



Flowering effort and reproductive phenology of intertidal colonizing *Zostera marina*

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ABSTRACT

The seagrass *Zostera marina* can inhabit the intertidal where the variable environmental conditions mean that the seagrass meadows are frequently in a continuous state of colonization, influencing, among others, their phenology and flowering effort. This could be especially notable in the southern limits of the species' distribution, where light exposure and temperature reach extreme values during the warmer seasons. In this study, we evaluate the flowering effort, phenology, photophysiology and vegetative development of recent colonizing *Z. marina* patches inhabiting the intertidal of a lagoon located near its southern limit of distribution, in the Ria de Aveiro (Portugal). To evaluate the effects of low tide exposure, tidepool and emerged seagrass patches were selected. Results showed short-lived differences in the phenology of the flowering of the patches and in their vegetative development, but in general, the photosynthetic activity, timing of flowering, densities of spathes and flowering shoots were similar in emerged and tidepool patches throughout the entire reproductive period (from June to August). The spathes per flowering shoot and the spathe and flowering shoot densities per unit of area of the colonizing patches were comparable to that obtained in other perennial intertidal meadows of this species and its timing was adjusted to that which occurred in areas of similar latitudes, ending when the temperature exceeded 25 °C. These results imply that intertidal colonizing *Z. marina* patches can flowering similarly to perennial meadows, regardless of whether or not they are submerged in pools at low tide, which implies a great adaptability of the species to intertidal areas. However, to fully understand the reproductive capacity of *Z. marina* in the Ria de Aveiro, future studies should determine the capacity of these flowers to form mature seeds, as well as their ability to germinate naturally in the area.

1. Introduction

Seagrass meadows are habitat-forming-species and play important ecological roles in coasts, estuaries and lagoons (Hemminga and Duarte, 2000). Among others, seagrasses are indicators of water quality (Denison et al., 1993), valuable carbon reservoirs (Fourqurean et al., 2012) and source of feed and refuge for several marine organisms (Duffy, 2006), many of them with fishery interest (Unsworth et al., 2018). Despite their importance, seagrasses have been seriously threatened by anthropogenic activities such as global warming (Waycott et al., 2009), especially during its period of sexual reproduction (Guerrero-Meseguer et al., 2020).

By sexual reproduction, seagrasses form seeds which can germinate

while are dispersed over the sea surface (Guerrero-Meseguer et al., 2018) or are buried in the sediment forming seed-banks until proper environmental conditions for germination occur (Orth et al., 2000). In this way, seagrasses are able of increasing their genetic variability and can develop phenotypes that allow them to survive against future changes in the environment (Kendrick et al., 2017). In most of the seagrass species, sexual reproduction is induced by warm temperatures (Walker et al., 2001). However, elevated values of temperature can increase the reproductive effort of seagrasses (Ruiz et al., 2017), but can also decrease intensity of flowering in the long term (Qin et al., 2020). Other factors such as salinity, photoperiod and light intensity can also affect the timing and flowering effort of seagrass meadows (Conacher et al., 1994; De Cock, 1980; McMillan, 1976; Setchell, 1922). This

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results in great spatial variability on the seagrass reproductive processes from biogeographic to local scale (von Staats et al., 2021), which is especially relevant in intertidal areas during the warmer seasons, where temperature and light exposure can reach extreme values simultaneous to the period of sexual reproduction. Thus, studying the reproductive cycle of the seagrass species in the intertidal is essential to determine their capacity to respond to rapidly changing conditions.

Zostera marina (L.) is one of the most widespread seagrasses of the northern hemisphere (Green and Short, 2003), especially due to its wide tolerance to temperature, salinity and photoperiod conditions (Olesen et al., 2015). This seagrass, colloquially known as “eelgrass”, is key species in many estuarine environments (Dennison et al., 1993; Thom et al., 2003), but also can form subtidal meadows in shallow lagoons and sheltered bays (Buia and Marzocchi, 1995; Kenworthy et al., 2014). Although *Zostera marina* is well adapted to intertidal conditions (Leuschner et al., 1998) and its flowering is positively related to temperature (Blok et al., 2018), temperatures above its optimal for photosynthesis could affect its vegetative growth and flowering effort (Qin et al., 2020). In addition, intertidal areas are usually subjected to numerous human activities related, among others, to shellfish collection, aquaculture, fishing, dredging and wastewater discharges, which can alter seagrasses due to the combination of multiple stressors (Grech et al., 2012; Orth et al., 2006). These conditions create very dynamic environments, where only opportunistic and colonising seagrasses, such as *Zostera* spp., can inhabit in permanent state of colonization, forming annual and semiannual populations (Kilminster et al., 2015). In this context, evaluating the reproductive capacity of seagrasses during the colonization stage is crucial to assess whether they are capable of maintain their natural recovery in the long term.

