TITLE: Positive effects of high salinity can buffer the negative effects of experimental warming on functional traits of the seagrass *Halophila ovalis*

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Abstract

Coastal ecosystems, and especially estuaries, are subject to environmental fluctuations that can be amplified by anthropogenic changes. Under a future scenario of global warming, temperature and salinity, two key environmental factors for plants, are likely to be altered and the persistence of macrophyte-dominated ecosystems can be compromised, particularly native or local seagrass communities. This study examined the response of the local seagrass *Halophila ovalis* to the joint effect of a short-term salinity increase and a transient temperature stress, through two indoor mesocosm experiments. Warming caused a decline in maximum quantum yield, TNC content in leaves and plant growth, and increased dark respiration, revealing clear detrimental symptoms of heat stress on plant metabolism and performance. Salinity increase in isolation favoured ramet survival.

However, in combination with warming, salinity had a positive effect on Gross P_{max}. This suggests that increased salinities might dampen the negative effects of high temperatures, buffering, to some extent, the impact of global warming in temperate estuaries.

Introduction

Global change is threatening ecosystems worldwide (Bellard et al., 2012; IPCC 2014) and is considered a major driver of the erosion of marine biodiversity (Poloczanska et al., 2013). Coastal ecosystems are particularly vulnerable to global change because they are exposed to a range of cumulative impacts, including, eutrophication, physical alterations, pollution and overfishing, all strongly linked to human population pressures on the coast in addition to climate change impacts (Halpern et al., 2015; Orth et al., 2006). Increased temperatures due to global warming and heatwaves (Smale et al., 2019) can impact coastal ecosystems, often in additive or synergistic ways with other stressors (e.g. Humanes et al., 2016; Ontoria et al., 2019a). This is of particular concern for ecosystem engineers, such as habitat forming corals, mangroves or seagrasses, due to the major cascading effects on biodiversity and ecosystem functions (Smale et al., 2019) including primary production, fisheries provision, carbon sinks and buffering acidification (Beaumont et al., 2007).

Estuaries are subject to environmental fluctuations, both gradual and abrupt. These pose significant physical forcing and influence ecological relationships (Day et al., 2012) making estuaries particularly vulnerable to climate change (Hallett et al., 2018). Organisms inhabiting estuaries generally tolerate salinity changes using a range of ecophysiological mechanisms via different metabolic pathways (Gupta and Huang, 2014). With progressive warming and heat waves, evaporation rates from estuaries are likely to increase, resulting in increases in salinity, especially where flushing with fresh

51 or marine water is limited. These two physical factors, temperature and salinity, are likely
52 to impact estuarine ecosystems beyond the range of variation already experienced (Hallett
53 et al., 2018).

54 Seagrasses, one of the most productive ecosystems on Earth (Hemminga and Duarte,
55 2000) and highly valued economically and ecologically (Orth et al., 2006), are a dominant
56 habitat in estuaries. Temperature clearly effects seagrass plant performance across a range
57 of scales, from the molecular to population level (Campbell et al., 2006; Marín-Guirao et
58 al., 2017; Ruiz et al., 2018; Ruocco et al., 2019). They are sensitive and vulnerable to
59 warming (e.g. Strydom et al., 2020) with negative effects on plant performance (e.g.
60 Collier et al., 2011; Ontoria et al., 2019a) and survival (Díaz-Almela et al., 2009)
61 documented. Changes in salinity also alter seagrass physiological functioning and,
62 consequently, influence plant growth and survival (Sandoval-Gil et al., 2012a; Salo and
63 Pedersen, 2014; Salo et al., 2014; Touchette and Burkholder, 2000). Seagrass die-off has
64 been observed with hypersalinity events (Wilson and Dunton, 2018). However, despite
65 the recent increasing interest in interactions between thermal and salinity tolerance
66 thresholds and acclimation mechanisms, this is still poorly understood for seagrasses,
67 particularly with hypersalinity. Temperature increases could affect plant performance not
68 only directly but also through its interaction with plant tolerance mechanisms to changes
69 in salinity (Piro et al., 2015). The higher respiratory demand under hypersaline conditions
70 (Johnson et al., 2018) could lead to synergistic impacts with temperature.

71 Estuaries in Mediterranean climate regions, such as southwestern Australia, experience
72 high temperatures and elevated salinities in summer and these are predicted to increase
73 with climate change (Hallet et al., 2018). *Halophila ovalis* is one of the most common
74 seagrass species found in estuaries of southwestern Australia. It is a fast growing,
75 colonizing species (*sensu* Kilminster et al., 2015) with a clear ability to recover quickly

from disturbances. *H. ovalis* has a wide tolerance range, occurring in waters between 10 ºC and 40 ºC (Ralph 1998) and from 5 to 45 psu (Hillman 1995; Tyerman 1982), which coincides with its broad distribution and abundance in estuarine environments. Previous short-term experiments (five days, Ralph 1998) with laboratory-cultured plants revealed that while *H. ovalis* has its optimum photosynthetic range between 25 and 30 ºC, its tolerance to salinity can range from 9 to 52 psu. However, there is a lack of knowledge about the responses to periods longer than five days to each one of these factors, as well as about their potential interaction.

The present study aims to explore the response of an estuarine seagrass, *H. ovalis*, to warming and salinity changes linked to climate change and, specifically, to assess whether temperature increases affect plant tolerance to salinity fluctuations. To do this, two indoor mesocosm experiments were performed to evaluate plant responses to changing salinity under thermal increase, at the physiological, individual and population levels. We hypothesize that the simultaneous occurrence of warming and high salinities will lead to deleterious effects on plant performance.

Material and Methods

The response of *H. ovalis* to increases in temperature under different salinity conditions was assessed through two independent mesocosms experiments, thus evaluating the responses not only to short-term (1 day) but also to medium-term (13 days) temperature and salinity exposure. The two experiments were required to enable measurements across a range of plant scales: physiological, individual and population levels and also with a range of treatments that were not possible in a single experiment due to technical and logistical constraints. In the first experiment (experiment A, hereinafter), photosynthesis-irradiance curves were performed after 1 day of exposure to five different temperatures

and two salinity conditions. In the second experiment, (experiment B, hereinafter), photochemical responses, carbon reserves, plant growth and survival were measured in plants subjected to two temperatures and two salinity conditions over 13 days (see experimental designs in Figure S1). The temperature conditions in this experiment were selected to represent temperature treatments where significant differences in the P-I relationship were detected in the first experiment as well as to simulate a heat wave event. Both experiments were conducted at the School of Science aquarium facilities at Edith Cowan University (Western Australia).

