation to environmental changes includes any organism response to the new environmental properties within a habitat (Woods and Harrison, 2002). According to this definition, acclimation induces an adjustment to a new environment. Such process can be induced by continuous or discontinuous biotic (e.g. presence of pathogens) or abiotic stimuli, (e.g. changes in temperature, salinity or drought events), and have been documented in animals, bacteria, fungi, and plants (Hilker et al., 2016). However, when the new condition represents a stress that occurs occasionally, it can even trigger an ecological stress

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**Fig. 1.** Study area. The blue square represents Porto Conte (cold site) and the red square Fiume Santo (warm site). The green circle indicates the power plant location, the MHW simulation area (common garden). The movement of the four grids (A, B, C, D) can be followed during the three experimental phases (early development, acclimation, and marine heat wave). The blue bordered grids correspond to the unacclimated, while the red bordered to the acclimated ones. The picture on the right represents a grid with the pots containing the seedlings. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Variation in temperature (from loggers) during the whole study period. Until T0 = early development phase; from T0 to T1 = acclimation; from T1 to T2 = MHW experiment time. Light blue = unacclimated and MHW absent (cold site); dark blue = unacclimated and MHW present (cold site + common garden); orange = acclimated and MHW absent (warm site); red = acclimated and MHW present (warm site + common garden). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**

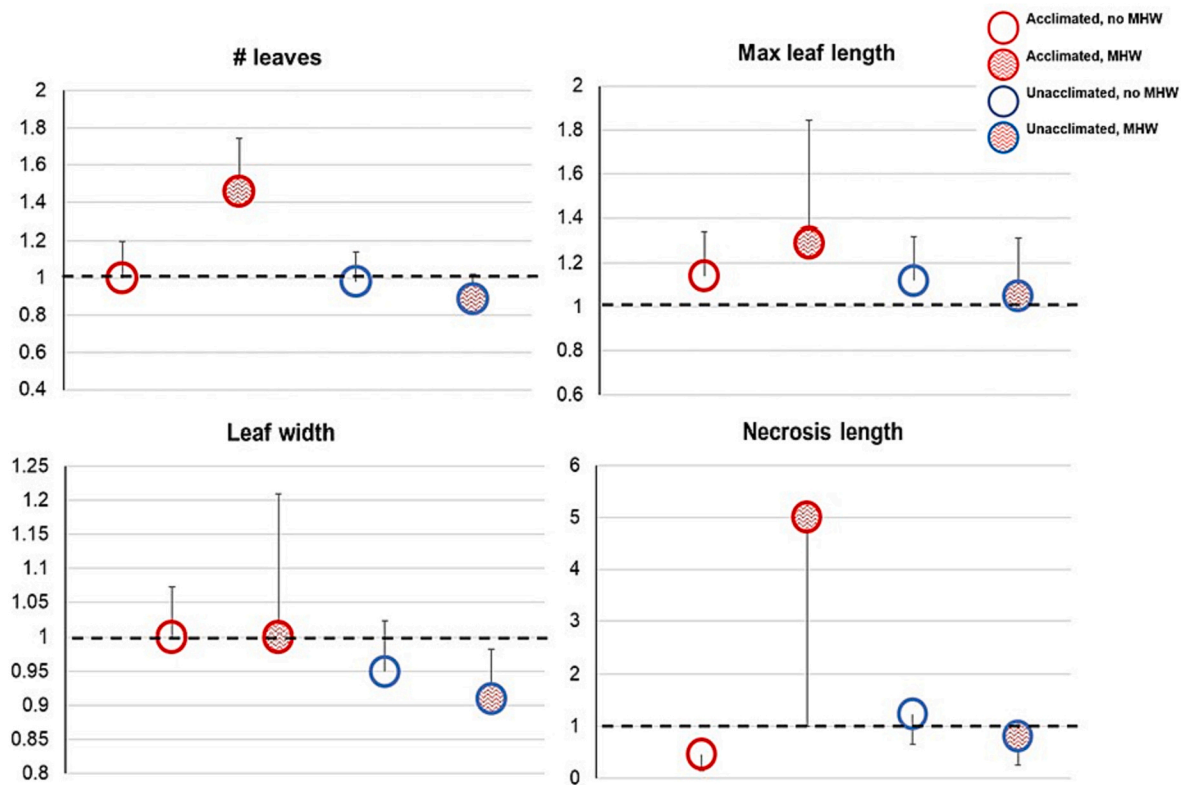
*Seedling morphology.* PERMANOVA results of the effect of site (cold = unacclimated vs warm = acclimated) on the morphology (# of leaves, maximum leaf length, leaf width and necrosis length) of T1 plants. \* indicates statistical significance.

PERMANOVAs	Site
Variables	<i>Pseudo-F</i> <sub>1,50</sub>
# of leaves	9.48*
Maximum leaf length	6.99*
Leaf width	6.40*
Necrosis length	5.59*

memory that may improve the response of the individual towards future stress experience (Karban, 2008; Conrath et al., 2015; Hilker et al., 2016). Then, if the information of the stimulus is stored after a lag phase

and individuals respond to low levels of subsequent stress with faster and stronger activation of defense, they are defined “primed” (Conrath et al., 2015; Hilker et al., 2016). To date, only few efforts have been focused on the thermo tolerance of plants (Saidi et al., 2011; Walter et al., 2013; Nguyen et al., 2020) and investigating the effects and mechanisms that occur during thermal-acclimation and priming has become pressing.