The aim of this study was to evaluate the sexual reproduction of colonizing *Z. marina* in the intertidal. To do this, recently colonizing eelgrass patches were monitored in the Ria de Aveiro lagoon (Portugal) to assess their reproductive effort and phenology throughout their flowering period. In this area, this species was considered extinct a decade ago, but, recently, several intertidal patches have colonized the lagoon (Guerrero-Meseguer et al., 2021). Additionally, to detect possible limitations in the flowering due to the extreme environmental conditions of the intertidal, the eelgrass photophysiology and vegetative

development were also monitored in tidepool and emerged patches along the period of sexual reproduction. The reproductive cycle of this species has never been studied in the Ria de Aveiro lagoon, so this study also contributes as a baseline data on the phenology of *Z. marina* in areas close to its distribution limit. This knowledge is essential to compare local changes in the reproductive effort of the eelgrass meadows of the Ria de Aveiro in the future due to anthropogenic factors, as well as to have a better understanding about the natural recolonization of intertidal eelgrass meadows at global scale.

2. Materials and methods

2.1. Study area and characterization of the eelgrass patches

The study was done in the Mira channel of the Ria de Aveiro lagoon (40°37.08'N, 8°44.34'W; Aveiro, Portugal, Fig. 1). Nine intertidal eelgrass patches were recently described in this channel in an area close to oyster farming after being extinct for a decade (Guerrero-Meseguer et al., 2021), so that *Z. marina* in this area can be considered as being in a state of colonization. The tide in this area propagates as a progressive and standing wave (tidal range from 2 to 3.5 m in spring), which causes the seagrass beds of the channel are exposed to air from 5 to 7 h in each semi-diurnal tidal cycle (Dias et al., 2000; Azevedo et al., 2016). However, some of the recent colonizing eelgrass patches remain submerged in tidepools even during low tide periods. Of the nine patches that colonized the area, only four formed sexual shoots and spathes and have enough size for proper replication (>20 m²), so these four patches were considered for the study. Since tidepool environmental conditions can differ from those of the patches which were emerged at low tide periods and this could alter the phenology and reproductive effort of the species, this fact was considered to check differences on the studied variables. In this way, two emerged eelgrass patches (E1 and E2, Fig. 1) and two tidepool eelgrass patches (T1 and T2, Fig. 1) were monitored for sexual reproduction in our study. The tidepool patches were submerged by 7.50 ± 0.783 cm of seawater at low tide. Under high tide periods, all the patches were at a similar depth.

The eelgrass patches of the Mira channel were visited month by month to detect the beginning of the flowering period and start the

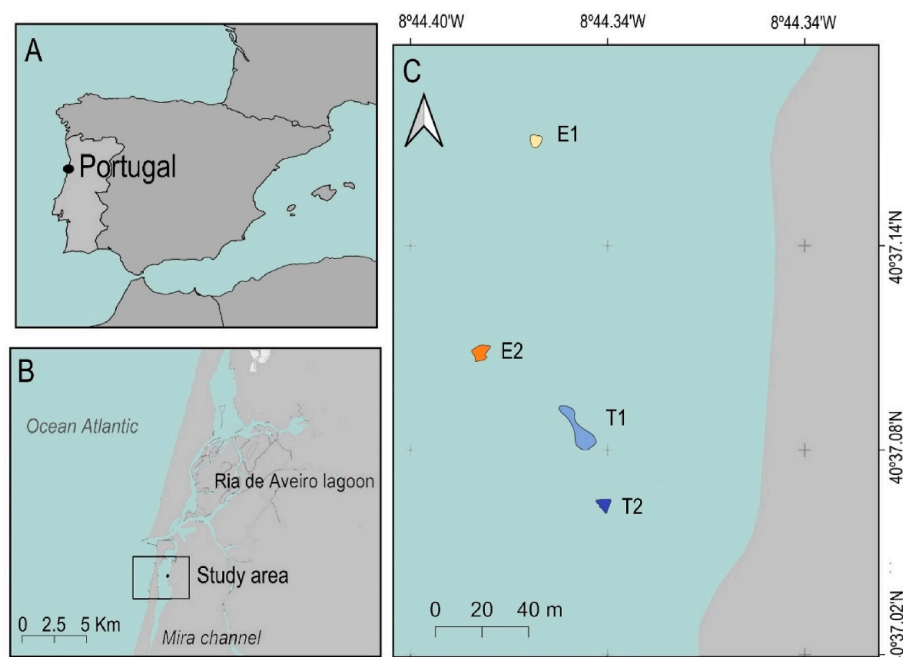


Fig. 1. Study area (A and B) and location of the emerged (yellow; E1, E2) and tidepool (blue; T1 and T2) patches of *Zostera marina* (C) monitored in the Mira Channel of the Ria de Aveiro lagoon. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

