Canopy-forming macroalgae can adapt to marine heatwaves

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ABSTRACT

Seawater warming and marine heatwaves (MHWs) have a major role on the fragmentation and loss of coastal marine habitats. Understanding the resilience and potential for adaptation of marine habitat forming species to ocean warming becomes pivotal for predicting future changes, improving present conservation and restoration strategies.

In this study, a thermo-tolerance experiment was conducted to investigate the physiological effects of *short* vs *long* MHWs occurring at different timing on recruits of *Gongolaria barbata,* a canopy-forming species widespread in the Mediterranean Sea. The recruits were collected from a population of the Marine Protected Area of Porto Cesareo (Apulia, Ionian Sea). Recruits length, PSII maximal photochemical efficiency (F_v/F_m), photosynthetic pigments content, concentrations of antioxidant compounds and total antioxidant activity (DPPH) were the response variables measured during the experiment.

Univariate asymmetrical analyses highlighted that all physiological variables were significantly affected by both the duration and the timing of the thermal stress with the only exception of recruits length. The higher F_v/F_m ratio, chlorophylls and carotenoids content, and antioxidant compounds concentration in recruits exposed to *long*-term stress likely indicate an acclimation of thalli to the new environmental conditions and hence, an increased tolerance of *G. barbata* to thermal stress.

Results also suggest that the mechanisms of adaptation activated in response to thermal stress did not affect the natural growth rate of recruits. Overall, this study supports the hypothesis that canopy-forming species can adapt to future climate conditions demonstrating a physiological acclimation to cope with MHWs, providing strong evidence that adaptation of marine species to thermal stress is more frequent than expected, this contributing to design tailored conservation and restoration strategies for marine coastal habitat.

2. Introduction

The frequency and intensity of extreme environmental conditions are increasing as a consequence of climate change (Galli et al., 2017). Marine heatwaves (MHWs), i.e., periods of extreme sea surface temperature that persist for days to months (Hobday et al., 2016), are becoming longer-lasting and more frequent, extensive and intense as consequence of global warming (Frölicher et al., 2018; Oliver et al., 2018; Collins et al., 2019). These events are contributing to the loss of marine organisms, mass mortality events and erosion of ecosystem resilience, as increasing temperatures are modifying key physiological, demographic and community scale processes (Wernberg et al., 2016; Garrabou et al., 2022). Their effects can be especially relevant for those organisms that, being sessile or having reduced motility or low dispersal ability, cannot move to colder water (Galli et al., 2017). Establishing the relationship between the biological responses of marine species and the different

levels of heat exposure is challenging and has been rarely addressed (Cheung et al., 2021; Hughes et al., 2021). Indeed, the high variability of responses observed among species and populations across different spatial and temporal scales, as well as the lack of empirical data on the effects of extreme (rare) events, is undermining our ability to understand this relationship (Garrabou et al., 2022).

Mass mortality outbreaks have been documented and extensively studied in a variety of macroinvertebrates of hard substrates including anthozoans, sponges, bryozoans, ascidians, bivalves and echinoderms (Garrabou et al., 2001, 2009, 2022; Crisci et al., 2011; Clemente et al., 2014; Rivetti et al., 2014, 2017; Corinaldesi et al., 2022). Recently, warming-induced effects have also been addressed for mussels (Anestis et al., 2007, 2010; Gazeau et al., 2014) and seagrasses (Marbà and Duarte, 2010; Marbà et al., 2022), increasing the understanding of thermal thresholds for their physiological activity and mortality.

Impacts are expected to be considerable in the near future, also for other important shallow water organisms such as canopy-forming algae (Schimpf et al., 2022), which are particularly sensitive to variations of environmental factors (Thibaut et al., 2005; Mangialajo et al., 2008; Sales et al., 2011; Gianni et al., 2013) and temperature fluctuations (Smale and Wernberg, 2013). For instance, the increased occurrence and intensity of MHWs have been proved to be problematic for kelps. Recent studies suggest a mechanistic link between MHWs and broad-scale kelp loss and highlight how warming can make ecosystem boundaries unstable, by forcing shifts to undesirable ecosystem states under episodically extreme climatic conditions (Smale et al., 2017; Filbee-Dexter et al., 2020). Similarly, climate change-driven impacts affect fucoid canopy algae (Buonomo et al., 2018) causing local extinctions and range shifts which cascade down on the associated ecosystems, changing community diversity and interactions, and key functions and services (Bracken et al., 2007; Smale et al., 2013; Tamburello et al., 2022).

However, while canopy-forming species re-distribution and latitudinal shifts, together with the loss of genetic variability, are often described as responses to extreme climatic events (Sunday et al., 2012; Smale and Wernberg, 2013), physiological responses to environmental changes have been poorly explored (Plouguerné et al., 2006; Le Lann et al., 2012), and only little is known about their potential adaptations to future climate conditions.

Evidences show that in response to variations of abiotic and biotic factors, macroalgae can produce and accumulate large quantities of secondary metabolites with the role of protection or defense compounds (Lobban and Harrison, 1994; Amsler, 2008; Sudatti et al., 2011). The production and accumulation of phenolic compounds is generally regarded as an indicator of stress due to variation in environmental factors (Pavia and Brock, 2000). Their concentration can vary within and among species, being affected by seaweed size, age, tissue type, environmental factors, and can have spatial and seasonal variations (Connan, 2004; Lopes et al., 2012). Since the increase of sea surface temperature, generally considered a stress condition, may cause changes in macroalgal metabolism, growth and reproduction, it is necessary to clarify the relationship between physiological response of species and sea warming (Celis-Plá et al., 2022).