Experiment A: Photosynthesis-Irradiance curves

Plant collection, experimental set up and incubation equipment

Plants for the experiment were collected in late spring-early summer 2017 in the Swan-Canning Estuary (southwestern Australia). The Swan-Canning estuary is a shallow estuary permanently open to the ocean. It is characterized by two distinct 'summer' and 'winter' phases due to the region's Mediterranean climate. During summer (December – March), air temperatures are high, and rainfall is typically low. Climatological (1961 – 1990) averages for the Perth metropolitan area, where the estuary is located, are in the range 29–31 ºC and 10–19 mm (Bureau of Meteorology, http://www.bom.gov.au). Water temperature and salinity are greatly influenced by these climatic conditions and in summer averages range between 22–24 ºC and 33–38 psu respectively (Hillman 1995; http://wir.water.wa.gov.au/Pages/Water-Information-Reporting.aspx). The dominant seagrass is *H. ovalis*, with the majority of meadows occurring in shallow, subtidal areas (< 2 m depth). Peak growth and reproduction are generally observed during summer months (Forbes and Kilminster, 2014). Contrastingly, during winter (June – August) air temperatures are cooler ~19–19.4 ºC whilst the majority (~80 %) of rainfall occurs and

126 averages between 125–126 mm (Bureau of Meteorology, http://www.bom.gov.au)
127 resulting in average temperature and salinity ranges of 14–15 ºC and 20-25 psu
128 respectively.
129 Fragments (ramets) of *H. ovalis* (including three or four pair of leaves, rhizomes, roots
130 and a growing tip) were carefully collected and transported in a cooler box filled with *in*
131 *situ* seawater to the laboratory. Dark adapted yields were measured from three randomly
132 chosen plants in the field using a diving PAM (pulse-amplitude modulated) fluorometer
133 (Walz, Germany). These measurements were used afterwards as a dark-adapted yield
134 reference to confirm that plants in the experimental tanks had acclimated to the laboratory
135 conditions after planting. Temperature and salinity at collection time were on average: 23
136 ºC and 34 psu and were used to set the acclimation conditions in the laboratory.
137 Plants were standardized to four leaf pairs with an apical meristem (the growing tip) and
138 were carefully cleaned by hand to remove epiphytes before planting. Ramets were then
139 planted into aquarium tanks (54 L capacity, 600 x 300 x 300 mm), previously filled with
140 10 cm depth of unsorted and washed quartz river sand and 52 L of *in situ* filtered seawater
141 at 20 ºC with a salinity of 34 psu. Water and sediments employed were not inoculated.
142 Each tank had its own independent sump tank containing a pump, filter (300 µm foam
143 block) and aquaria heaters which controlled water temperature. Incident light on plants
144 (180 µmol photons m^{-2} s^{-1}, based on saturating irradiance for *H. ovalis* according to
145 Strydom et al., 2017) was provided by marine aquarium Light Emitting Diode (LED)
146 modules with a full spectrum light (MarinTech™ Pty Ltd) on a 12 h: 12 h light: dark
147 photoperiod. After two days of acclimation, temperature was progressively increased or
148 decreased (1 ºC day^{-1}) in the tanks until reaching the experimental temperatures (20 ºC,
149 24 ºC, 28 ºC, 31 ºC and 34 ºC). This range of temperatures was established in order to
150 cover the natural temperature range this species experiences during spring and summer

151 in the area where it was collected based on ten years of water quality monitoring by the
152 state government agency (https://www.water.wa.gov.au/maps-and-
153 data/monitoring/water-information-reporting). The highest temperature treatment was
154 above what is experienced on average in the estuary and was chosen in order to achieve
155 the temperature expected to be experienced during a heat wave event, up to 5 ºC above
156 average summer temperatures that was previously observed in the region (Pearce and
157 Feng, 2013). Simultaneously, salinity was progressively increased (2 psu day^{-1}) from 34
158 psu (salinity during acclimation days) until reaching 40-42 psu (high salinity) in half of
159 the tanks, keeping the other half at 34 psu (low salinity) (See Figure S2 A for further
160 details on application of the treatments). This high salinity is above what is generally
161 experienced in this estuary. Thus, ten different conditions were set up: five different
162 temperatures with each temperature treatment having two salinity treatments. Once the
163 experimental conditions were reached in the tanks, plants were left for 24 hours before
164 measuring maximum quantum yield on five randomly chosen plants to confirm their
165 acclimation. Only one set of five measurements from one salinity by temperature could
166 be undertaken during one day, therefore the measurements of each treatment were
167 staggered over a two week period (Figure S2 A). Yields obtained were equal or higher to
168 those measured in the field at the time of collection (0.65-0.71) in all cases, hence the
169 plants were considered acclimated.
170
171 **P-I determinations, curve fitting and extraction of photosynthetic parameters**
172 Seagrass respiration and photosynthesis were measured via the consumption or
173 production of O_2 after 24 hours incubation period under each corresponding experimental
174 condition. Plants were incubated in sealed transparent acrylic chambers, with a diameter
175 of 52 mm and a length of 150 mm (volume = 318 ml). Water within the chamber was

176 circulated using a small submersible pump with a flow rate of 7000 ml hr^{-1}. Dissolved
177 oxygen concentrations within the chambers were measured using FireSting™ 3 mm
178 robust REDFLASH technology sensors (Pyroscience) inserted through the chamber wall
179 and connected through a 4-channel meter to a computer recording O_2 concentrations (mg
180 L^{-1}). To maintain a stable temperature (± 0.25ºC) chambers were submerged in a 300 L
181 tank containing 150 L of seawater, which was circulated through a chiller-heater unit set
182 to the appropriate experimental temperature. For temperature treatments above 30 ºC
183 saltwater was made using aquarium salt and milli-Q water to remove the additive effect
184 of microbial respiration. The internal temperature of the chamber was also measured
185 using a submersible temperature sensor connected to the FireSting O_2 machine. Light was
186 provided by full spectrum LED light units (GrowPro 320; MarinTechTM Pty Ltd)
187 suspended above the chambers, providing light intensities from 30 to 300 µmol photons
188 m^{-2} s^{-1}. Prior to each incubation, the oxygen electrodes were calibrated using a 2-point
189 method (0 % and 100 % air saturated water) calibration as per the manufacturer's
190 instructions. For P-I determinations, four-five *H. ovalis* ramets (apical meristem with four
191 preceding leaf pairs connected to rhizome) were placed into the chambers. Five replicate
192 chambers with plants and a blank (no plant material) were established and placed into the
193 temperature-controlled tank. The chambers were then covered in aluminum foil to
194 exclude light and the inlet of each chamber was connected to its individual pump, to allow
195 the chamber to be flushed whilst the plant material was left to dark adapt for 30 minutes.
196 When dark adapted, the chamber outlet was connected to the pump to create a sealed
197 system. Once sealed, the dissolved oxygen concentration in each chamber was monitored
198 every second. Monitoring continued in the dark for at least 20 minutes after the slope of
199 dissolved oxygen vs time stabilized. The foil was then removed, and photosynthetic rates
200 were measured for 10 minutes (once the slope had stabilized) at each of the 7 light

intensities (30, 60, 90, 120, 180, 240, and 300 µmol photons m^{-2} s^{-1}). At the end of the experiment whole plants were removed from the chambers and were separated into above (leaves) and below ground (roots and rhizomes) tissue. Fresh weight was recorded before drying the plants (48 hours at 60 ºC) and reweighing for dry weight determination.

For each replicate chamber (and control) at all unique temperature-light intensity combinations, oxygen concentration was plotted against time after discarding the first 2 minutes of data, which was considered a stabilisation period. The portion of the remaining data used to determine the rate, was confined to that where the R^2 value was greater than 0.9. At the two lowest light intensities, due to noise, a lower R^2 value of 0.5 was used. Rates of oxygen exchange were normalised to g DW of seagrass hr^{-1}. Oxygen concentrations within the control chamber (containing no seagrass) were measured throughout the experiment as a procedural control, data was accepted if there was a stable line with little variation, neither decreasing nor increasing in oxygen concentration. This was achieved in all trials.

Photosynthetic parameters were extracted from the photosynthesis-irradiance (P-I) curves using the least-squares method. For each incubation, P-I curves were fitted to the data using the hyperbolic tangent model equation of Chalker (1981):

$$P = \text{Gross } P_{max} \times \tanh\left(\frac{\alpha \times I}{GPmax}\right) - R,$$

where P is the net rate of photosynthesis (mg O_2 g DW^{-1} h^{-1}), Gross P_{max} is the maximum gross photosynthesis (mg O_2 g DW^{-1} hr^{-1}), α is the photosynthetic efficiency estimated as the slope for the linear portion (light-limited portion) of the P-I curve, I is irradiance (µmol photons m^{-2} s^{-1}), and R is the rate of oxygen consumption in the dark (mg O_2 g DW^{-1} h^{-1}).