study. Additionally, patch size, sediment grain size and organic matter content of each eelgrass patch were measured in those previous visits to have initial measurements of these parameters. Patch size was assessed by recording the coordinates of their perimeters with a GPS (GPSMAP 78, Garmin, Spain). Then, Geographical Information System software (QGIS, [QGIS.org](https://qgis.org) (2020) was used to calculate the area of each eelgrass patch. Two cores (10 cm diameter) per patch were used to determine grain size and organic matter content (OM) of each patch. Grain size was determined by drying the sediment at 60 °C for 24 h and sieving it through sieves with different mesh sizes following the Wentworth scale (Wentworth, 1922) and classified following Folk's scale (Folk, 1954). Organic matter content was determined in 1 g of the finest fraction of the sediment (<0.25 mm) dried at 60 °C for 24 h. Then, organic matter content was estimated as the weight loss on ignition (450 °C, for 4 h).

Flowering began in the patches in early June. Therefore, we use the data collected at the end of May 2020 to describe the environmental conditions of the patches. In May 2020, patches sizes were 28.5 m² in E1, 46.6 m² in E2, 135.9 m² in T1 and 29.9 m² in T2. The sediment grain size of the patches varied from fine sand in E1 to muddy sand – sandy mud in E2, T1 and T2. The percentage of organic matter content in the sediment of the patches were $2.18 \pm 0.14\%$, $1.84 \pm 0.25\%$, $1.35 \pm 0.39\%$ and $1.94 \pm 0.31\%$ in E1, E2, T1 and T2, respectively.

The patches were visited in periods of low tide from May until August of 2020. Temperature, salinity, and photosynthetically active radiation (PAR) ($n = 4$) were measured in situ over the study period always between 10:00 and 12:00 a.m. LT (local time), by using a portable meter (HQ40, Hach, Germany) and light meter (ULM-500, Waltz, Germany), respectively. PAR radiation was measured on the surface of the eelgrass patches. No permits were required for the described study, which complied with all relevant regulations. The field measurements made in the eelgrass meadows in the present study were not lethal, complying with the guidelines and legislation for vulnerable species of the IUCN Policy Statement on Research Involving Species at Risk of Extinction.

2.2. Eelgrass reproductive effort and flowering phenology

The reproductive effort and the flowering phenology of each eelgrass patch were monitored every 15 days from the beginning of the flowering period until its end. Reproductive effort was assessed by measuring in situ the spathe and flowering shoot densities and the spathes per flowering shoot. Spathe and flowering shoot densities were determined by counting, respectively, the number of spathes and flowering shoots in a 50 × 50 cm quadrat ($n = 4$). Then, spathe and flowering shoot densities were calculated as the number of spathes and flowering shoots per square meter. Additionally, flowering frequency was calculated as the number of spathes per each flowering shoot. Due to the small size of the patches, four 50 × 50 cm quadrats per patch and date were representative of the reproductive effort of the entire eelgrass patch.

Eelgrass flowering phenology was measured by visually determining the sexual reproduction stage of each sexual spathe according to its morphology (de Cock, 1980). In this way, four sexual reproduction stages were visually identified: Stage 0, Stage I, Stage II and Stage III. The Stage 0 corresponded with very small, closed green spathes in formation and no internal sex organs were appreciated (i.e., anthers or pistils). The Stage I was assigned to yellow and closed spathes which showed sexual organs in the interior (Fig. 2). Stage II was assigned to open spathes in which the pistils or/and the anthers were emerged and hatched to facilitate fecundation (de Cock, 1980) (Fig. 2). Finally, Stage III corresponded to spathes which contained seeds in the interior. Then, the percentage of spathes in each sexual reproduction stage was calculated with respect to the total number of spathes of each quadrat. Broken, empty, and unhealthy-looking spathes were considered aborted/dehiscent spathes and were included in the calculation of the percentages of each reproductive stage. Due to the recent state of colonization of the eelgrass patches in the lagoon, its phenology was determined visually to avoid pulling the spathes. Thus, the number of



Fig. 2. Spathes of a *Zostera marina* emerged patch. The image shows three spathes in different stages of sexual reproduction (Stage I and Stage II).

seeds and their state of maturity was not distinguished into the spathes because most of them were covered by epiphytes. For the same reason, empty spathes due to abortions were not differentiated from those dehiscent by the release of mature seeds.