The slow-growing seagrass *Posidonia oceanica* (L.) Delile is a marine clonal angiosperm endemic to the Mediterranean that forms very extended meadows. It supports important goods and services such as nursery grounds, nutrient cycling, blue carbon sequestration, sediment stabilization, trophic transfer to adjacent habitats and towards higher trophic levels and coast protection from erosion (Hemminga and Duarte, 2000; Fonseca and Koehl, 2006; Larkum et al., 2006). *P. oceanica* in early spring releases buoyant fruits which are transported by winds and currents until germination occurs (Balestri, 2017). The seedlings are the



**Fig. 3.** Change ( $\pm$ SE) of the morphological variables at the end of experiment (T2). The red circles represent the acclimated seedling; the blue ones the unacclimated. The circles filled with red waves represent the seedling which experienced the MHW. Value = 1 indicates no change; values > 1 indicate an increase; values < 1 indicate a decrease. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 2**

*Seedling morphology.* PERMANOVA results on the change (T2-T1) in # of leaves, maximum leaf length, leaf width, and necrosis length due to Acclimation (acclimated vs unacclimated), MHW (present vs absent) and their interaction. At the bottom pairwise tests for the significant Acclimation  $\times$  MHW interaction: AC = acclimated, UNAC = unacclimated; MHW P = marine heat wave present and MHW A = marine heat wave absent. Bold refers to significant interactions. \* indicates statistical significance.

PERMANOVAs	Acclimation = A	MHW = M	A $\times$ M
Variables	<i>Pseudo-F</i> <sub>1,28</sub>	<i>Pseudo-F</i> <sub>1,28</sub>	<i>Pseudo-F</i> <sub>1,28</sub>
# of leaves	4.48*	1.73	<b>6.53*</b>
Maximum leaf length	4.82*	3.44	<b>4.61*</b>
Leaf width	2.05	0.38	1.00
Necrosis length	0.39	1.38	1.67

Variables	A $\times$ M
Pair-wise tests	
# of leaves	MHW A MHW P ACCLIMATED UNACCLIMATED
Maximum leaf length	MHW A MHW P ACCLIMATED UNACCLIMATED

most vulnerable life stage in seagrasses (Balestri et al., 2009), being highly susceptible to environmental stressors (Hernán et al., 2016; Guerrero-Meseguer et al., 2020), MHWs included (Pazzaglia et al., 2022). This iconic seagrass adapts its morphology according to the thermal environment (Pansini et al., 2021; Stipcich et al., 2022a), but is also severely threatened by climate change and ECEs (Marbà and Duarte, 2010; Jordà et al., 2012; Hernán et al., 2017; Chefaoui et al., 2018). Both field (Stipcich et al., 2022b) and mesocosm (e.g. Marín-Guirao et al., 2016) studies have found that the thermal environment

**Table 3**

*Seedling leaf FAs.* PERMANOVA results on the change (T2-T1) in lipid content (SFA, MUFA, PUFA, PUFA/SFA, and %TFA) of seedling leaves due to Acclimation (acclimated vs unacclimated), MHW (present vs absent) and their interaction. At the bottom pairwise tests for the significant Acclimation  $\times$  MHW interaction: AC = acclimated, UNAC = unacclimated; MHW P = marine heat wave present and MHW A = marine heat wave absent. Bold refers to significant results of interest. \* indicates statistical significance.

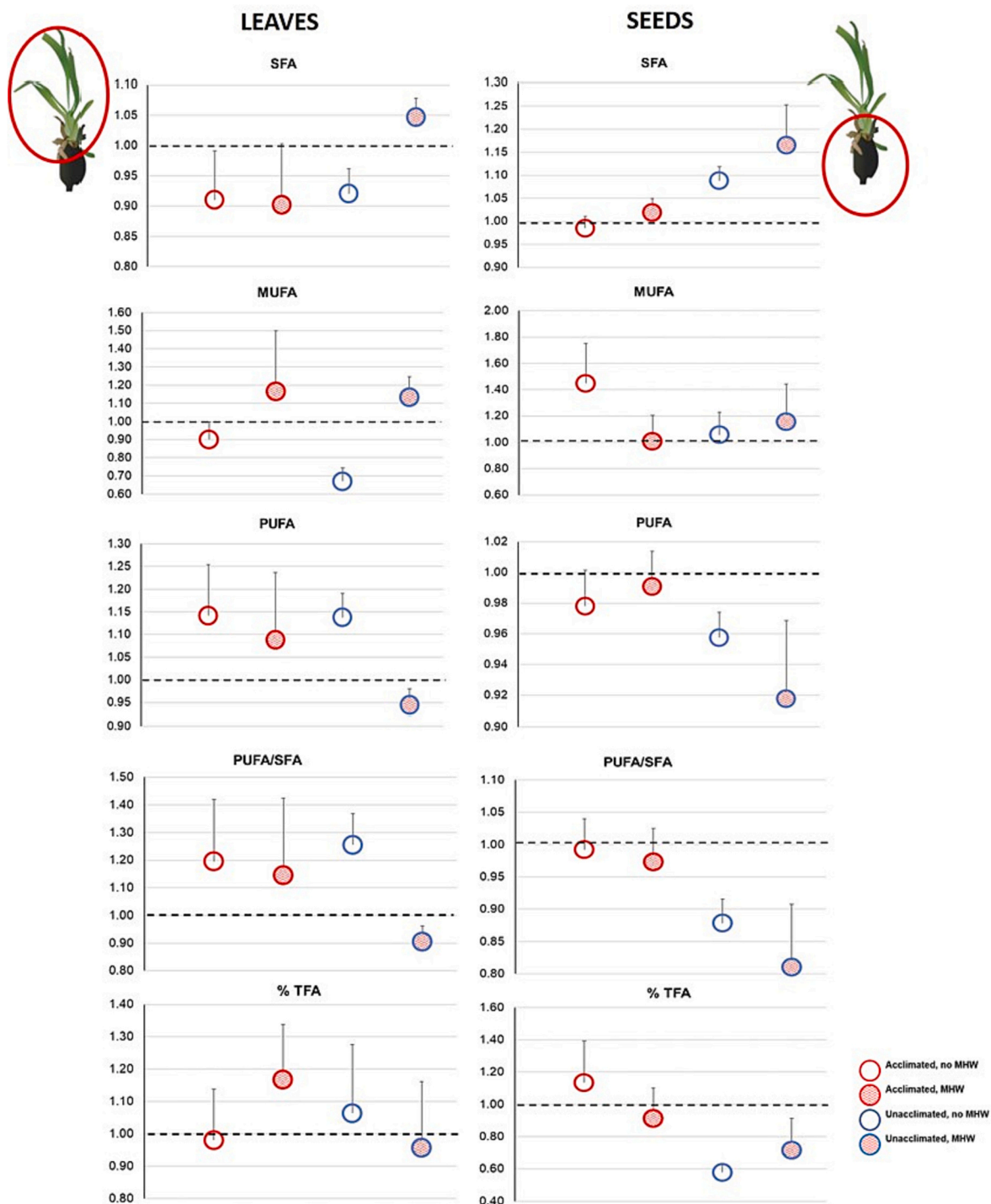
PERMANOVAs	Acclimation = A	MHW = M	A $\times$ M
Variables	<i>Pseudo-F</i> <sub>1,21</sub>	<i>Pseudo-F</i> <sub>1,21</sub>	<i>Pseudo-F</i> <sub>1,21</sub>
SFA	6.03*	3.52	<b>4.51*</b>
MUFA	1.79	<b>13.71*</b>	1.01
PUFA	2.96	<b>8.30*</b>	2.64
PUFA/SFA	1.09	<b>5.44*</b>	3.06
% TFA	0.38	0.14	2.05