Locating and protecting those populations showing climate adaptation strategies, can contribute to species persistence and conservation. In addition, restoring degraded areas with individuals that live in highly variable environments and hence, are likely to be pre-adapted to future conditions, can increase the chance of bringing in climate change-resilient individuals (Reside et al., 2018). However, these conservation and restoration strategies need science-based information to be effective. In this study, we explored the physiological effects of *short* vs *long* MHWs occurring at different timing on recruits of *Gongolaria barbata* (Stackhouse) Kuntze, a Mediterranean widespread canopy-forming species exposed to fragmentation and loss, by means of a thermo-tolerance experiment. The aim was to test the potential for adaptation of recruits of *G. barbata* to MHWs and future ocean warming for predicting upcoming changes and designing tailored conservation

and restoration strategies.

3. Materials and methods

3.1. Study area and specimen collection

Field sampling was carried out in the Marine Protected Area (MPA) of Porto Cesareo, located along the Ionian coast of the Salento Peninsula (Apulia, Italy). Natural stones (n = 47) with recruits of *G. barbata* (n = 1–5 for each stone) were collected from the general reserve zone (zone B) of the MPA (40°14′47.1″N, 17°54′33.0″E), in the internal coast of the lagoon delimited by La Strea peninsula, where an abundant population of the species is documented (Parenzan, 1984). The coast within the MPA is characterized by stretches of sandy coast alternating to stretches of shallow rocky coast, rich in outcropping rocks. Sampling activities were conducted at 0.5–1.0 m depth at the end of June 2021. At the sampling time seawater temperature was between 27 and 28 °C (temperature data logger: Hobo TidbiT v2, Onset) and light irradiance was around 600 nm (light data logger: Odyssey® Submersible PAR Logger). Samples were then transported at ~26 °C in dark conditions inside coolboxes filled with filtered seawater (FSW, 25 μm mesh filters) to the laboratory aquaria of the Ischia Marine Center in Ischia (Stazione Zoologia Anton Dohrn, Napoli).

3.2. Experimental setup and maintenance

A 2000 L reservoir and 'ad hoc' mesocosms, comprising eighteen independent closed circuit plexiglass aquaria (10 L each), were set up in the facility of the Ischia Marine Center. Seawater was pumped into the reservoir for room temperature acclimation (25 °C) and then pumped through 25 μm vacuum filters into the experimental aquaria. For each 10 L tank an aerator and a recirculating pump were used in order to keep the water within the tank properly oxygenated and mixed. To keep the temperature constant and avoid fluctuations due to alteration in the room temperature, all aquaria were partially submerged in 4 thermostatic baths in which the temperature was maintained using four aquarium chillers/heaters (Teco TK150, Italy). MHWs were simulated in the culturing tanks using 100 W aquarium heaters properly regulated. A day/night cycle of 12/12 h was used using 4 phytostimulant aquarium led lamps (DMC SilverMoon Universal, Italy). Half of the seawater volume in each aquarium was changed every two days taking care to avoid further stress to thalli, and the Von Stosch (1963) culture medium enriched with GeO_2 (2 mg L^{-1}) was added to inhibit diatom contamination (Lewin, 1966). Temperature and salinity were recorded daily.

3.3. Experimental design

To test the effect of MHWs on recruits of *G. barbata*, a two fixed factorial design with asymmetrical controls was used. A *control* condition not exposed to MHWs was compared against two orthogonal factors: i) MHWs *duration*, fixed factor with two levels: *short* MHW and *long* MHW; ii) MHWs *timing*, fixed factor with two levels: an *early* (summer) MHW and a *late* (summer) MHW. Every treatment was replicated in three separate tanks with three stones each, apart from the *control* condition where an additional tank with two stones was used. At the beginning of the experiment, the number of individuals (i.e., replicates) per treatment ranged between 35 and 46 (Fig. 1). The experiment lasted from the 14th of July to the 12th of September 2021. After two acclimation days in which all tanks were at 25 °C, temperature for the *control* condition was set following the seasonal average trend registered at La Strea lagoon from 1982 to 2021, ranging between 25.5 and 26.5 °C. Accordingly, temperatures simulating MHWs were set increasing by 2 °C the average values based on the detection of historical MHWs events. Thus, they ranged between 27.5 and 28.5 °C respectively for the *early* and *late* MHWs. The *duration* of the MHWs was set to 13 and 26 days respectively for the *short* and the *long* treatment, reproducing MHWs
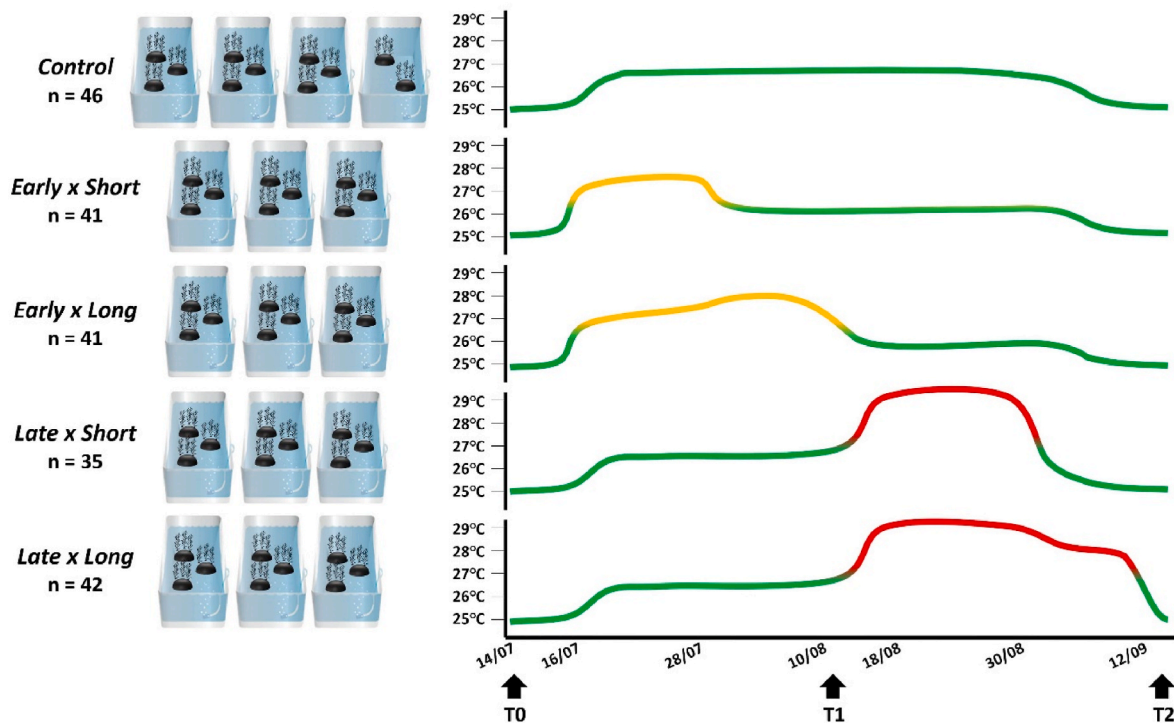
Fig. 1. The figure shows, for each treatment (i.e., *control, early short, early long, late short* and *late long*), the number of recruits (i.e., "n = "), stones and tanks used (left side of the figure). The plots in the right side show the temperatures (y axis) set at each time of the experiment (x axis): from the 14/07 to the 16/07 all treatments were exposed to the same acclimation temperature (25 °C); on 16/07, the *early short* and *early long* MHWs started; on 28/07 the *early short* MHW ended; on 10/08 the *early long* MHW ended; on 18/08, the *late short* and *late long* MHWs started; on 30/08 the *late short* MHW ended; on 12/09 the *late long* MHW ended. Temperatures for the *control* and treatment tanks while non exposed to MHWs followed the seasonal average trends. The bottom arrows (T0, T1 and T2) represent the sampling time of the measurement of the response variables on the recruits.