2.3. Eelgrass vegetative development

The vegetative development of the colonizing eelgrass patches (eelgrass coverage and vegetative shoot density) was measured before (May 2020) and after the flowering period (August 2020) to check differences between the tidepool and the emerged eelgrass patches during the sexual reproduction period. The coverage of *Z. marina* was calculated in four quadrats of 50 × 50 cm. To do this, each quadrat was divided into 25 sub-quadrants of 10 × 10 cm and then, a scale from 0, with an absence of *Z. marina*, to 4, when all the quadrat (10 × 10 cm) were covered by *Z. marina* was attributed for each sub-quadrant. Then, the final value of coverage for each quadrat was obtained by the accumulation of the 25 estimates (Dethier et al., 1993). Shoot density was calculated by counting the number of vertical rhizomes of each patch into 4 sub-quadrants per each quadrat of 50 × 50 cm ($n = 4$). Shoot density was represented as number of shoots per square meter.

2.4. Eelgrass photophysiology

To evaluate the photosynthetic activity of the eelgrass patches during the sexual reproduction period, maximum quantum yield (F_v/F_m) and rapid light curves (RLCs) were measured with a pulse amplitude modulated fluorometer (JUNIOR-PAM, Waltz, Germany) once per month (June, July and August). All the measurements were done between 10:00 and 12:00 LT on sunny days with a light irradiance greater than 2200 PAR.

The measurements were done on two haphazardly selected leaves from four vertical shoots in each eelgrass patch ($n = 4$). The average value of the two leaves measured by vertical shoot was considered for the analyses. Measurements were always made on leaves free of epiphytes and at the base of the youngest leaves of each vertical shoot, which showed an intense green color and an F value greater than 200. All the measurements were made by using a 60° leaf clip of the JUNIOR-

PAM, ensuring the same orientation of the saturating pulse over the eelgrass leaves. Leaves for F_v/F_m measurements were dark-adapted for 5 min by fastening the eelgrass leaves with the magnetic leaf-clip of the JUNIOR-PAM and by covering their corresponding vertical shoots with aluminium foil and with an opaque plastic box. Then, the leaves were exposed to a saturating light pulse to record the F_v/F_m of the Photosystem II (PSII). The intensity and width of the saturation pulse was previously adjusted to ensure a distinct plateau of maximum quantum yield at a set distance from the blade (Genty et al., 1989). In this way, a saturation intensity setting of 2 with a width of 0.8 was used in all the measurements.

RLCs measurements were performed in ambient light (no dark adaptation period). The irradiances for RLCs ranged between 0 and $2700 \mu\text{mol m}^{-2} \text{s}^{-1}$ and were each one applied for 10 s followed by a saturating pulse of 0.8 s. The $rETR$ values were plotted against the light irradiances to produce a curve fitting the exponential model proposed by Platt et al. (1980). Then, derived parameters of RLCs include photosynthetic efficiency (α), dynamic photoinhibition parameter (β), relative electron transport rate maximum ($rETR_{\text{max}}$) and the minimum saturation irradiance (E_K) that were calculated following Ralph and Gademann (2005).

2.5. Data analyses

Data from eelgrass reproductive effort and flowering phenology, vegetative development and photophysiology were analysed using a two-way ANOVA with *Patch* as an orthogonal and fixed factor with four levels (i.e., E1, E2, T1 and T2) and *Time* as a random factor and 4 replicates per patch. In the case of the flowering effort and percentages of spathes in each sexual reproduction stage, five levels were used for the factor time: 09 June, 25 June, 08 July, 29 July and 10 August. As eelgrass development was measured before and after the flowering period, in this case two levels were used for the factor time (before and after). For the eelgrass photophysiology, as measurements were made once per month during the sexual reproduction period, three levels were used for the factor time (June, July and August).

Bartlett tests were previously done to check for homogeneity of variances, and when test was significant ($p < 0.05$) data were transformed to remove heterogeneity. When this was not possible, untransformed data were analysed and results were considered robust if significant at $p < 0.01$, to compensate for the increased probability of type I error (Underwood, 1997). Statistical analyses were done with the R statistical software (v. 2.15.0; R Core Team, 2020). Statistical tests were conducted at a significance level of $\alpha = 0.05$. All results are expressed as mean $\pm$ standard error (SE).

3. Results

3.1. Evolution of environmental conditions in eelgrass patches during the reproductive period

The PAR, temperature, and salinity of each eelgrass patch during the flowering period has been summarized in Table 1.

3.2. Eelgrass reproductive effort and flowering phenology

The flowering of the colonizing *Z. marina* patches of the Ria de Aveiro lagoon lasted from June to August of 2020. The flowering shoot density, spathe density and spathes per flowering shoot were similar among patches but showed temporal variability (Supplementary Table 1). The patches produced a total average of 5.625 ± 1.080 flowering shoots $\cdot \text{m}^{-2}$, being the period of maximum production at the beginning of the flowering period (Fig. 3A). The spathe density in the area was 48.75 ± 5.256 spathes $\cdot \text{m}^{-2}$, showing a spathes per flowering shoot of 7.232 ± 0.637 spathes per flowering shoot and reaching maximum production in mid-June (Fig. 3B).