Variable	A $\times$ M
Pair-wise test	
SFA	MHW A MHW P ACCLIMATED UNACCLIMATED.

where *P. oceanica* lives affects the resilience to heat events, but the effects of heat on seedlings are poorly understood. However, this is critical since seeds will be considered an essential component in *P. oceanica* meadow restoration protocols, with higher rates of flower and seed production projections due to global warming (Diaz-Almela et al., 2006). Thus, investigating an improved performance and seedling success under climate change scenarios is particularly necessary and timely.

Besides changes in morphology of *P. oceanica* shoots in response to heat stress (Stipcich et al., 2022b), biochemical changes have been also



**Fig. 4.** Change (+SE) of the FA groups in the seedling leaves and seeds at the end of experiment (T2). The red circles represent the acclimated seedling; the blue ones the unacclimated. The circles filled with red waves represent the seedling which experienced the MHW. Value = 1 indicates no change; values > 1 indicate an increase; values < 1 indicate a decrease. Images of seedlings: <https://vecta.io/symbols/tag/posidonia#> (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

considered valuable early warnings to heat stress (Roca et al., 2016; Franzitta et al., 2021). In particular, changes in fatty acid (FA) composition in seagrasses are considered key metabolic indicators used to evaluate the response to heat stress (Beca-Carretero et al., 2018a) and other environmental factors (Hazel and Williams, 1990; Viso et al., 1993; Beca-Carretero et al., 2019; Franzitta et al., 2021). FAs are essential compounds that confer fluidity, flexibility and selective permeability to cellular membranes, and they have a functioning role in

the photosynthesis acting as major sources of metabolic energy, and also serving as chemical messengers (e.g. Routaboul, 2002; Wallis et al., 2002; Couturier et al., 2020). Changes in FA composition can have important effects not only on the physiology of the organism itself (Rabbani et al., 1998), but also on the entire trophic chain (Havelange et al., 1997; Beca-Carretero et al., 2020; Franzitta et al., 2021). Particularly, the increase temperature during a MHW event induced in adult plants (Beca-Carretero et al., 2018a and 2021; Franzitta et al., 2021) a