events that actually occurred in the summer season. Mean intensity (°C) and cumulative duration (days) of MHWs events were calculated using the R package 'heatwaveR'. Daily data of Sea Surface Temperature (SST) from 1982 to 2021 were retrieved from the geoportal Copernicus (Copernicus Climate Change Service, 2019). The Copernicus dataset provides global SST data in the raster format (NetCDF) based on multiple sensors with a spatial resolution of $0.05° \times 0.05°$ and no spatial gaps. From each raster file, we extracted daily SSTs for the pixel corresponding to the lagoon of Porto Cesareo using the Open Source software QGIS (QGIS Development Team, 2022).

Fig. 1 graphically represents the experimental conditions set for each treatment throughout the experiment.

3.4. Recruits length measurements

To determine recruits growth, the length of thalli (i.e., the distance from the holdfast to the thallus tip in mm) was measured randomly taking 18 individuals from each treatment exposed to MHWs and 20 individuals from the *control* condition (i.e., 2 individuals from each stone, to ensure replicates independence; one among the *control* stones was not taken into account since it hosted only one individual). Photographic sampling of the stones, using a stereo microscope (Leica Z16 APO), equipped with a Leica DFC 300FX camera connected to a computer with the Leica LAS program (Leica Application Suite, Version 4.5) was taken three times during the experiment: at the beginning of the experiment during the acclimation period (T0), after the *early* MHWs and before the *late* ones (T1) and at the end of the experiment, after the *late* MHWs (T2) (Fig. 1). Images were analyzed through the Open Source software ImageJ (Schroeder et al., 2021).

3.5. Maximal PSII photochemical efficiency in vivo measurements

Measurements of chlorophyll *a* fluorescence emission have been carried out on recruits thalli *in vivo* before the harvesting (between T1 and T2) to assess the healthy status of photosystem II following the stress conditions in the five experimental treatments. The measurements were conducted on four recruits for experimental conditions by a pulse amplitude modulated fluorometer (Junior-PAM, Walz Gmbh, Effeltrich, Germany) equipped with a fiberoptics of 1.2 mm diameter.

Thalli were darkened for 15' to determine the maximum PSII photochemical efficiency (F_v/F_m); this time is considered sufficient for macroalgae to oxidise (i.e., maintain open) the reaction centres of photosystem II (Porzio et al., 2020). The basal fluorescence signal (F_0) was obtained by a weak beam of blue light of about 1–2 μmol photons $m^{-2}\,s^{-1}$, while the maximal fluorescence in the dark-adapted state (F_m) was measured by applying a 0.8-s light saturating pulse of 3000 μmol photons $m^{-2}s^{-1}$. The F_v/F_m ratio was calculated as $(F_m–F_0)/F_m$, according to Kitajima and Butler (1975). F_v/F_m ratio represents the maximum PSII potential yield, and it is a common index to assess the health/stress status of the PSII as well as the occurrence of photoprotective/photoinhibition processes (Beer et al., 2000; Porzio et al., 2018).

Rapid light response curves (RLC) were performed by exposing thalli at different light intensities (0–820 μmol photons $m^{-2}\,s^{-1}$) to assess the efficiency of the photosynthetic apparatus in utilising the absorbed light in photochemistry (Porzio et al., 2020) (Supplementary Figure S1).

At each irradiance level, a saturating light pulse was applied, and the maximal fluorescence (F_m') was determined. The difference between F_m' and F_t is assumed to be the result of electron transport activity and used to calculate the quantum yield of PSII (Φ_{PSII}) according to Genty et al. (1989) as $\Phi_{PSII} = (F_m'-F_t)/F_m$ and electron transport rate (ETR) following Schreiber (2004) as: ETR = Φ_{PSII} *PAR*0.5*AF and represents

the PSII electron transport rate per absorbed photon. AF (absorbing factor) was calculated according to Beer et al. (2000). Three RLCs were performed for each experimental treatment.