Table 1

PAR radiation ($\mu\text{mol} \cdot \text{photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), salinity (PSU) and temperature ($^{\circ}\text{C}$) over the flowering period in the *Z. marina* patches of the Ria de Aveiro.

	Date	Patch E1	Patch E2	Patch T1	Patch T2
PAR	09-June	2321.1 $\pm$ 27.0			
	25-June	2358.1 $\pm$ 37.9			
	08-July	2431.3 $\pm$ 74.0			
	29-July	2249.9 $\pm$ 40.5			
	10-August	2446.3 $\pm$ 23.7			
	August				
Salinity	09-June	31.00 $\pm$ 1.26	30.83 $\pm$ 1.41	31.05 $\pm$ 1.17	30.20 $\pm$ 1.17
	25-June	23.91 $\pm$ 0.05	27.71 $\pm$ 1.21	32.53 $\pm$ 1.22	28.63 $\pm$ 1.28
	08-July	30.92 $\pm$ 1.02	32.52 $\pm$ 1.05	32.71 $\pm$ 1.48	30.32 $\pm$ 1.19
	29-July	23.61 $\pm$ 1.24	32.01 $\pm$ 1.70	33.72 $\pm$ 0.99	30.51 $\pm$ 1.30
	10-August	27.97 $\pm$ 0.98	28.57 $\pm$ 1.24	34.94 $\pm$ 1.01	30.03 $\pm$ 0.99
	August				
Temperature	09-June	24.81 $\pm$ 0.08	24.43 $\pm$ 0.12	26.31 $\pm$ 0.09	24.61 $\pm$ 0.10
	25-June	21.62 $\pm$ 1.05	23.22 $\pm$ 0.08	22.52 $\pm$ 0.07	20.11 $\pm$ 0.08
	08-July	19.12 $\pm$ 0.10	18.12 $\pm$ 0.07	18.94 $\pm$ 0.08	18.55 $\pm$ 0.10
	29-July	21.85 $\pm$ 0.03	21.45 $\pm$ 0.09	21.10 $\pm$ 0.05	20.06 $\pm$ 0.08
	10-August	30.45 $\pm$ 0.15	28.53 $\pm$ 0.13	27.43 $\pm$ 0.09	25.52 $\pm$ 0.11
	August				

The percentages of spathes in the stages 0, I and II were similar among patches over the time, showing these three stages maximal production in June (Fig. 3C, D and 3E, Supplementary Table 1). Similarly, the percentage of aborted/dehiscent spathes did not show changes among meadows over the time, showing the lowest values at the end of July (Supplementary Table 1). However, the percentage of spathes in the Stage III was significantly different among patches over the time (Fig. 3F, Supplementary Table 1). At the beginning of June, the Patch E1 had a lower percentage of spathes in the Stage III than the other patches, but at the end of June all the patches had similar percentages. At the beginning of July, the percentage of spathes in Stage III was higher in the emerged patches (Patches E1 and E2) than in the tidepool patches (Patches T1 and T2; Fig. 3F) but at the end of July all the patches showed similar percentages.

3.3. Eelgrass vegetative growth during flowering period

Eelgrass coverage significantly varied among patches over the time (Supplementary Table 2). Before the flowering period, the Patch E2 had a lower coverage than the other patches (Fig. 4A). However, after the flowering period, the Patch T2 showed a lower coverage than the Patches E1 and T1 (Fig. 4A). The vegetative shoot density was significantly variable among patches over the study period. Before the flowering period, all the patches showed similar number of vegetative shoots, but afterward, the emerged patches E1 and E2 showed a higher number of shoots than the tidepool patches T1 and T2 (Fig. 4B, Supplementary Table 2).

3.4. Eelgrass photophysiology during flowering period

The F_v/F_m was significantly variable among patches over the study period (Supplementary Table 3). In June, the Patch E1 showed lower values of F_v/F_m than the Patches E2 and T1. In July, the F_v/F_m was higher in the Patch E2 than in the Patches T1 and T2 but in August, the Patch E2 showed lower values than the patches T1 and T2 (Fig. 5).