224 NP_{max} was defined as the maximal net rate of photosynthesis (mg O_2 g DW^{-1} h^{-1}) and was
225 calculated as:

226 $$NP_{max} = \text{Gross } P_{max} - R$$

227 The half-saturating irradiance (I_k; µmol photons m^{-2} s^{-1}) was calculated as:

228 $$I_k = (NP_{max} + R) / \alpha$$

229

230 ***Experiment B: Photochemical responses and other plant traits***

231 **Plant collection, mesocosm system and experimental set up**

232 Undamaged healthy *H. ovalis* ramets were carefully collected by hand from a shallow,
233 undisturbed meadow (< 0.5 m deep) in spring-early summer 2017 in Peel Harvey Estuary
234 (southwestern Australia). Similar to the Swan-Canning estuary, the Peel-Harvey Estuary
235 is a shallow, permanently open estuary and the salinity and temperature regimes reflect
236 the hot, dry conditions during summer. Between December to March, rainfall and
237 temperature range between 12.5–19.6 mm and 28–31 ºC, respectively (Bureau of
238 Meteorology, http://www.bom.gov.au). In the estuary, water temperatures average 21–23
239 ºC, and becomes increasingly hypersaline over the summer from 35 psu in December to
240 43 psu by March (http://wir.water.wa.gov.au/Pages/Water-Information-Reporting.aspx).
241 Since the estuary was opened to the ocean via the Dawesville channel in 1994, salinity
242 has become more stable and closer to marine values (~ 35 psu) except during winter when
243 most of the rainfall occurs (Water and Rivers Commission, 1998, link:
244 https://www.water.wa.gov.au/__data/assets/pdf_file/0019/5356/10564.pdf) and this has
245 favoured the establishment of *H. ovalis* meadows, the dominant seagrass species in the
246 estuary.

247 At the time of collection, the average field temperature was 20 ºC and salinity 34 psu.
248 Plants were transported to the laboratory and kept under constant temperature (20 ºC)

249 overnight before planting. Nine ramets of *H. ovalis* plants (each ramet standardized to
250 two pairs of leaves, rhizome, roots and a growing tip) were planted in aquarium tanks as
251 described above. The ramets were shorter than in the experiment A as the plants were
252 naturally more fragmented in the field.

253 In order to determine plant response to warming when growing under different levels of
254 salinity, *H. ovalis* plants were exposed to two levels of temperature (control and high)
255 and two levels of salinity (low and high) simultaneously in a short-term experiment
256 lasting for 13 days. Control temperature condition was set as the average field temperature
257 (24 ºC) in late spring while 30 ºC was set up as the high thermal treatment. The latter
258 temperature is above the average summer temperatures and was selected to simulate the
259 temperature that might be reached in these estuarine environments during extreme
260 warming events such as heat waves based on Pearce and Feng (2013). The salinity
261 measured in the field during plant collection was 34 psu and it was considered the low
262 salinity treatment. The high salinity tested (40-42 psu), in turn, is within the range
263 experienced in some parts of the estuary, but not where the seagrass was collected. Water
264 temperature and salinity were monitored every two days by a WTW^{TM} conductivity meter
265 throughout the experiment. Plants were acclimated at 24 ºC (progressive increasing of 1
266 ºC day^{-1}) and 34 psu for seven days, after which temperature and salinity were increased
267 progressively (1 ºC day^{-1} and 2 psu day^{-1}, respectively) in the appropriate treatment tanks
268 until reaching the temperature and salinity experimental treatments. It took 13 days to
269 reach treatment conditions, then the plants were exposed for another 13 days to different
270 temperature and salinity treatments as follows: control temperature and low salinity (24
271 ºC, 34 psu), high temperature and low salinity (30 ºC, 34 psu), control temperature and
272 high salinity (24 ºC, 40-42 psu) and high temperature and high salinity (30 ºC, 40-42 psu).
273 Temperature and salinity during the acclimation and experimental period were

maintained within ±1 ºC and ±1 psu (Figure S2 B and C). For each treatment, four replicate tanks were established (total n=16 independent tanks) and treatments were randomly allocated. Incident light (200-220 µmol photons m^{-2} s^{-1}, above the saturation irradiance for these plants) was measured using an underwater quantum sensor (MicroPAR) and maintained on a 12 h: 12 h light: dark photoperiod.

Plant traits assessment

Chlorophyll a fluorescence parameters

In each tank, chlorophyll *a* fluorescence parameters were measured in three randomly selected shoots from three independent ramets using a diving PAM fluorometer. The effective quantum yield (AF/F_m') and maximum quantum yield (F_v/F_m) of PSII measurements were obtained in the middle portion of the leaf (one leaf of the second or third pair of leaves behind the apical tip) by the saturation pulse method after an overnight dark adaptation. After being exposed to illumination conditions for three hours, an increasing photosynthetic photon flux density was applied directly to the leaf at intervals of 10 s to perform rapid light curves (RLCs), which were fitted to the equation described by Jassby and Platt (1976) using SigmaPlot (version 11) in order to estimate the photosynthetic efficiency (α), half-saturating irradiance (I_k) and maximum absolute electron transport rate (ETR_{max}). The absorption factor (AF) was determined following Beer and Björk (2000) and averaged for the population (AF=0.6) before performing RLCs.

Carbohydrates content

Total non-structural carbohydrates (TNCs) analyses were performed separately in leaves and rhizome material. All ramets from each tank were pooled thus having a total of four replicate samples of each tissue per treatment. Samples were analyzed for soluble sugars

and starch content following the anthrone assay described in Marín-Guirao et al., (2013), based on Invers et al., (2004) and Yemm and Willis, (1954).

Plant growth and survival

At the beginning of the experiment, all the ramets were tagged to be able to identify the new tissue produced in the experiment. All newly produced material (leaves, petioles, rhizomes and roots) from each ramet was dried at 60 ºC for 48 h and weighed. Plant growth was expressed as mg DW $ramet^{-1}$ day^{-1}. At the end of the experiment, the number of surviving ramets in each tank was estimated and expressed in percentage relative to the initial number of ramets planted.

Statistical analyses (Experiments A and B)

PERMANOVA analyses based on a similarity matrix created from the Euclidean distances between samples was performed to test the statistical significance of the effects of temperature and salinity (fixed factors) on seagrass response parameters. For the experiment A, we had 10 experimental conditions (with five replicates for each), resulting from the combination of five temperatures (20 ºC, 24 ºC, 28 ºC, 31 ºC and 34 ºC) and two salinities (low and high, i.e. 34 psu and 40-42 psu). For experiment B, the two factors had two levels (temperature: 24 ºC and 30 ºC, salinity: 34 psu and 40-42 psu), resulting in four experimental conditions, with four replicate tanks for each.

PERMANOVA analyses were run separately for each experiment and each response variable (experiment A: photosynthetic and respiration rates, half-saturating irradiance and photosynthetic eficiency; experiment B: F_v/F_m, AF/F_m', ETR_{max}, half-saturating irradiance and photosynthetic efficiency, TNC content in leaves and rhizomes, plant growth rate, and ramets survival). This test was selected as it is produced by permutation and meeting the assumptions of a normal distribution was not required (Anderson 2001).