After measurements, all samples were collected and stored at $-20\ °C$ for the analyses of photosynthetic pigments, and antioxidants.

3.6. Biochemical determinations on thalli

The determinations of concentrations of chlorophylls, carotenoids, flavonoids, polyphenols, tannins and total antioxidant capacity were carried out to understand thalli's capability to withstand the thermal stress. The assays were performed by collecting ten individuals for each treatment. Samples were preventively powdered in liquid nitrogen, using a mortar and pestle, and then processed.

3.6.1. Photosynthetic pigment content

Photosynthetic pigments, i.e., total chlorophylls and total carotenoids were extracted in 100% acetone from 40 mg of sample. Then, the extracts were centrifuged at 5000 rpm for 5 min. The absorbance of supernatants was read at 662 nm, 645 nm, 630 nm and 470 nm for chlorophyll *a*, chlorophyll *b*, chlorophylls *c1 + c2* and total carotenoids *x + c*, and total carotenoids, respectively, and quantified according to Jeffrey and Humphrey (1975) and Etemadian et al. (2017).

3.6.2. Polyphenols, flavonoids, tannins and total antioxidant activity analysis

For determining total polyphenols, flavonoids, tannins and total antioxidant activity, 0.20 g of powdered material was extracted in 2 mL of aqueous 80% methanol. All the extracts were incubated for 1 h at 4 °C, centrifuged at 11.000 rpm for 5 min and stored at 4 °C.

Total polyphenols were quantified according to Costanzo et al. (2022). 274 µL of extracts were mixed and shaken with the same volume of the 10% Folin–Ciocâlteu reagent and, after 3 min, were mixed with 1.452 µL of 700 mM sodium carbonate (Na_2CO_3) solution. Samples were kept for 2 h in darkness at room temperature. Then, the absorbance of samples was read at 765 nm, and the total polyphenol content was expressed as mg of gallic acid equivalents per gram of fresh weight (mg GAE g^{-1} FW) by a calibration curve built with gallic acid as standard.

Total flavonoids were assessed following Moulehi et al. (2012) and Sun et al. (2010). Extracts (250 µL) were mixed with 75 µL of 5% $NaNO_2$ (sodium nitrite) and, after 6 min, with 150 µL of 10% $AlCl_3$ (aluminium chloride) and 500 µL of NaOH (1 M), adjusting the final volume with distilled water. The absorbance of samples was read at 510 nm. Total flavonoid content was calculated using a catechin standard curve and expressed as mg catechin equivalent per gram of fresh weight (mg CAT eq g^{-1} FW).

Tannins were determined according to Sun et al. (1998), adopting slight changes to the procedure. Briefly, 1.25 mL of 1% methanol vanillin solution and concentrated H_2SO_4 (1:1 v/v) were mixed with 500 µL of sample extract. After 15 min, the absorbance was measured at 500 nm. Then, total condensed tannins were expressed as mg catechin equivalents per gram of fresh weight (mg CAT eq g^{-1} FW) using a calibration curve built with catechin.

Finally, the free radical scavenging activity of samples was evaluated by applying the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay as reported by Dudonné et al. (2009). Extracts (67 µL) were added to 2 mL of 6×10^{-5} M DPPH methanolic solution, shaken and incubated at 37 °C for 20 min. The absorbance of samples was read at 515 nm and used to calculate the percentage of inhibition of DPPH radicals using the following equation:

$$\text{Inhibition } (\%) = \left[\left(Ab_{blank} - Ab_{sample} \right) / Ab_{blank} \right] \times 100$$

where Ab_{blank} is the absorbance of the blank and Ab_{sample} is the absorbance of the tested Sample extract. Trolox was used as the positive control.

3.7. Statistical analyses

To test for differences among treatments, recruits length at T2, the PSII maximal photochemical efficiency (F_v/F_m), photosynthetic pigment content, and concentrations of antioxidant compounds were analyzed by means of separate Analyses of Variance (ANOVA), followed by pairwise comparisons when significant results were obtained. Since the experiment followed an asymmetrical design (see Underwood, 1997), all data (i.e., differences *among all* treatments) were analyzed by a one-way ANOVA. Then, the four *non-control* treatments (i.e., *among others*) were analyzed as a two-way ANOVA with the *duration* (*short* VS *long* MHWs) and *timing* (*early* VS *late* MHWs) as factors (Table 1). Pairwise comparisons were also carried out when significant results were obtained.

Recruits length measurements at T0 were tested for differences *among all* treatments using a two-way ANOVA. The random factor *tanks* nested in treatments was also included to identify possible bias in the allocation of replicates among treatments.

The analysis on recruits length at T1 instead was carried out excluding *late* treatments since they were not exposed to any thermal stress yet. Thus, a one-way ANOVA was carried out *among all treatments* and between the two *duration* treatments (*short* VS *long* MHWs).

All statistical analyses were based on matrices of Euclidean distances and carried out with PRIMER-E 7 software. Table 2 shows for each response variable the sample size, the sampling time, the statistical analysis performed and the factors included in the analysis.

To visualize distances among treatments and how the ecophysiological response varied across them, the non-metric multidimensional scaling (nMDS) based on Euclidean distances among transformed (square root transformation) and standardized data was performed. In addition, the k-means clustering algorithm on the obtained ordination was applied to detect overall patterns in the response of recruits to the different treatments.

The dataset produced for this study and used for performing all statistical analyses is available in Mendeley Data repository (Fabbrizzi et al., 2023).