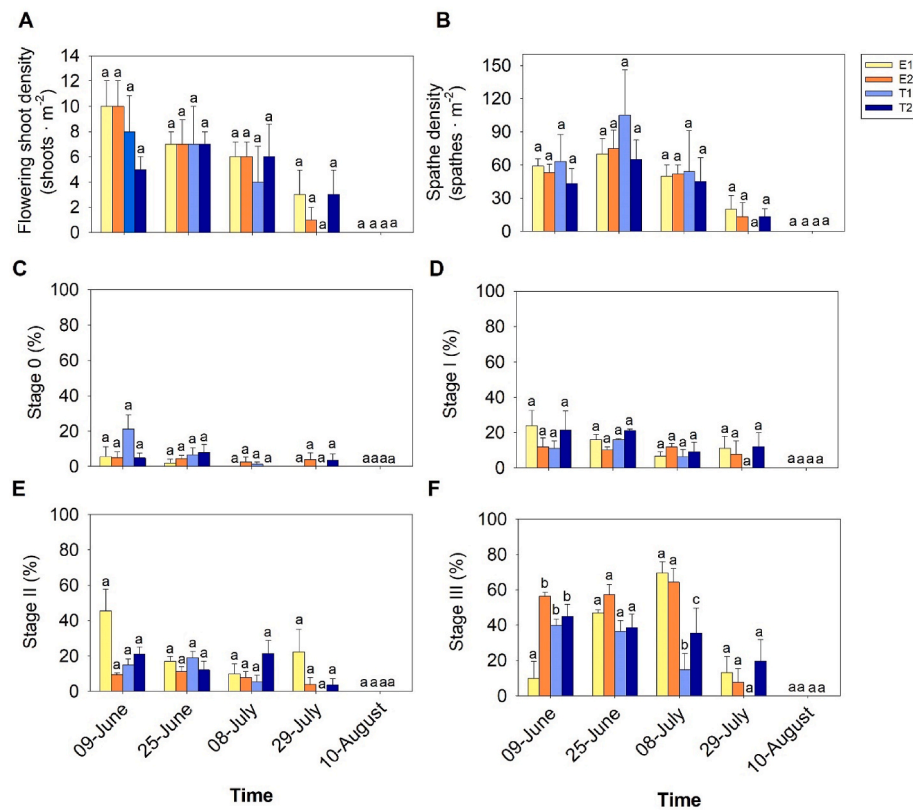


Fig. 3. Flowering shoot (A) and spathe (B) densities and percentages of each sexual reproduction stage observed in the emerged (E1 and E2) and tidepool (T1 and T2) colonizing *Zostera marina* patches of Ria de Aveiro from June to August 2020 (Stage 0, C; Stage I, D; Stage II, E and Stage III, F; mean $\pm$ SE, $n = 4$). Different letters above error bars indicate significant differences among patches over the time.

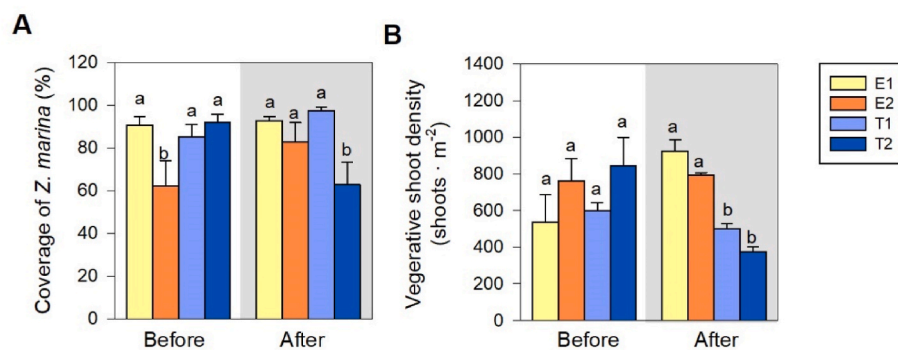


Fig. 4. Coverage (A) and vegetative shoot density (B) (mean $\pm$ SE, $n = 4$) of the emerged (E1 and E2) and tidepool (T1 and T2) *Z. marina* patches before (white background; May 2020) and after (Gray background; August 2020) the flowering period. Different letters above error bars indicate significant differences among patches over the time.

Regarding the RLCs, none of the measured parameters (α , β , $rETR_{max}$ and E_K) showed significant differences among the eelgrass patches, but all of them had differences over the time (Supplementary Table 3). In August the patches showed higher averages of α and β than in the other months (Fig. 6A and B) but had a lower E_K average (Fig. 6D). However, the $rETR_{max}$ average was higher in July than in June and August (Fig. 6C).