The PERMDISP test was performed to test the homogeneity of dispersions. All variables with the exception of plant growth rate had even dispersions, in this example the significance level (α) was modified from p=0.05 to p=0.01. When fixed factors (temperature, salinity and/or the interaction) had a significant effect on the variable, pair wise comparisons among all pairs of levels of this given factor were performed to identify significant differences between treatments. When the number of permutations was too low (< 999, Anderson et al., 2008), a Monte Carlo test was conducted in order to establish an alternative p-value for analysis validation. All analyses were done using the Primer v6 statistical package (Clarke and Gorley, 2006) in conjunction with the Windows PERMANOVA+ module (Anderson et al., 2008).

Results

Experiment A: Photosynthesis-Irradiance curves

Typical Michaelis-Menten-like curves with no photoinhibition were observed in each experimental condition for the *H. ovalis* plants (Figure 1). All of the photosynthetic parameters extracted from the photosynthesis-irradiance (P-I) curves were significantly affected by the experimental factors, either temperature only, salinity only, both and/or an interaction between the two (Figure 2, Table 1). Maximum gross photosynthesis values ranged from 2.6 to 6.6 mg O_2 g DW^{-1} hr^{-1}, and was significantly affected by both factors and by their interaction (Table 1). At low salinity, the effect of temperature, albeit significant, was unclear, with highest values at 20 ºC and 31 ºC (3.9 and 4.1 mg O_2 g DW^{-1} hr^{-1}, respectively) and lowest at 24 ºC and 28 ºC (2.6 mg O_2 g DW^{-1} hr^{-1}) (Figure 2A). In contrast, at high salinity, temperature had a clear and positive effect on maximum gross photosynthesis, reaching 6.6 mg O_2 g DW^{-1} hr^{-1} at 34 ºC.

348 Respiration rates ranged from 0.84 to 2.61 mg O_2 g DW^{-1} hr^{-1} (Figure 2B). Temperature
349 had a positive and significant effect on respiration, resulting in an increase of 81 % at the
350 highest temperature (34 ºC) relative to the lowest (20 ºC), while salinity did not
351 significantly affect respiration (Table 1). Half-saturating irradiance (I_k) ranged from 62
352 to 126 µmol photons m^{-2} s^{-1} (Figure 2C). Salinity affected I_k significantly (Table 1) with
353 higher I_k at higher salinities. Temperature, in contrast, did not affect I_k (Table 1).
354 Photosynthetic efficiency (α) ranged from 0.03 to 0.07 mg O_2 g DW^{-1} hr^{-1} /µmol quanta
355 m^{-2} s^{-1} (Figure 2D). No individual effects of temperature nor salinity were found, but there
356 was a significant interaction (Table 1) due to the opposite response of α with temperature,
357 decreasing at low salinity (from 0.07 to 0.04 mg O_2 g DW^{-1} hr^{-1} /µmol quanta m^{-2} s^{-1}) and
358 increasing at high salinity (from 0.03 to 0.07 mg O_2 g DW^{-1} hr^{-1} /µmol quanta m^{-2} s^{-1}).
359
360 ***Experiment B: Photochemical responses and other plant traits***
361 Most of the plant traits measured were significantly altered by the thermal treatment (30
362 ºC). Thus, maximum quantum yield (F_v/F_m) was depressed (by 40%) and maximum
363 electron transport rate (ETR_{max}) increased 28 % (in both cases relative to control values,
364 Figure 3A and 3B; Table 2). There was a significant interaction for photosynthetic
365 efficiency (α) (Figure 3C; Table 2), it increased 38 % with higher temperature when plants
366 were grown at the low salinity but not under high salinity . However, effective quantum
367 yield ($\Delta F/F_m'$) and half-saturating irradiance (I_k) were not affected by temperature (Figure
368 S3 A and B; Table 2). Higher temperatures significantly reduced TNCs in leaves by 28
369 % (Figure 3D; Table 2) but not in rhizomes (Figure S3 D; Table 2). Moreover, plant
370 growth dropped at high temperature (55 % less than controls, Figure 3E; Table 2), but did
371 not modify plant survival. The only significant effect caused by salinity was in ramet
372 survival, which increased 21 % (Figure 3F; Table 2).

Discussion

In this work, the response of *H. ovalis* to temperature and salinity, both individually and in combination, was assessed in two indoor mesocosm experiments. Overall, our exploratory results show negative effects of the high temperatures but, interestingly, high salinities seem to buffer, to some extent, the impacts of short-term warming.

Symptoms of thermal stress were detected in most plant traits evaluated. The sensitivity of the photosynthetic apparatus to warming was reflected by the decline of F_v/F_m to values below those commonly accepted for healthy plants (0.7-0.8, Campbell et al., 2006; Ralph 1998) after 13 days of exposure to high temperature (30 ºC), which indicates photochemical damage. However, this damage seemed to have no negative consequences in maximum gross photosynthesis and maximum electron transport rate. This lack of response in maximum gross photosynthesis might be attributed to the short exposure (1 day) to thermal conditions in the P-I experiment (experiment A). However, it is consistent with previous studies on other fast-growing seagrass species inhabiting estuarine environments (e.g. *Cymodocea nodosa*, Marín-Guirao et al., 2018; *Halodule uninervis*, Collier et al., 2011; *Halophila johnsonii,* Fernández-Torquemada et al., 2005), where photosynthesis was either unaffected or affected positively by increasing temperatures. As our plants were photosynthetically active throughout the whole range of temperatures tested (20-34 ºC) in the P-I experiment, the critical temperature threshold for photosynthesis was not reached, and a photosynthetic thermal optimum for this species cannot be drawn from here. The observed increase of ETR_{max} at high temperature following 13 days exposure (experiment B) is consistent with the enhancement of photosynthetic rates suggesting high rates of inorganic carbon assimilation. Moreover, ETR_{max} enhancement could also respond to the activation of alternative electron sinks

398 that can be activated under stressful conditions to alleviate PSII damage (Sharkey 2005,
399 Marín-Guirao et al., 2016), as indicated by the reported decline in F_v/F_m in our
400 experiment.

401 As expected, the respiration rate did increase with temperature, as has been observed in
402 other species (e.g. *Cymodocea serrulata*, *Halodule uninervis* and *Zostera muelleri*,
403 Collier et al., 2017; *Posidonia oceanica* and *C. nodosa*, Marín-Guirao et al., 2018;
404 *Thalassia hemprichii* and *Enhalus acoroides*, Pedersen et al., 2016). This increase in
405 respiration seemed to have an overall negative effect at the individual plant scale, as
406 indicated by a decrease in growth rate and carbohydrate content in the leaves at high
407 temperature. This can be attributed, most likely, to the use of internal resources to cope
408 with the energetic demands of increased respiration. In fact, reductions in growth have
409 been reported for many species of seagrasses worldwide when the optimal temperature is
410 exceeded (Collier and Waycott, 2014; Collier et al., 2011; Olsen et al., 2012; Ontoria et
411 al., 2019a, 2019b; Traboni et al., 2018). Our exploratory results support the well known
412 observation that the optimum temperature for photosynthesis is usually higher than for
413 growth (Lee et al., 2007). A reliable explanation is that, under thermal stress, despite the
414 photosynthetic rate being maintained, a greater amount of carbon fixed by photosynthesis
415 is used by respiration at the expense of the carbon allocation to growth and storage.
416 Despite reduced growth, warming did not affect plant survival over the 13 days period of
417 exposure. This has not been the case in other studies where increased mortality of *H.*
418 *ovalis* was observed following 6 days of thermal stress (Collier et al., 2014). These
419 differences can be attributed not only to the different temperatures used (30 ºC in this
420 study, 40-43 ºC in Collier et al., 2014) but also to the different environments the plants
421 live in (temperate areas here, tropical ones in Collier et al., 2014). Differential,
422 intraspecific tolerance to heat stress have been evidenced in Mediterranean seagrass

423 species in relation to the different thermal regimes under which different populations
424 inhabit (e.g. depth gradients, Marín-Guirao et al. 2017; or across geographical gradients,
425 Marín-Guirao et al., 2018) highlighting the need for considering local adaptation (or
426 acclimation) when predicting impacts of changing environmental conditions.
427 Furthermore, our study has shown that the eurythermal species *H. ovalis* (den Hartog
428 1970; Hillman 1985) tolerates thermal shock up to 30 ºC for 13 days, at least in terms of
429 mortality, widening the previous 4 days tested by Ralph (1998).