Table 1

Analysis to test differences among treatment in asymmetrical design experiments.

Source of Variation	Degree of Freedom (df)			Num. df	Den. df
Among all[1]	$(a - 1)$ = 4			4	$5\ (n - 1)$
Controls VS *Others*[2]		$(b - 1)$ = 1		1	$(n_c - 1) + (n_o - 1)$
Among others[3]		$(c - 1)$ = 3		3	$4\ (n - 1)$
Duration (D)[4]			$(d - 1) = 1$	1	$4\ (n - 1)$
Timing (T)[4]			$(e - 1) = 1$	1	$4\ (n - 1)$
D x T[4]			$(d - 1)\ (e - 1) = 1$	1	$4\ (n - 1)$
Residual[1]	$5\ (n - 1)$				
Total[1]	$5n - 1$				

a = 5 levels *among all treatments* (i.e., *controls, early short, early long, late short* and *late long*).

b = 2 levels *controls* VS *others* (i.e., *early short, early long, late short* and *late long*).

c = 4 levels *among others* (i.e., *early short, early long, late short* and *late long*).

d = 2 levels *duration* (D) (i.e., *short* VS *long*).

e = 2 levels *Timing* (T) (i.e., *early* VS *late*).

n Number of replicates of *all* treatments (see Table 2).

N_c Number of replicates of *controls* treatments.

n $_o$ Number of replicates of *others* treatments.

[1] One-way ANOVA of all data.

[2] Sum of squares by subtraction of among others from *among all* treatments.

[3] Sum of squares by addition of those in ^{d}.

[4] Two-way ANOVA omitting *controls*.

Table 2

Response variable measured indicating sample size, sampling time, statistical analysis performed and the factors included in the analysis.

Variable name	Definition	Sample size	Sampling time	Statistical analysis	Source of Variations
Recruits length	Distance from the holdfast to the thallus tip in mm	n = 20 (*controls*) n = 18 (*others*)	T0 T1 T2	Two-way ANOVA One-way ANOVA ANOVA for asymmetrical designs	*Among all* and *tanks* *Among all* and *duration* (i.e., *early short* VS *early long* MHWs) See Table 1
F_v/F_m	PSII maximal photochemical efficiency	n = 4 (*all*)	Between T1 and T2	ANOVA for asymmetrical designs	See Table 1
Photosynthetic pigment content	Total chlorophylls Total carotenoids	n = 10 (*all*)	After T2	ANOVA for asymmetrical designs	See Table 1
Polyphenols	Concentration of polyphenols	n = 10 (*all*)	After T2	ANOVA for asymmetrical designs	See Table 1
Flavonoids	Concentration of flavonoids	n = 10 (*all*)			
Tannins	Concentration of tannins	n = 10 (*all*)			
DPPH	Total antioxidant activity	n = 10 (*all*)			

4. Results

4.1. Recruits length

Since no significant differences *among all* treatments emerged in recruits length at T0 we assumed that our replicates were correctly interspersed among treatments. Significant differences occurred in the factor *tanks*, which, being a random factor, is supposed to capture the natural variability of the population (Supplementary Table S1).

Results from both T1 and T2 show that recruits length was not significantly affected by heatwaves (Supplementary Tables S2–S3).

4.2. Maximal PSII photochemical efficiency

Analysis on the F_v/F_m ratio revealed significant differences *among all* treatments, identifying two groups based on the *duration* of MHWs exposure (Supplementary Table S4, P < 0.001). Recruits exposed to both the *short*-term MHWs (i.e., *early short* and *late short*), showed lower values in the maximum PSII photochemical efficiency when compared to recruits exposed to the *long* MHWs treatments, which did not significantly differ from the *controls* (Fig. 2).

4.3. Biochemical determination on thalli

4.3.1. Photosynthetic pigment content

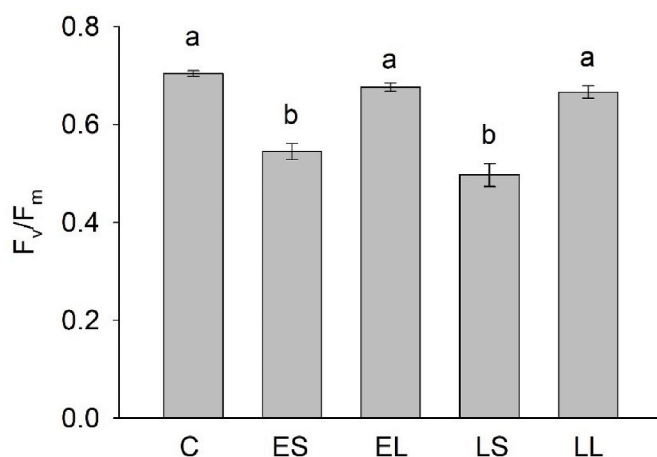Looking at total chlorophylls and carotenoids content, significant

Fig. 2. Mean ± SE of maximal PSII photochemical efficiency (F_v/F_m) of recruits under the *control* condition (C) and the *early short* (ES), *early long* (EL), *late short* (LS) and *late long* (LL) treatments. Lowercase letters indicate homogeneous groups among data according to the ANOVA test results. Data are represented using SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA).

differences *among all* treatments were identified, recognizing three groups based on both the *duration* and the *timing* of MHWs exposure, respectively without and with interactions between the two factors (Supplementary Tables S5–S6, P < 0.001). The chlorophylls content significantly increased with the duration of the MHW, and with the age of the recruits (i.e., it was higher in recruits exposed to later heatwaves compared to those exposed to the earlier ones). Recruits exposed to the *early short*-term MHW had a chlorophylls content comparable to that of controls (Fig. 3A). The concentration of carotenoids increased due to longer and later MHWs, with a pattern similar to that observed for chlorophylls. Yet, while doubling the duration of the MHW affecting younger recruits (i.e., *early* MHW) increased the concentration of carotenoids by 20%, the longer duration of the MHW affecting older recruits (i.e., *late* MHW) caused a moderate increase (i.e., less than 10%) (Fig. 3B).