4. Discussion

This study demonstrated that the intertidal colonizing patches of *Z. marina* of the Ria de Aveiro were able to sexually reproduce in the lagoon regardless of whether they were emerged or submerged in periods of low tide. Emerged and tidepool eelgrass patches showed similar

densities of spathes and flowering shoots, spathes per flowering shoot, photosynthetic activity, and coverage over the sexual reproduction period, which lasted from June to August. Although there were some differences between the tidepool and emerged patches regarding sexual reproduction stages, these differences were short-lived and, in general, flowering phenology evolved similarly in all the patches. In addition, the flowering shoot density per unit of area in the Ria de Aveiro was similar to the obtained in other eelgrass meadows from the southern limit of Europe in the south of Portugal (Cabaço and Santos, 2010) and in other shallow areas from the Pacific coast of USA (Phillips et al., 1983). Similarly, the production of spathes per flowering shoots in the lagoon was in the range of this seagrass species (Phillips et al., 1983; Silberhorn et al., 1983; Phillips and Backman, 1983; Morita et al., 2007; Cabaço and Santos, 2010). This suggests that flowering effort of intertidal colonizing

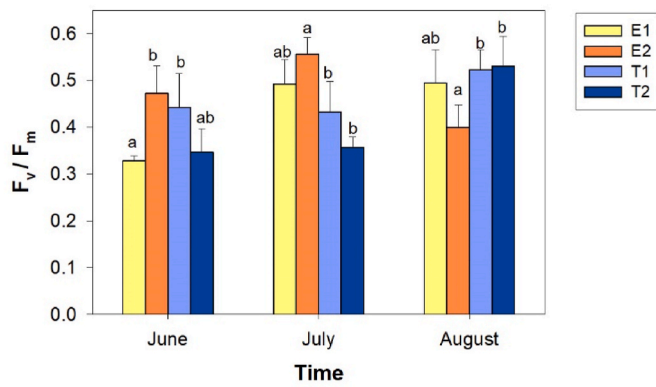


Fig. 5. Maximum quantum yield (F_v/F_m ; mean $\pm$ SE, $n = 4$) of the PSII measured in the leaves of the emerged (E1 and E2) and tidepool (T1 and T2) eelgrass patches of the Ria de Aveiro over the flowering period. Different letters above error bars indicate significant differences among patches over the time.

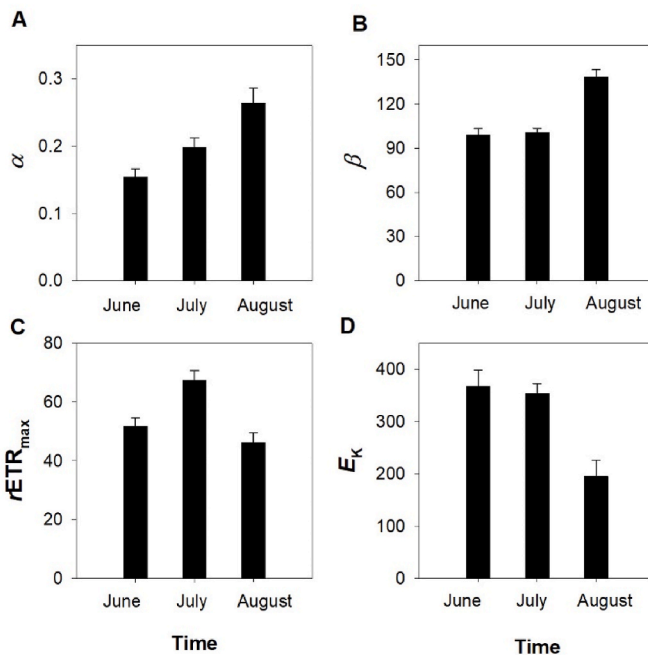


Fig. 6. Temporal variability in parameters α , β , $rETR_{max}$ and E_K ($\mu\text{mol electrons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) obtained from the RLCs of the eelgrass patches over the flowering period (mean $\pm$ SE, $n = 4$).

eelgrass is possible at the same level as in other perennial populations, providing an important reservoir for the genetic exchange of the species in dynamics environments.

Coinciding with other European populations located at similar latitudes (Blok et al., 2018; Cabaço and Santos, 2010; Infantes and Moksnes, 2018), flowering of *Z. marina* in the Ria de Aveiro reached its peak between June and July. However, unlike other populations located at higher latitudes where the eelgrass flowering can last until October (Hootsmans et al., 1987; Infantes and Moksnes, 2018), in the Ria de Aveiro, flowering had finished at the beginning of August. This difference in the duration of flowering corroborates that the timing of flowering in *Z. marina* meadows varies along latitude and temperature gradients, lagging towards cooler northern locations (Blok et al., 2018). Although eelgrass shows great adaptation to warming at high latitudes, its flowering and formation of mature seeds rarely continues after long periods above 25 °C (Blok et al., 2018; Qin et al., 2020; Silberhorn et al., 1983). Temperatures above the optimal for photosynthesis increases

respiration rates in seagrass meadows, leading to negative carbon balance in the plants (Lee et al., 2007). This fact can be observed in the progression of the electron transport rates ($rETR_{max}$) of the eelgrass patches during our study, which were lower when seawater temperature exceeded the 24 °C (June and August) than when temperature ranged from 18 to 22 °C (July). In addition, the RLCs showed higher α and β but lower E_K values in August than in the other months, which suggest a greater photoinhibition in the eelgrass patches in August (Ralph and Gademann, 2005).