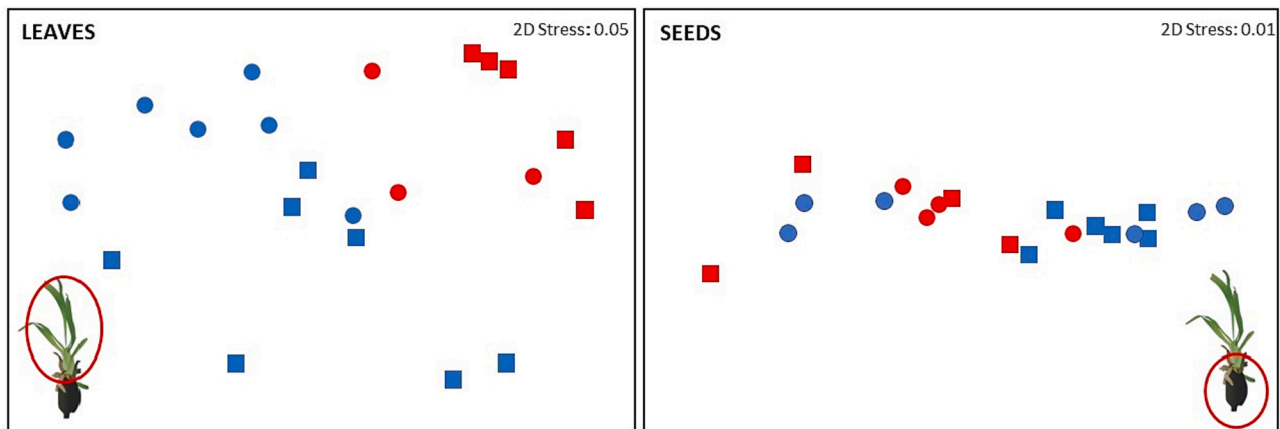


Fig. 5. Fatty acids in the seedling leaves (left) and seeds (right). nMDS of the interaction between Acclimation and Marine Heat Wave (MHW): red = acclimated; blue = unacclimated; squares = MHW present; circles = MHW absent. Images of seedlings: <https://vecta.io/symbols/tag/posidonia#> (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decrease in polyunsaturated fatty acids (PUFA) and an increase in saturated fatty acids (SFA), thus affecting membrane functionality, photosynthetic activity and energetic reserves. Fatty acid composition of *P. oceanica* seedlings has not been described to date, nor how heat events may affect their composition and, therefore, seedlings performance.

This study aimed to investigate i) whether the thermal environment affects the early development of *P. oceanica* seedlings and ii) whether the effects of a field-simulated MHW on *P. oceanica* seedlings change depending on an acclimation period. This was investigated based on morphological and biochemical (FA composition) responses. The hypotheses were that acclimated seedlings would have a higher resilience to MHW, with a higher shoot development and higher unsaturated FA composition than the unacclimated. Additionally, we aimed at identifying the most important FAs (considered as groups and single acids) contributing to *P. oceanica* acclimation to heat waves in the very first stages of development. Results contribute to key insights into *P. oceanica* acclimation processes in the warming scenario.

## 2. Materials and methods

### 2.1. Seed collection and seedling germination

The study was conducted in north-western Sardinia (Italy, Fig. 1) from late June to the end of September 2021. In this area, Porto Conte (hereafter the ‘cold site’) on the western and Fiume Santo (hereafter the ‘warm site’) on the northern coast are characterized by different thermal regimes because of the Mediterranean Sea circulation (Zavatarielli and Mellor, 1995; Olita et al., 2013). To characterize the two sites, differences in the SST climatology and MHWs occurrence were estimated in the last 30 years (see Stipcich et al., 2022b for data), using the rerdap (Chamberlain, 2021) and the heatwaveR (Schlegel and Smit, 2018) package in R.

During the second and third week of June, *P. oceanica* fruits were collected from two beaches in North Sardinia (Monti Russu and San Silverio, Fig. S1) and transported in a tank with an oxygen pump to the laboratory where seeds were extracted. Two days after collection, seeds were placed in small plastic pots (6 cm in diameter) filled with coarse gravel (three seeds per pot) and wrapped with an elastic net in order to prevent loss of material. The pots were then tied to metal grids (40 × 40 cm) and transported to the cold site (Fig. 1). During the transport (about 1 h), the metal grids containing the pots were put in a tank with an oxygen pump. Underwater the grids were caged by a transparent net to avoid effects of grazers, fixed at 2.5 m of depth on dead mat of *P. oceanica*, and left there for the early development until the 10th of August (T0), when 52 viable seedlings were overall selected to be part of

the experiment.

### 2.2. Experimental set up

At T0 (the end of the early development phase), the viable seedlings were split into two groups: 26 seedlings were attributed to the acclimated treatment and 26 to the unacclimated (Figs. 1 and 2). The seedlings that had to be acclimated were transferred, with the same procedure as above, to the warm site (Fig. 1) where they were fixed at the same depth (2.5 m) until the 13th of September (T1, 34 days of acclimation). The unacclimated seedling remained at the cold site (Figs. 1 and 2).

At T1 (the end of the acclimation treatment), six seedlings from each treatment were randomly collected and kept frozen  $-20^{\circ}\text{C}$  for biochemical analysis. Then, 10 seedlings (three in each pot tied to the grids) of either group (acclimated and unacclimated) were transported as above to a common garden in front of Fiume Santo power plant which releases seawater on the shore line ( $12\text{--}24\text{ m}^3\text{ s}^{-1}$ , about  $6\text{--}8^{\circ}\text{C}$  above normal temperature) creating a marked seawater temperature gradient, and thus a natural laboratory (Fig. 1). This warm water flow was used to simulate a MHW that lasted 15 days. Here, because of the warm water stratification, the grid with the seedling pots was attached to a second larger floating grid (100 cm × 100 cm) fixed at the sea bottom using four concrete blocks, allowing the seedlings to stay in the warm water 1 m below the sea surface. On the 27th of September (T2), all the seedlings were collected and transported to the laboratory and kept frozen. On each grid for the whole experiment time, a temperature logger (HOBO Pendant Temp/Light MX2202) was fixed.

### 2.3. Seedling data collection

T0, T1, and T2 *P. oceanica* seedlings were analyzed for morphological variables (number of leaves, maximum leaf length, width and the necrosis length of the longest leaf per seedling) and FA content.