430 Salinity on its own (that is, at control temperatures, 20-24 ºC in the short-term P-I
431 experiment and "low", 24 ºC, in the moderate exposure experiment) had no negative
432 effects on plant performance. This was relatively unexpected, as other studies have
433 documented enhanced respiration with increased salinity, as for instance *P. oceanica*, *C.*
434 *nodosa* or *Zostera japonica* (Marín-Guirao et al., 2011; Sandoval-Gil et al., 2012b; Shafer
435 et al., 2011). Notwithstanding, respiration reduction at high salinities of 40-60 psu were
436 described in a congeneric species (*H. johnsonii*, Fernández-Torquemada et al., 2005).
437 Photosynthetic efficiency (α) in our experiment A decreased with salinity at control
438 temperatures, indicating a decline in plant functioning at high salinity conditions in the
439 absence of warming. However, photosynthesis was stimulated by high salinities even
440 with only moderate warming. Fernández-Torquemada et al., (2005) found that
441 photosynthesis was favored by acute salinity exposure up to 40 psu (on *H. johnsonii*), but
442 this was opposite to the response of a larger more persistent species, *P. oceanica*, which
443 reduced photosynthetic rate with high salinity (Sandoval-Gil et al., 2014).

444 Interactive effects of temperature and salinity in maximum gross photosynthesis and in α
445 (photosynthetic efficiency at low irradiances) were identified in the short term
446 experiment. Thus, on the one hand, while maximum gross photosynthesis remained more

447 or less constant with rising temperature, it was stimulated at high salinity when
448 temperature was 28 ºC and above.

449 Plants exposed to high salinity can activate different acclimation mechanisms including
450 changes in the cellular ion and solute concentrations through modification of the cell wall
451 (Sandoval-Gil et al., 2012b; Touchette 2007). Although not assessed in this study, these
452 types of plant responses combined with others induced by high temperatures (e.g.
453 antioxidant enzymes, Tutar et al., 2017), may have led to the observed increase in
454 photosynthesis, which is in turn necessary for the metabolic cost of these protective
455 mechanisms. If this was the case, then the TNCs content in leaves of plants grown under
456 high salinity and high temperature, would not have as big a decline. Our results were
457 suggestive of a trend of dampening the reduction in carbohydrates caused by high
458 temperature with high salinity but were no conclusive (p-value of 0.085). However, at the
459 population level, plant survival was higher at high salinity. These observations suggest
460 that this species could likely have developed mechanisms to acclimate to increased
461 salinity, as has been reported for other seagrass species exposed to potentially stressful
462 conditions (e.g. transcriptomic mechanisms to cope with heat stress, Marín-Guirao et al.,
463 2017), such as the optimization of phenotype variation in response to stress or the pre-
464 adaptation of genes in anticipation of stress. This is not surprising as *H. ovalis* is a
465 euryhaline species with a distribution across a wide range of environments, in terms of
466 the salinity regime. Any of those above-mentioned mechanisms may be one of the traits
467 explaining the tolerance of this species to such different salinities, an ability that has been
468 documented previously for other seagrass species invading a wide range of environments
469 (e.g. *Halophila stipulacea*, Oscar et al., 2018).

470 The notion that some consequences of climate change such as increased salinity can
471 dampen other effects such as warming has great interest and warrants pursuing further.

This finding is based on the two complementary experiments presented here which were designed to broadly explore responses and performance at different plant scales (from physiology to population, *sensu* O'Brien et al., 2018) to increased temperature and salinity. Although slightly different exposure levels and durations were used, and the populations of *H. ovalis* were collected in different estuaries, there was consistency in the responses giving confidence in the general value of our exploratory results for this species, at least for those populations inhabiting the temperate area where we worked. It is largely accepted that mesocosms have the advantage of working under controlled conditions, allowing to assess individual and combined effects of different stressors, but they do not fully replicate the complexity of the natural environment. So, field observations or natural experiments examining the responses of seagrass meadows to heatwave events across a salinity gradient would be very valuable to complement this work in the future.

Conclusions

The assessment of the effects of global change on ecosystems in fluctuating environments is a relatively unexplored field of research. Based on our findings, *H. ovalis* populations living in variable salinity environments, such as in the estuaries of southwestern Australia, may be negatively impacted by more frequent and extreme warming events. Extrapolating these exploratory results to real world, suggest that if warming, in turn, results in high salinity conditions through increased evaporation and reduced rainfall, high salinities might buffer plant survival and reduce the negative effect of temperature on plant performance. The experimental nature of this work contributes to an improved understanding of how different factors can interact to influence aquatic plants in coastal ecosystems. This is timely and relevant considering the range of multiple stressors involved in the decline of coastal ecosystems worldwide.

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Tables and figures

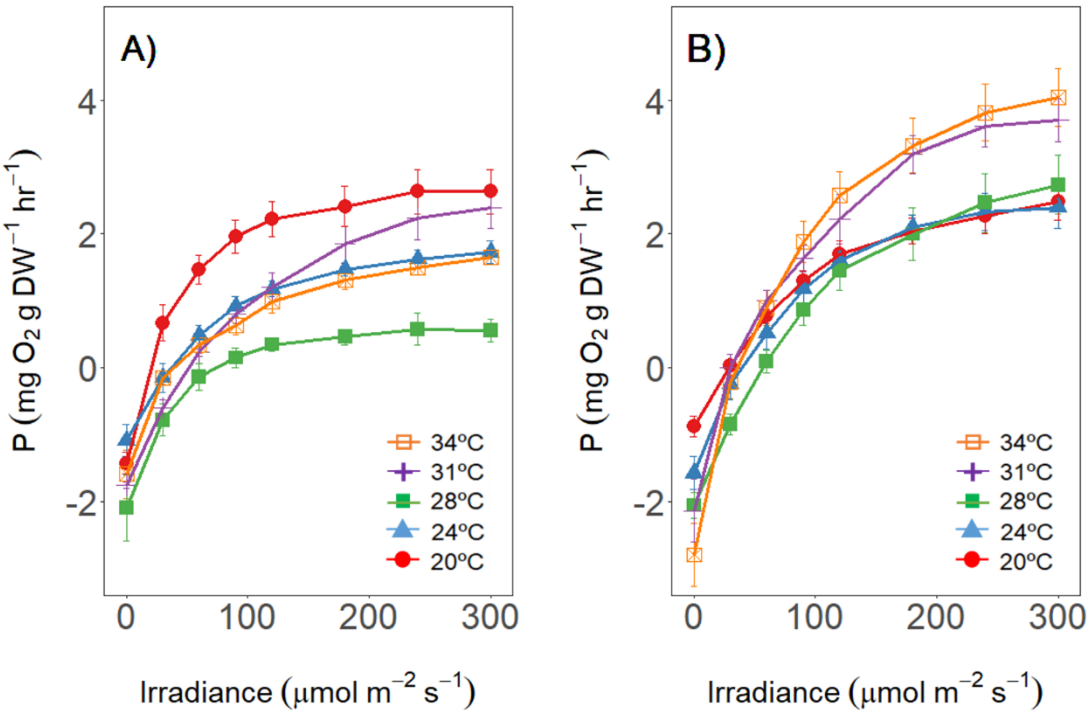


Figure 1. Photosynthesis-Irradiance curves of *Halophila ovalis* plants at five temperatures (20 ºC, 24 ºC, 28 ºC, 31 ºC and 34 ºC) and two salinities: A) low (34 psu) and B) high (40-42 psu), obtained from experiment A.