4.3.2. Polyphenols, flavonoids, tannins and total antioxidant activity

The ANOVA tests for the concentration of polyphenols, flavonoids and tannins, and for the total antioxidant capacity always highlighted significant differences *among all* treatments (Supplementary Tables S7–S10, P < 0.001) with interactions between the *duration* and *timing* factors. Treatments were separated in four groups based on the concentration of polyphenols and flavonoids (Fig. 4A and B), while three groups were identified looking at the concentration of tannins and the total antioxidant capacity (Fig. 4, C-D).

The concentration of polyphenols increased proportionally to the duration of heatwaves, highlighting a greater difference between *long* and *short* MHWs when exposure occurred on older recruits (i.e., *late* MHWs). Yet, the overall concentration of polyphenols of recruits exposed to *early* heatwaves or to a *late short* heatwave, reduced compared to unmanipulated *controls* (Fig. 4A).

Flavonoids concentration increased due to longer heatwaves compared to the shorter ones at both timing of disturbance. Yet, while the content of flavonoids of recruits affected by *early* heatwaves was either comparable or higher than that of unmanipulated *controls*, it drastically reduced in recruits affected by later heatwaves, with a modest increase due to duration (Fig. 4B).

Tannins concentration reduced proportionally to the timing of exposure to MHWs, with a stronger reduction in later treatments (Fig. 4C). Although the analysis indicates an interactive effect of both duration and timing of MHW on tannins concentration (Supplementary Table S9), post-hoc analyses did not indicate significant differences between recruits exposed to *long* or *short* heatwaves at each time of treatment.

Finally, DPPH capacity was affected by the duration of the heatwaves only in later exposed recruits (i.e., *late short* MHW). Older recruits did not significantly differ from the *control* ones when exposed to *long* heatwaves (i.e., *late long* MHW). While DPPH capacity similarly decreased for *short*- and *long-early* MHWs, it reduced more drastically (or by 10%) only due to the *late short* MHW, when compared to the *controls*
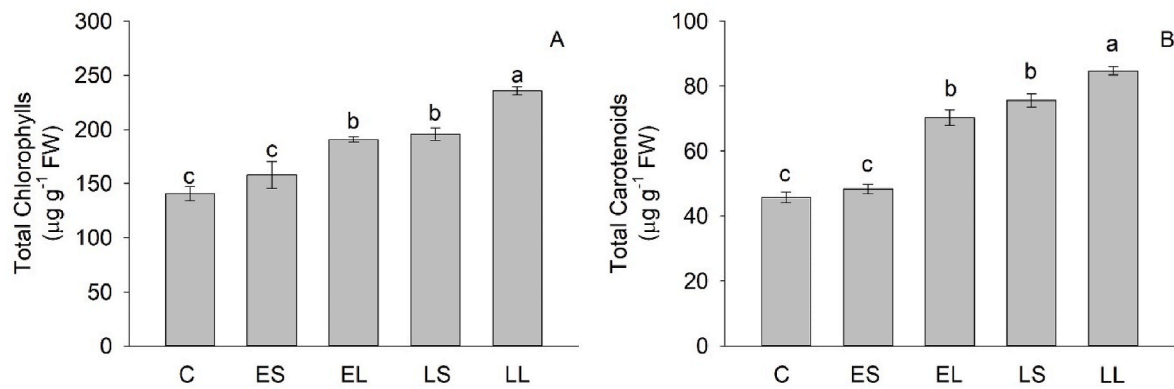
Fig. 3. Mean ± SE of total chlorophylls (Fig. 3A) and total carotenoids (Fig. 3B) of recruits under the *control* condition (C) and the *early short* (ES), *early long* (EL), *late short* (LS) and *late long* (LL) treatments. Lowercase letters indicate homogeneous groups among data according to the ANOVA test results. Data are represented using SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA).
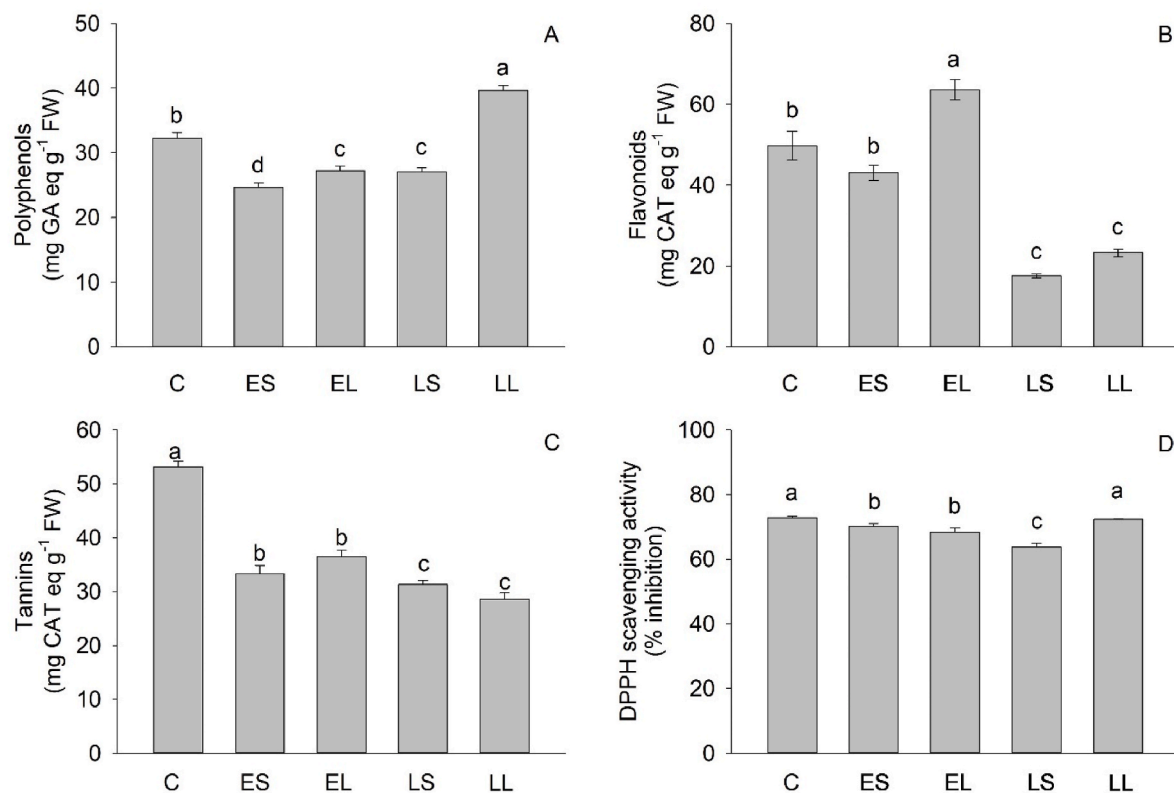