The FA content and composition was estimated on seedlings collected at each time and kept frozen at  $-20^{\circ}\text{C}$ . After 24–48 h the biomass was dried at  $40^{\circ}\text{C}$  until leaves and seeds were perfectly dried, and later kept frozen at  $-20^{\circ}\text{C}$  in hermetically sealed bags containing silica gel (see protocol in Beca-Carretero et al., 2020). Before analyses, biomass was ground into a fine powder using a bead mill homogenizer Beadmill 4 (Fisher Scientific, USA) machine at  $5\text{ m s}^{-1}$  for 3 min. The content and composition of FAs in seedlings were determined by modifying a protocol previously applied in seagrass species (Beca-Carretero et al., 2018a): extraction of FAs was done using  $\sim 10\text{ mg}$  of powdered leaf material. To quantify total and individual FA content, a

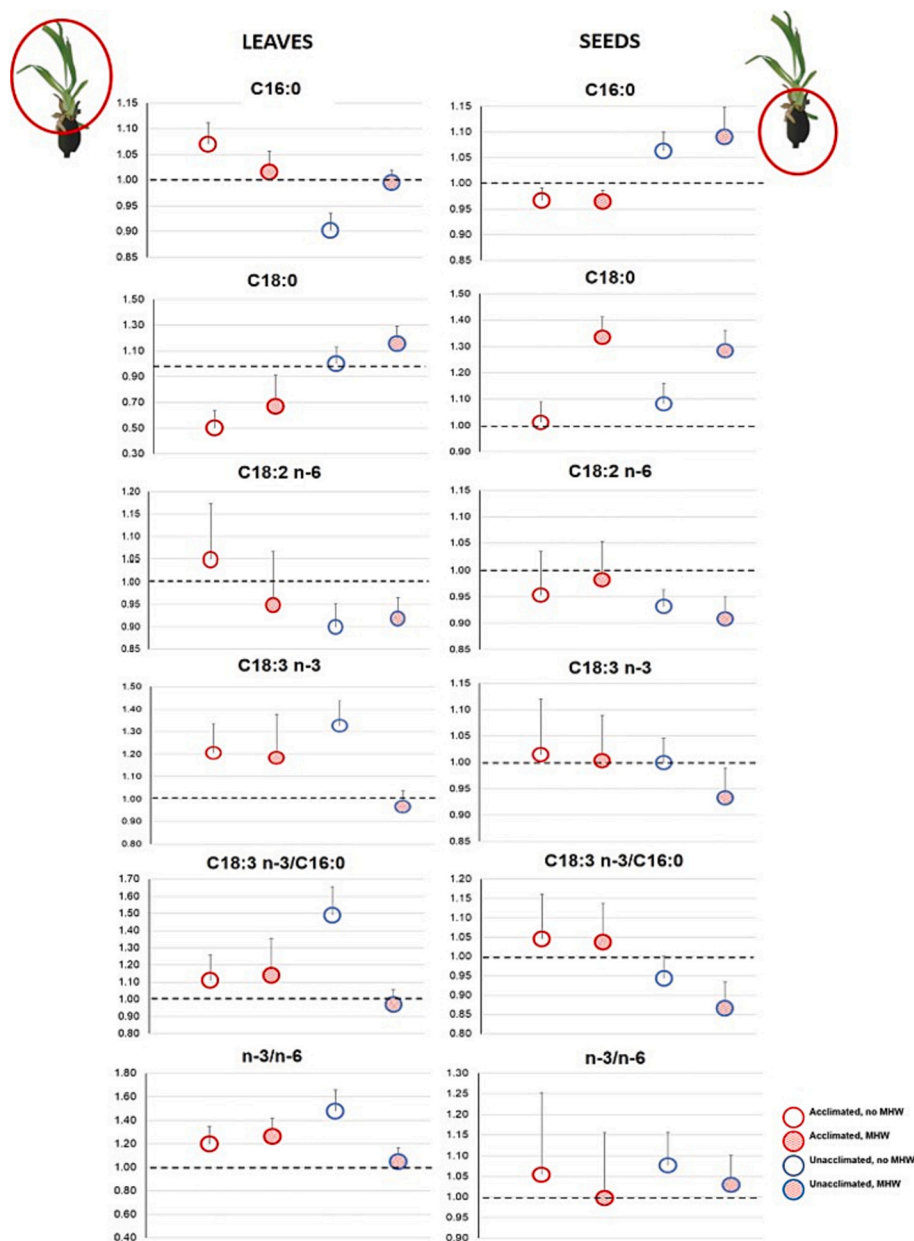


Fig. 6. Change (+SE) of the single fatty acids and their ratio in the seedling leaves and seeds at the end of experiment (T2). The red circles represent the acclimated seedling and the blue ones the unacclimated. The circles filled with red waves represent the seedling which experienced the MHW. Value = 1 indicates no change; values > 1 indicate an increase; values < 1 indicate a decrease. Images of seedlings: <https://vecta.io/symbols/tag/posidonia#> (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

known quantity of 0.1 mL of the saturated fatty acids (SFAs; 15:0 in 0.4 mg mL<sup>-1</sup>) of hexane (99 %, Sigma Aldrich, catalog no. 89680) was added as an internal standard before starting the direct transmethylation. Fatty acid methyl esters (FAMES) were obtained by direct transmethylation with 2 mL dry methanol containing 2 % (v/v) H<sub>2</sub>SO<sub>4</sub>. Extraction of FAMES was conducted at 78 °C for 2 h under continuous stirring conditions. To prevent oxidation, vials were sealed with nitrogen gas before heating. After transmethylation, 1 mL of Milli-Q water was added and later the FAMES were extracted using 0.25 mL of n-hexane. Identification of FAMES was achieved by co-chromatography with authentic commercially available FAME standards of fish oil (Menhaden Oil, catalog no. 47116; Supelco). Analysis of FAMES was conducted using an Agilent 7890A/5975C gas chromatography/mass selective detector (GC/MSD) Series (Agilent Technologies, USA) equipped with a flame ionization detector and a fused silica capillary column (DB-WAXETR, 0.25 mm × 30 m × 0.25 μm, Agilent Technologies, Catalogue no.122-7332).

#### 2.4. Data analysis

A permutational analysis of variance (PERMANOVA; Anderson, 2001) for each morphological and biochemical variable measured at T1 was run using a similarity matrix based on the Euclidean distance of untransformed data to determine whether the warm site, used for the acclimation, affected the growth of the seedlings. The analysis consisted of one fixed factor (site) with two levels (warm and cold).