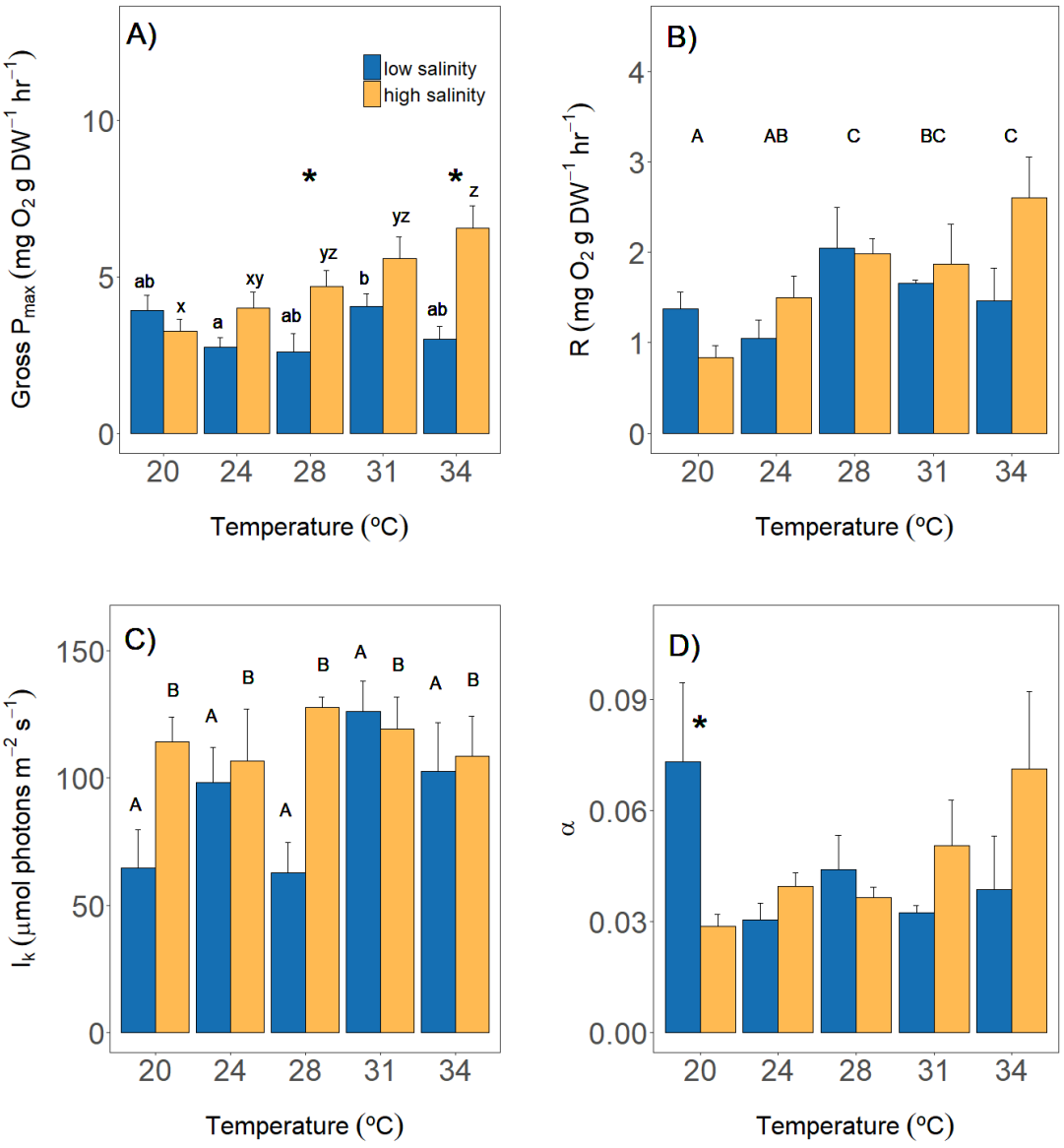


Figure 1. Photosynthetic parameters obtained from P-I curves for *Halophila ovalis* plants in experiment A: A) maximum gross photosynthesis (Gross P_{max}), B) respiration rate (R), C) half-saturating irradiance (I_k) and D) photosynthetic efficiency (α) at five temperatures (20 ºC, 24 ºC, 28 ºC, 31 ºC and 34 ºC) and two salinities (low (34 psu) -blue bars-, and high (40-42 psu) - orange bars-) in the experiment A. Capital letters over the bars represent significant differences between thermal or salinity treatments (independent from the second factor); lower case letters represent significant differences between thermal treatments at each salinity separately (a and b for low salinity treatment and x, y and z for high salinity treatment). Asterisks indicate significant differences between salinity treatments at that temperature.

Table 1. Results of PERMANOVA (univariate analysis) testing for the significance of temperature (20 ºC, 24 ºC, 28 ºC, 31 ºC and 34 ºC) and salinity (low, 34 psu and high, 40-42 psu) effects on photosynthetic parameters extracted from P-I curves obtained from experiment A. Bold values indicate significant effects ($p<0.05$).

Source	df	MS	Pseudo-F	P	Unique perms	
Individual test						
Gross P_{max}						
Temperature (T)	4	4.334	3.472	**0.0151**	9949	
Salinity (S)	1	26.919	21.565	**0.0001**	9830	High > Low
T x S	4	5.440	4.358	**0.0068**	9965	
Residual	36	1.248				
Respiration						
Temperature (T)	4	1.744	3.856	**0.0098**	9952	
Salinity (S)	1	0.659	1.457	0.2333	9865	
T x S	4	0.921	2.037	0.1018	9947	
Residual	36	0.452				
I_k						
Temperature (T)	4	1235.200	1.260	0.3055	9961	
Salinity (S)	1	6738.500	6.876	**0.0131**	9840	High > Low
T x S	4	2167.100	2.211	0.0876	9955	
Residual	36	979.980				
alpha						
Temperature (T)	4	0.001	1.027	0.4018	9946	
Salinity (S)	1	0.000	0.043	0.8375	9817	
T x S	4	0.002	3.111	**0.0223**	9961	
Residual	36	0.001				