Fig. 4. Mean ± SE of polyphenols (Fig. 4A), flavonoids (Fig. 4B), tannins (Fig. 4C) DPPH radical scavenging activity (Fig. 4D) of recruits under the *control* condition (C) and the *early short* (ES), *early long* (EL), *late short* (LS) and *late long* (LL) treatments. Lowercase letters indicate homogeneous groups among data according to the ANOVA test results. Data are represented using SigmaPlot 12 software (Jandel Scientific, San Rafael, CA, USA).

(Fig. 4D).

The 2-dimension nMDS (Euclidean distance, stress = 0.05) represented in Fig. 5 shows the distances between treatments according to the ecophysiological variables considered. Through the k-means clustering, three clusters were detected, evidenced in the nMDS using differently colored ellipses. Cluster 1 contains all data points from *control* treatment as well as data points from the *early short* one. This cluster is characterized by high concentrations of flavonoids and tannins. Cluster 2 contains all data points corresponding to the *early long* treatment. nMDS shows that this cluster is relatively close to and thus similar to cluster 1 but it is discriminated from the latter by higher concentrations of flavonoids. Cluster 3 groups all together data point from *late* treatments characterized by the highest concentrations of polyphenols and

photosynthetic pigments (i.e., Chlorophylls and Carotenoids).

5. Discussion

The thermo-tolerance experiment carried out in this study shed light on the overlooked effects of climate change on habitat-forming species. Results showed that physiological variables (i.e., PSII maximal photochemical efficiency, photosynthetic pigment content, concentrations of antioxidant compounds and DPPH radical scavenging activity) are strongly influenced by both the *duration* and the *timing* of thermal stress events, demonstrating the potential for adaptation of this species to future climate conditions. Three main findings emerged from the analyses performed. Firstly, from a physiological point of view, it is
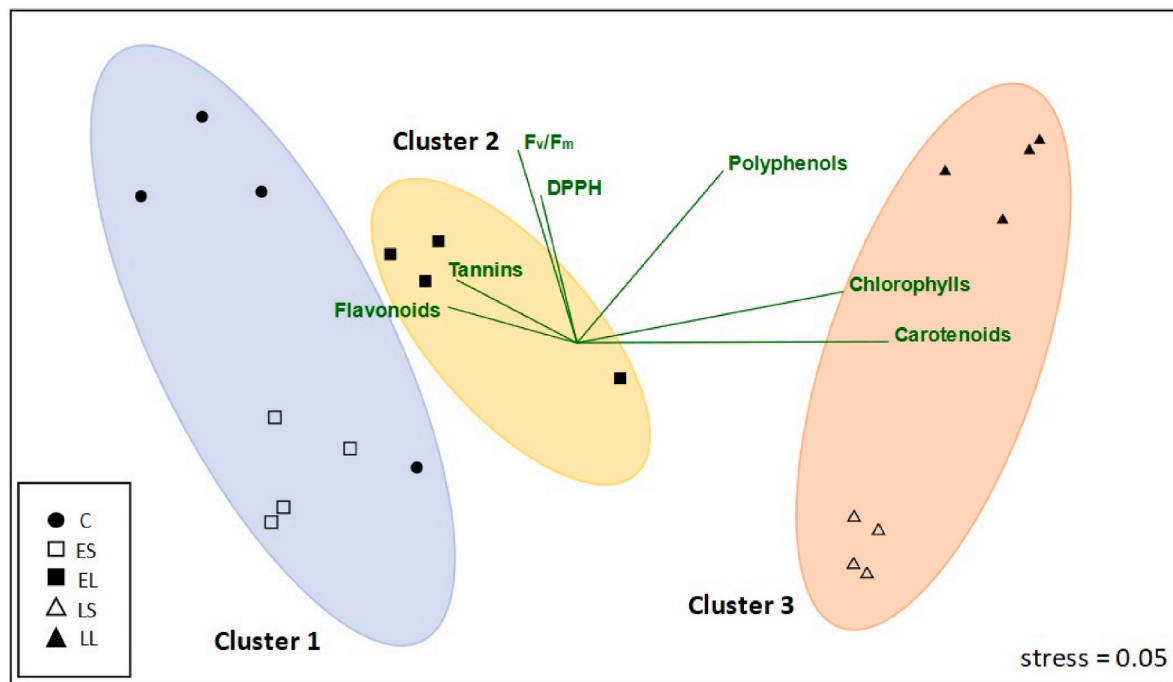
Fig. 5. 2-D nMDS (Euclidean distance, stress = 0.05) performed on the response variables. Colored ellipses correspond to the three clusters detected through the k-means clustering analysis.