To evaluate the effects of the MHW depending on the acclimation, all the morphological and biochemical response variables measured on T2 plants were calculated as the change with respect to T1 plants. Then, a PERMANOVA was run on each variable using a similarity matrix based on the Euclidean distance of untransformed data considering Acclimation (acclimated vs unacclimated) and MHW (present vs absent), orthogonal and fixed factors. Overall, the variables considered were maximum leaf length, leaf width, number of leaves, and necrosis length per seedling for the morphology, and the lipid content in the leaves and seeds grouped as SFA, MUFA, PUFA, PUFA/SFA, and %TFA for the biochemical level. Significant differences were investigated using a

**Table 4**

*Seedling leaf FAs.* SIMPER test results on the effect of Acclimation and MHW on seedling leaves, which have identified the most contributing FAs to dissimilarities (in parenthesis the percentage contribution, 90 % cut off) and PERMANOVA results on the change (T2-T1) in the FA content (C 16:0; C 18:2 n-6; C 18:3 n-3; C 18:0; C 18:3 n-3/C16:0; n-3/n-6) of the seedling leaves due to Acclimation (acclimated vs unacclimated), MHW (present vs absent) and their interaction. At the bottom pairwise tests for the significant Acclimation × MHW interaction: AC = acclimated, UNAC = unacclimated; MHW P = marine heat wave present and MHW A = marine heat wave absent. Bold refers to significant results of interest. \* indicates statistical significance.

SIMPER test	Contribution of variables		
AC vs UNAC	C 18:3 n-3 (47.95); C16:0 (20.35); C18:2 n-6 (12.75); C18:0 (9.77)		
MHW P vs A	C 18:3 n-3 (71.85); C16:0 (10.86); C18:2 n-6 (5.54)		

PERMANOVAs	Acclimation = A	MHW = M	A × M
Variables	<i>Pseudo-F</i> <sub>1,21</sub>	<i>Pseudo-F</i> <sub>1,21</sub>	<i>Pseudo-F</i> <sub>1,21</sub>
C 16:0	10.58*	0.46	<b>6.42*</b>
C 18:2 n-6	2.86	0.58	
C 18:3 n-3	0.39	5.90*	<b>4.64*</b>
C 18:0	<b>29.45*</b>	3.22	0.01
C 18:3 n-3/C16:0	0.61	4.42*	<b>5.66*</b>
n-3/n-6	0.01	1.89	3.45

Pair-wise tests	Variables	A × M	
C 16:0		MHW A	AC > UNAC
		MHW P	AC = UNAC
		ACCLIMATED	MHW P = A
C 18:3 n-3		UNACCLIMATED	MHW P > A
		MHW A	AC = UNAC
		MHW P	AC = UNAC
C 18:3 n-3/C16:0		ACCLIMATED	MHW P = A
		UNACCLIMATED	MHW P < A
		MHW A	AC = UNAC
		MHW P	AC = UNAC
		ACCLIMATED	MHW P = A
		UNACCLIMATED	MHW P < A

**Table 5**

*Seedling seed FAs.* PERMANOVA results on the change (T2-T1) in the lipid content (SFA, MUFA, PUFA, PUFA/SFA and %TFA) of seedling seeds due to Acclimation (acclimated vs unacclimated), MHW (present vs absent) and their interaction. At the bottom pairwise tests for the significant Acclimation × MHW interaction: AC = acclimated, UNAC = unacclimated; MHW P = marine heat wave present and MHW A = marine heat wave absent. Bold refers to significant F values. \* indicates statistical significance.

PERMANOVAs	Acclimation = A	MHW = M	A × M
Variables	<i>Pseudo-F</i> <sub>1,19</sub>	<i>Pseudo-F</i> <sub>1,19</sub>	<i>Pseudo-F</i> <sub>1,19</sub>
SFA	<b>7.61*</b>	1.48	0.23
MUFA	0.64	1.30	3.21
PUFA	3.02	0.25	0.95
PUFA/SFA	<b>6.96*</b>	0.69	0.22
% TFA	<b>9.23*</b>	0.11	2.10

posteriori pair-wise test.

Then, using all FAs analyzed in leaves and seeds, SIMPER tests (90 % of cut off) were run to identify the single FA most contributing to the dissimilarities. FA composition was used to visualize the overall changes in *P. oceanica* seedlings due to the effects of the MHW on the acclimated and unacclimated seedlings by a non-metric multidimensional scaling (nMDS) based on the Euclidean distance. In order to understand the contribution of each single FA among the ones identified by the SIMPER test and the ratio between some of them, PERMANOVAs were run using the same model considered above: two orthogonal and fixed factors, Acclimation (acclimated vs unacclimated) and MHW (present vs absent).

**Table 6**

*Seedling seed FAs.* SIMPER test results on the interaction between Acclimation and MHW in the seedling seeds, which have identified the most contribution variables to dissimilarities (in parenthesis the percentage of the contribution, 90 % cut off) and PERMANOVA results on the change (T2-T1) in the SIMPER FAs content (C 16:0; C 18:2 n-6; C 18:3 n-3; C 18:0; C 18:3 n-3/C16:0; n-3/n-6) of the seeds due to Acclimation (acclimated vs unacclimated) MHW (present vs absent) and their interaction. Bold refers to significant F values. \* indicates statistical significance.