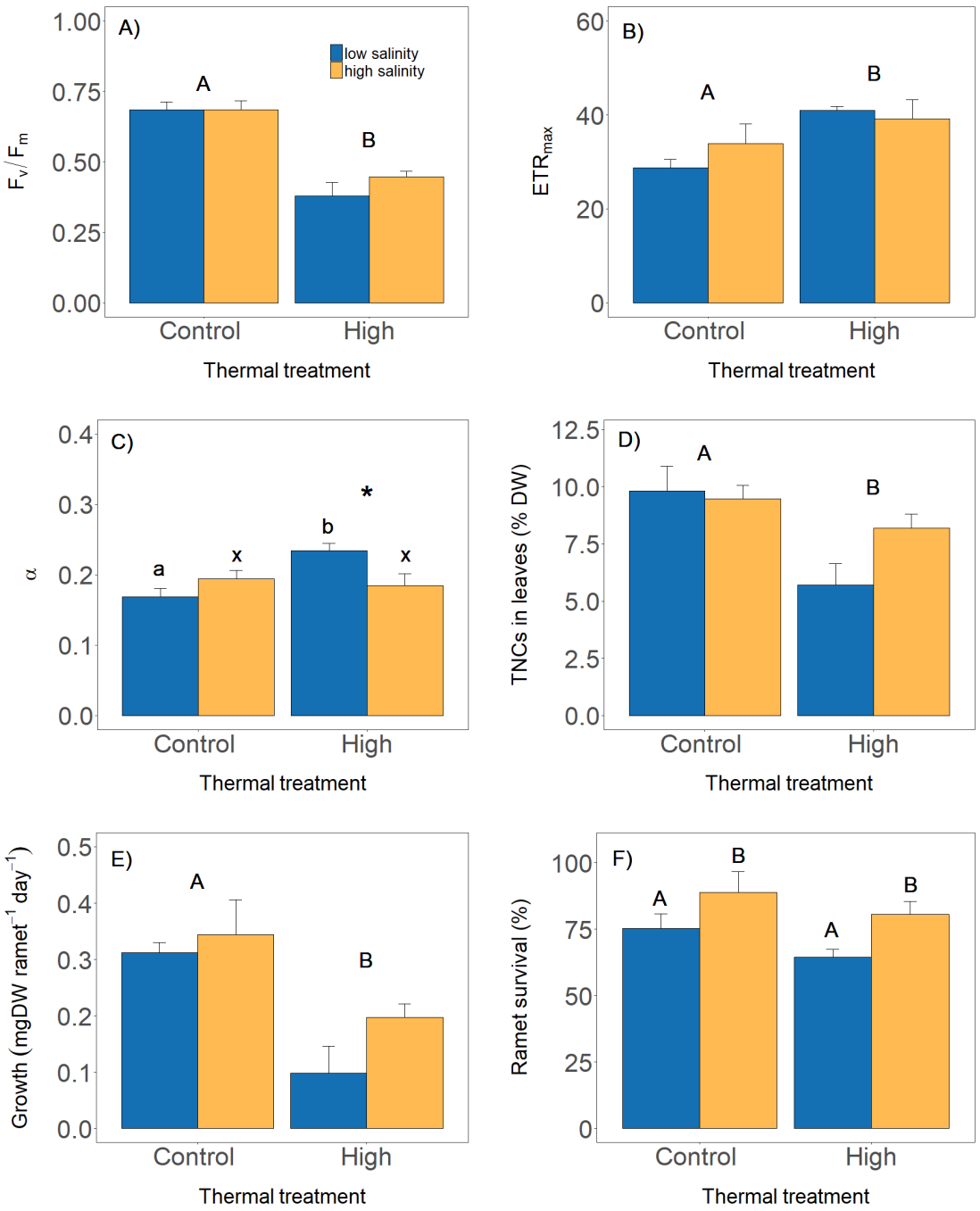


Figure 2. *Halophila ovalis* plant traits measured in experiment B: A) maximum quantum yield, B) maximum absolute electron transport rate, C) photosynthetic efficiency (α), D) total non-structural carbohydrates (TNCs) content in leaves, E) growth rate and F) ramet survival, measured in plants (mean ± SE, n=4) exposed to two thermal treatments (control (24 ºC) and high (30 ºC)) and two levels of salinity (low (34 psu) -blue bars-, and high (40-42 psu) - orange bars-) for 13 days. Capital letters over the bars represent significant differences between thermal or salinity treatments (independent from the second factor); lower case letters represent significant differences between thermal treatments at each salinity separately (a and b for low salinity treatment and x for high salinity treatment). Asterisks indicate significant differences between salinity treatments at that temperature.

744
745 Table 2. Results of PERMANOVA (univariate analysis) testing for the effect of
746 temperature and salinity on plant functional traits of *Halophila ovalis* after growing for
747 13 days under experimental conditions in experiment B. Bold values indicate significant
748 effects ($p<0.05$ for all variables except growth where $p<0.01$).
749

Source	df	MS	Pseudo-F	P	Unique perms	
Individual test						
F_v/F_m						
Temperature (T)	1	0.30	62.56	**<0.001**	9854	Control > High
Salinity (S)	1	0.00	1.01	0.333	9814	
T x S	1	0.00	1.01	0.322	9816	
Residual	12	0.00				
AF/F_m'						
Temperature (T)	1	0.01	2.05	0.177	9824	
Salinity (S)	1	0.00	0.30	0.584	9818	
T x S	1	0.01	4.20	0.063	9828	
Residual	12	0.00				
ETR_{max}						
Temperature (T)	1	303.10	8.06	**0.014**	9843	High > Control
Salinity (S)	1	11.49	0.31	0.589	9824	
T x S	1	49.04	1.30	0.274	9830	
Residual	12	37.63				
alpha						
Temperature (T)	1	0.00	4.63	0.054	9837	
Salinity (S)	1	0.00	0.84	0.373	9832	
T x S	1	0.01	8.24	**0.018**	9821	
Residual	12	0.00				
I_k						
Temperature (T)	1	3049.7	2.03	0.189	9854	
Salinity (S)	1	2363.1	1.57	0.239	9859	
T x S	1	1380.8	0.92	0.370	9845	
Residual	12	1505.2				
TNCs leaves						
Temperature (T)	1	28.96	12.66	**0.003**	9823	Control > High
Salinity (S)	1	4.56	1.99	0.185	9816	
T x S	1	7.98	3.49	0.085	9849	
Residual	12	2.29				
TNCs rhizome						
Temperature (T)	1	0.01	0.01	0.905	9834	

Salinity (S)	1	0.03	0.03	0.862	9839	
T x S	1	0.31	0.33	0.578	9848	
Residual	12	0.95				
Growth						
Temperature (T)	1	0.13	18.16	**0.002**	9851	Control > High
Salinity (S)	1	0.02	2.39	0.145	9835	
T x S	1	0.00	0.61	0.448	9811	
Residual	12	0.01				
Survival						
Temperature (T)	1	370.41	2.98	0.111	9741	
Salinity (S)	1	879.88	7.09	**0.026**	9663	High > Low
T x S	1	6.65	0.05	0.823	9757	
Residual	12	124.13				