noteworthy that the photosynthetic apparatus of *G. barbata* seems to be more sensitive to abrupt thermal stress than long ones. The maximum PSII photochemical efficiency (F_v/F_m) was lower after *short*-term exposure in both *early* and *late* MHWs, while it recovered to control values under the *long* ones. The return to control values of F_v/F_m may indicate the occurrence of mechanisms conferring an acclimation to new environmental conditions. The fast light response curves (RLCs) confirm the hypothesis that thalli exposed to *late long* MHWs are more performant in response to increasing light intensities than those exposed to the *late short* ones, suggesting a better light utilization in PSII photochemistry (Supplementary Figure S1).

Secondly, the "acclimation status" is likely reached by regulating the content of photosynthetic pigments, which act as photoprotective compounds. Indeed, the higher pigment concentration found in both the *late* treatments could be interpreted as a defense strategy aimed at dissipating excess thermal energy, especially considering the photoprotective role of carotenoids (Demmig-Adams and Adams, 2006; García-Plazaola and Becerril, 1999). Goss and Jakob (2010) suggested that the xanthophyll cycle, in which pigments are part of the whole carotenoid pool, represents an essential mechanism of photoprotection in plant cells. Under certain stress conditions, de-epoxidation of violaxanthin to zeaxanthin occurs through antheraxanthin. Zeaxanthin is used to dissipate excess energy from excited chlorophylls.

Thirdly, it seems that *G. barbata* recruits modulate the production of secondary metabolites with antioxidant action to face environmental constraints, thus avoiding an overproduction of reactive oxygen species (ROS). In particular, higher levels of polyphenols were found in recruits exposed to *late long* MHWs, conditions associated with higher longer-lasting temperatures. Such evidence can be regarded as a physiological adjustment to quench the excess of temperature, with polyphenols acting as ROS scavengers minimizing the effect of thermal stress. In other words, increased synthesis of these compounds was rapidly triggered in recruits exposed to the highest stress level as a defense strategy against ROS production. This response can contribute to the acclimation of thalli to the new environmental condition (Swanson and Fox, 2007; Cruces et al., 2012), increasing the thermo-tolerance of *G. barbata* recruits. The recovery of radical scavenging activity (DPPH) to control

values under *late long* MHWs suggests that polyphenols strongly contribute to the antioxidant capacity, enabling the recruits to endure the putative oxidative stress.

Conversely, low concentrations of tannins were found in *late* MHWs treatments. The downregulation of tannins may result from a stress condition which likely exceed the capacity of algae to synthesize these compounds (Cruces et al., 2012). At the same time, the higher levels of tannins in the *control* treatment, can be assumed as a distinct trait of this species, also considering that in other brown seaweed, the level of tannins can account for up to 20% of the dry weight of the seaweed (Swanson and Druehl, 2002).

The flavonoid profile under the different treatments was less clear; indeed, flavonoid concentration raised in recruits exposed to the *early long* MHWs, suggesting a threshold-dependent production of these compounds, tolerating temperatures not exceeding 28 °C.

However, despite the outcomes emerged from this study, it is noteworthy that our experiment investigated the effect of one specific predictive variable on *G. barbata* recruits. The physiologic adaptations in benthic organisms is challenging as many responses can be triggered by multiple factors acting simultaneously, resulting in either synergistic or antagonistic effects (Lotze and Worm, 2002). Results suggest that the mechanisms of adaptation activated in response to thermal stress did not affect the natural growth rate of recruits (i.e., no significant differences in recruits length emerged at the end of the experiment). Anyway, this response could be at the expense of other defense mechanisms impairing the capacity of recruits to cope with different stressors. For instance, the low tannins concentration observed in *late* MHWs treatments might pose a threat since tannins are important deterrent compounds against herbivory (Haavisto et al., 2017), making recruits more vulnerable to the grazing pressure. Cumulative effects of climate change and human stressors exert continuous and dynamic impacts on marine ecosystems inducing ecological responses at multiple levels of biological organization (Gissi et al., 2021). Understanding the interactions among multiple stressors, the role of ecological memory (Dal Bello et al., 2017) and the importance of species functional traits is urgently needed to identify species vulnerability to MHWs (Turner et al., 2020; Garrabou et al., 2022; Harvey et al., 2022) and hence, their potential for adaptation. In

addition, long-term data, including historical and real time data, collected at the appropriate temporal and spatial scale, are critically needed to correctly depict both long-term responses and short-term abrupt shifts (Rilov et al., 2020; Gissi et al., 2021). A recruits thermo-tolerance test cannot provide conclusive evidences to depict the complexity of adaptation processes. Future efforts should be directed in combining laboratory tests and in-situ investigations, especially for quantifying spatial-temporal variations in key physical, chemical, biological and meteorological variables to be selected to test recruits resilience to climate change. Defining the range of conditions under which this habitat-forming species can live is pivotal to assess if it will become dominant over other low-tolerant species or expand its biogeographical distribution domain.

Despite the uncertainties related to the limitations above described, overall, this study supports the hypothesis that canopy-forming species can adapt to future climate conditions demonstrating a physiological acclimation to cope with MHWs, providing strong evidence that adaptation of marine species to thermal stress is more frequent than expected, this contributing to design tailored conservation and restoration strategies for marine coastal habitat.

Credit author statement

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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 data that support the findings of this study are openly available in Mendeley Data repository at http://doi.org/10.17632/f9gnk9vkdd.2.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117218.

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