SIMPER test	Contribution of variables		
AC vs UNAC	C18:2 n-6 (46.05); C 18:3 n-3 (34.00); C16:0 (9.57); C18:0 (3.95)		
MHW P vs A	C18:2 n-6 (40.32); C 18:3 n-3 (34.85); C16:0 (11.11); C18:0 (6.57)		

PERMANOVAs	Acclimation = A	MHW = M	A × M
Variables	<i>Pseudo-F</i> <sub>1,19</sub>	<i>Pseudo-F</i> <sub>1,19</sub>	<i>Pseudo-F</i> <sub>1,19</sub>
C 16:0	<b>14.67*</b>	0.18	0.25
C 18:0	0.01	3.59	0.19
C 18:2 n-6	1.53	0.01	0.47
C 18:3 n-3	0.66	0.55	0.28
C 18:3 n-3/C16:0	<b>4.60*</b>	0.44	0.29
n-3/n-6	0.13	0.45	0.01

To facilitate the visualization of any change between T2 and T1 in all the variables considered in the study, mean values (+propagated SE) were used for graphical representation. Propagated SEs were calculated with an online error propagation calculator (<https://www.eoas.ubc.ca/courses/eosc252/error-propagation-calculator-fj.htm>).

### 3. Results

#### 3.1. Temperature conditions

During the early development phase (in the cold site, up to T0), temperature changed from 18.83 °C to 25.80 °C, being on average 22.47 °C (Fig. 2). During acclimation (T0 – T1) the difference in temperature between the warm and cold site was on average 2.03 °C. During the MHW experiment (T1–T2), the difference in temperature between the common garden (MHW) and the warm site was 1.94 °C, while between the common garden (MHW) and the cold site was 2.68 °C (Fig. 2).

According to the SST climatology of the two sites, the seedlings from the cold site (unacclimated) during the MHW experiment experienced a 15-day heat wave with 2 days of strong, 10 days of severe, and 3 days of extreme intensity, while the seedlings from the warm site (acclimated) experienced a wave with 10 days of severe and 5 of extreme intensity.

#### 3.2. Seedling morphology

Treatment used for acclimation affected *P. oceanica* seedling development: significant morphological differences between seedlings from the cold and warm sites (unacclimated and acclimated, hereafter) were found by T1 for all variables (Fig. S2 and Table 1): seedlings from the cold site were more developed, as higher values were found for the number of leaves, max leaf length, leaf width, and necrosis length per seedling, compared to those from the warm site.

At the end of the experiment (T2), it was evident that the acclimation also changed the effect of the MHW on the number of leaves, since those of acclimated seedlings were more than those in unacclimated (Fig. 3 and Table 2). Furthermore, for the maximum leaf length a significant acclimation × MHW effect was found, but no alternative hypotheses were detected (Table 2).

#### 3.3. Fatty acid composition

Acclimation did not change the FA composition of *P. oceanica* seedlings at T1 (Tables S1 and S3), but affected the influence of the MHW on

seedlings at T2 (Tables 3 and S4). In fact, FA groups in the leaves of the seedlings depended on the acclimation, as a higher SFA content in the unacclimated seedlings that experienced the MHW was found (Fig. 4 and Table 3). For MUFA, PUFA and PUFA/SFA no dependence on the acclimation was detected since only the effect of the MHW was significant (Table 3).

Nevertheless, specific FAs in *P. oceanica* seedling leaves reported more clear responses to treatments than grouped. The FAs which gave a higher contribution to the dissimilarities between treatments were the n-3 PUFA  $\alpha$ -linolenic acid (C18:3 n-3), the n-6 PUFA linolenic acid (C18:2 n-6), and the SFAs palmitic acid (C16:0) and stearic acid (C18:0) (Figs. 5 and 6 and Table 4). Specifically, the MHW did not affect C16:0 and C18:3 n-3 in acclimated seedlings, which increased and decreased in unacclimated seedling after the MHW respectively, while the ratio C18:3 n-3/C16:0 decreased in unacclimated seedlings after the MHW (Fig. 6 and Table 4).

The lipid content in *P. oceanica* seedling seeds varied consistently to the leaf patterns, but with a smaller magnitude, as acclimation affected the SFA, PUFA/SFA and in % TFA although independently of the MHW (Fig. 4 and Table 5). However, the specific FAs that contributed to the dissimilarities were the same as in leaves (SIMPER test and nMDS, Fig. 5 and Table 6). The unacclimated seedlings that did not experience the MHW seemed to have a more homogenous lipid content than those of all the other treatment combinations, while acclimation alone only affected C16:0 and C18:3 n-3/C16:0 by the end of the experiment (Fig. 6 and Table 6).

#### 4. Discussion

This study investigated for the first time the effects of a field acclimation in the morphology and FA composition in both leaves and seeds of seagrass seedlings. It provided information about how thermal conditions affect *P. oceanica* seedling early development and how the acclimation increases their resilience to a following MHW. Extensive previous evidence corroborating the hypothesis that the thermal context where plants live affects their tolerance to heat was investigated in adult seagrasses (Winters et al., 2011; Beca-Carretero et al., 2018b; Marín-Guirao et al., 2019; Nguyen et al., 2020; Bennett et al., 2022; Stipcich et al., 2022a and 2022b), and only little information was available regarding the influence of thermal environment on *P. oceanica* seedling development (Guerrero-Meseguer et al., 2017 and 2020; Pazzaglia et al., 2022).