Supplementary material

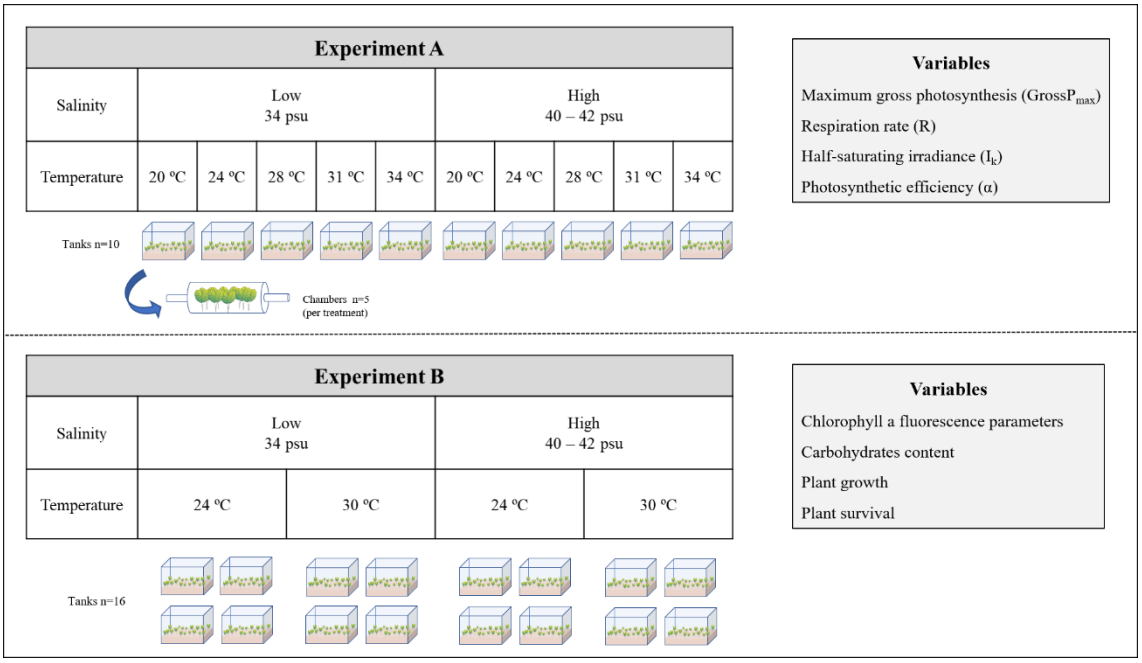
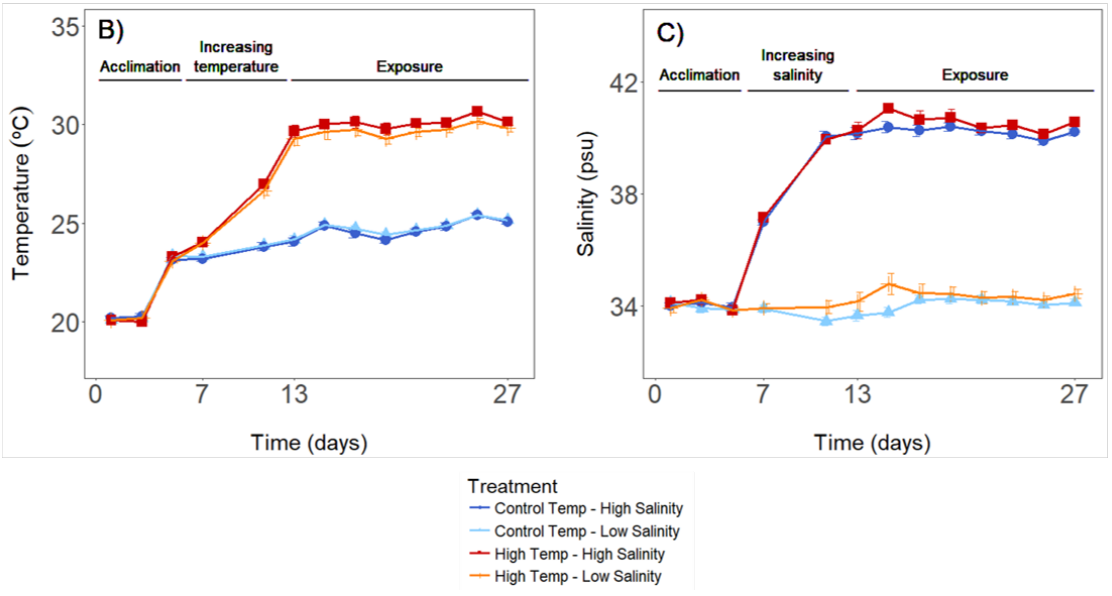


Figure S1. Experimental designs. Experiment A: 10 treatments as a result of the combination of five temperatures (20 ºC, 24 ºC, 28 ºC, 31 ºC and 34 ºC) and two salinities (low, 34 psu and high, 40-42 psu). Five replicate chambers per each treatment. Period of exposure to the experimental conditions: 1 day. Experiment B: 4 treatments as a result of the combination of two temperatures (control, 24 ºC and high, 30 ºC) and two salinities (low, 34 psu and high, 40-42 psu). Four replicate tanks per treatment. Period of exposure to experimental conditions: 13 days.

A)

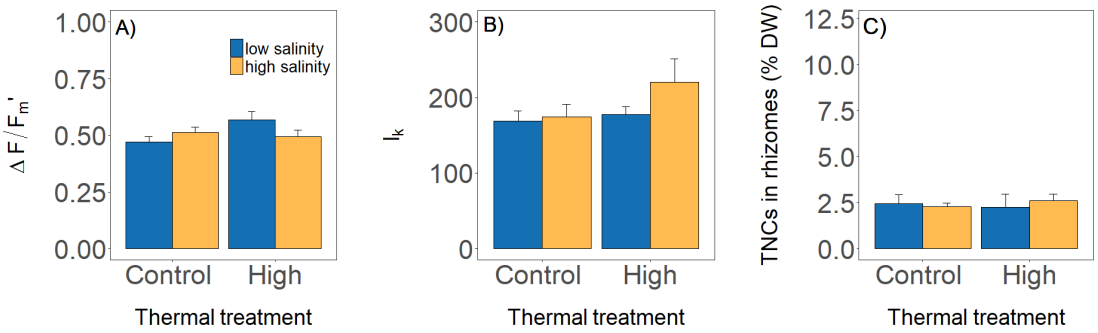
Treatment T (°C)	Treatment S (psu)		Acclimation	Increasing temperature (1 °C/day) and salinity (2 psu/day) →																
20	34	Temp	20 °C	20 °C	**20 °C**															
		Sal	34 psu	34 psu	**34 psu**															
20	40-42	Temp	20 °C	20 °C	20 °C	20 °C	20 °C	**20 °C**												
		Sal	34 psu	36 psu	38 psu	40 psu	42 psu	**42 psu**												
24	34	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	**24 °C**												
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	**34 psu**												
24	40-42	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	24 °C	**24 °C**											
		Sal	34 psu	34 psu	36 psu	38 psu	40 psu	**42 psu**	**42 psu**											
28	34	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	**28 °C**								
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	**34 psu**								
28	40-42	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	**28 °C**								
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	36 psu	38 psu	40 psu	**42 psu**	**42 psu**								
31	34	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	29 °C	30 °C	31 °C	**31 °C**					
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	**34 psu**					
31	40-42	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	29 °C	30 °C	31 °C	**31 °C**					
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	36 psu	38 psu	40 psu	**42 psu**	**42 psu**					
34	34	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	29 °C	30 °C	31 °C	32 °C	33 °C	34 °C	**34 °C**		
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	**34 psu**		
34	40-42	Temp	20 °C	21 °C	22 °C	23 °C	24 °C	25 °C	26 °C	27 °C	28 °C	29 °C	30 °C	31 °C	32 °C	33 °C	34 °C	**34 °C**		
		Sal	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	34 psu	36 psu	38 psu	40 psu	42 psu	**42 psu**		



763

764 Figure S2. A) Implementation of the different temperature and salinity treatments prior
765 to sampling in experiment A. Grey squares highlight thermal and salinity conditions at
766 which plants were collected and placed into the chambers for the P-I determination. B)
767 Temperature and C) salinity trends measured during acclimation, treatment application
768 and exposure period in experiment B.

769



770

771 Figure S3. *Halophila ovalis* plant traits measured in experiment B: A) Effective quantum
772 yield ($\Delta F/F_m'$), B) photosynthetic efficiency (α) and B) half-saturating irradiance (I_k) and
773 C) TNCs content in rhizomes measured in plants (mean ± SE, n=4) exposed to two
774 thermal treatments (control (24 ºC) and high (30 ºC)) and two levels of salinity (low (34
775 psu) – blue bars -, and high (40-42 psu) – orange bars-) for 13 days.