Evidence gained here supports the theory that the thermal environment can affect the growth of *P. oceanica* seedlings since the thermo acclimation has initially constrained their early development (T1) in terms of number of leaves, max leaf length and leaf width. The leaf necrotic portion was also higher in seedlings from the cold site and this result highlights the need to answer the question if necrosis should be seen as a sign of natural leaf senescence or as response to a heat stress. Although the warmer temperature experienced during the first stages of development appears to be tolerated by seedlings (no mortality was recorded) in line with a critical heat threshold (29 °C) previously identified (Guerrero-Meseguer et al., 2017), it would seem to represent a disadvantage condition initially, as slowing the seedling development rate, but it may also affect its resistance to any subsequent disturbance events. Conversely, even though acclimation treatment has initially inhibited the morphological development of *P. oceanica* seedlings, it increased their resilience to the following MHW, as in acclimated seedlings a higher number of leaves and leaf length was found at T2.

Evidence of an acclimation positive effect on *P. oceanica* seedlings was also provided from changes in FA composition. Similar to patterns observed in other marine and terrestrial primary producers (Britton et al., 2020; Beca-Carretero et al., 2021), SFA in *P. oceanica* seedlings appeared the most sensitive FA group, acclimated seedlings having similar values either they experienced a MHW or not: this likely indicates the absence of the need of readjusting the investment of the

excess of energy generated during the photosynthesis in storage (Goncharova et al., 2004), thus representing a mechanism of acclimatization to warming. In addition, the reported increase in SFA in unacclimated seedlings after the MHW, is likely to contribute to the maintenance of the correct fluidity of the membrane lipids when temperature rises (Millar et al., 2000). A change in proportion of PUFA, MUFA and PUFA/SFA in seedling leaves was found only after the MHW (where temperature reached higher values than during acclimation), suggesting that these groups cannot be used to study acclimation effects. In particular, PUFA content followed a contrasting pattern to SFA as it decreased in unacclimated seedlings after the MHW, mostly due to changes in C18:3 n-3. PUFAs constitute the cellular membranes (in particular the thylakoid membranes of chloroplasts) promoting their fluidity, the electron transport, and therefore the photosynthetic activity (Gombos et al., 1994; Sanina et al., 2004; Beca-Carretero et al., 2018a) and this reduction can explain the lower performance of unacclimated seedlings to the MHW.

Therefore, the observed differences in n-3 PUFA relative to SFA between acclimated and unacclimated plants under MHW can indicate that acclimation generated resilience and increased the capacity of plants to retain more n-3 PUFA which are critical to favor optimal photosynthetic activity and physiological state. Results for C18:3 n-3/C16:0 support this ratio as a new candidate for thermal stress biomarker in primary producers: indeed, since C18:3 n-3 is the most abundant PUFA with the highest unsaturation level and C16:0 is the most abundant SFA present in seagrasses, their relative proportion is likely to accurately detect changes in unsaturation/saturation levels of photosynthetic structures. Lastly, because C18:3 n-3 is an essential compound found in plants that cannot be synthesized by herbivores (Holman, 1986; Parrish, 2009), the effects of warming on its abundance can trigger changes in nutritional values of plants and shifts in the trophic chain.

Responses of the FA groups to the increased temperature were similar in leaves and seeds, although delayed in the former and with a lower extent in the latter (similarly to Guerrero-Meseguer et al., 2017), likely suggesting a higher plasticity in the leaves and a lower thermal resistance in the seeds. Nevertheless, seeds which are known to supply N and P to the seedling to ensure rapid growth rate (Balestri et al., 2009), seem to maintain their reserve function (e.g. Vaughton and Ramsey, 2001) during the whole seedling development.

Overall, the results of this study suggested that acclimation in *P. oceanica* seedlings can be initially considered a heat stress, but it may also trigger a higher future tolerance to the following heat stress. This process likely occurs commonly in nature depending on the thermal environment where the seedling germinates and thus it may be a process shaping the adaptation of seagrass individuals. In this study no other factors, besides the thermal environment, were considered: among these, light condition that seedling experienced may resemble the depth and sea bottom context where the seeds fall, as no shade was provided to the experimental units, and how seedling performance can be affected by irradiance will remain to be investigated. Furthermore, whether seedling transport across sites affected their performance can only be assumed since transfers were particularly short.

In conclusion, in combination with other studies that provide evidence that the heat stress can induce flowering in *P. oceanica* (Marín-Guirao et al., 2019), our results lead to support the ability to acclimatize to heat as a driver to cope with warming conditions of the Mediterranean Sea, providing an encouraging outlook in contrast to more pessimistic projections (Chefaoui et al., 2018; Pruckner et al., 2022). Future restoration projects could take advantage of a thermo acclimation by inducing an increased resistance to heat, simply treating seedlings with temperature conditions (either in mesocosms or in field common gardens) before transplants, although there remains the need of defining proper methodologies, specifically regarding the duration and the intensity of the temperature treatment, likely adjusted to the seed origin and the recipient field. Further experiments are also needed to identify if the acclimation effects on *P. oceanica* seedlings would vanish during a

lag phase before the MHW or, if they were thermo primable, for how long a memory for the thermo stress would be maintained (Walter et al., 2013; Hilker et al., 2016). Nevertheless, because *P. oceanica* meadows flowering rates has increased recently (Balestri et al., 2017; Arias-Ortiz et al., 2018) and seeds will be more readily available in the future, the cultivation and transplantation of seedlings could become more feasible for *P. oceanica* restoration efforts (Terrados et al., 2013), especially if supported by experimental investigations on acclimation effects.

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## CRedit authorship contribution statement

P.S., G.C. and A.P. conceived the ideas and designed the experiments; P.S., A.P., D.B.S. and P.B. collected the data; P.S. analyzed the data; P.S. and G.C. led the writing of the manuscript. All authors have contributed critically to the drafts, gave final approval for publication and agreed to be accountable for all aspects of the work.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marpolbul.2022.114230>.

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