The optimum temperature for photosynthesis of *Z. marina* can vary geographically (Lee et al., 2007), but in areas where the maximum temperature is close to 30 °C, such as in the Ria de Aveiro, the optimum for this species is in the range of 19–22 °C (Evans et al., 1986; Penhale, 1977; Wetzel and Penhale, 1983). Moreover, in the Ria de Aveiro lagoon, the eelgrass patches are exposed to air from 5 to 7 h in each semi-diurnal tidal cycle (Dias et al., 2000; Azevedo et al., 2016) and elevated temperatures in shallow areas can further affect photosynthesis and seagrass productivity due to high solar radiation and wide temperature fluctuations (Bulthuis, 1987; Ralph et al., 1998; Seddon and Cheshire, 2001). Since in this study the PSII measurements of the leaves were taken at low tide periods, the lower values of F_v/F_m values of the eelgrass patches observed (between 0.3 and 0.6) in comparison with those obtained in other studies of the same species (>0.5–0.7; i.e. Ralph and Gademann, 2005; Park et al., 2016; Yang et al., 2017) could be a result of these environmental conditions. Therefore, it was expected that the flowering in the Ria de Aveiro would end in August in our study, coinciding with the period of maximum temperatures in the area.

During the first stages of flowering, in which the pistils and anthers were developing in *Z. marina*, all the patches showed a similar evolution. At the end of the flowering period, the production of spathes containing seeds and the vegetative development were lower in the tidepool than in the emerged patches but, in general, all patches showed a very similar flowering phenology. Nevertheless, this variability was short-lived and thus, it could be due to punctual worse environmental conditions in the tidepools than in the emerged areas. A decrease in vegetative shoot density is a commonly employed strategy of seagrasses to reduce self-shading within the remaining shoots (Dalla Via et al., 1998; Enríquez and Pantoja-Reyes, 2005; Collier et al., 2016), in which the plant promotes shoot mortality to be able of receiving more light irradiance and compensate the negative carbon balance by photosynthesis (Lee et al., 2007; Ralph et al., 2007; McMahon et al., 2013; Wong et al., 2021). Moreover, the area where the *Z. marina* patches grow in the Ria de Aveiro is in the access to an oyster-growing area; thus, some of the patches are continuously trampled by workers, who even added shells over one of the studied eelgrass patches to facilitate their passage. Trampling in highly visited areas, such as the Ria de Aveiro, has potentially to negatively affect the vegetative shoot density of *Z. marina* (Travaille et al., 2015). In addition, we noted a significant increase of the macroalga *Ulva* spp. over the leaves of the eelgrass patches during our study, especially in those submerged in tidepools, which could have limited even more the light incidence in these submerged patches. Although we do not have measurements that corroborate the possible cause of a short-term decrease in seed production and vegetative development in the patches, seagrass vegetative shoots and sexual spathes can be easily torn off under these stress conditions.

Contrary to expectations (Cabaço and Santos, 2012), the eelgrass patches of the Ria de Aveiro did not seem to show a stress-induced flowering response under the influence human activities occurring in the area. On the other hand, even though a greater reproductive effort was expected for *Z. marina* in the intertidal than in subtidal, the eelgrass patches of the Ria de Aveiro did not show a higher flowering density than that found in other subtidal zones (Phillips et al., 1983; von Staats et al., 2021). This, together with the fact that the differences found in vegetative growth and in the production of spathes with seeds were of very short duration, suggests that the intertidal eelgrass patches were well adapted to the current conditions of the Ria de Aveiro and that their

reproductive capacity could continue over time regardless the effect of air exposure during low tide. Nevertheless, there are no historical data on the flowering of *Z. marina* in the Ria de Aveiro that allows us to compare if any factor could already limit the flowering capacity of the species in the lagoon. Recent studies have confirmed that the increase in temperature due to global warming can limit the reproductive effort of *Z. marina* in the long-term (Qin et al., 2020). Thus, the high temperatures reached in summer in the Ria de Aveiro could have somehow slowed down the flowering density in the lagoon. In areas where summer temperatures are milder than in Portugal, such as North of France (Jacobs and Pierson, 1981), Canada (Keddy, 1987) or Denmark (Olesen and Sandjensen, 1994), the flowering shoot density in eelgrass meadows can be ten times greater than the reported in the Ria de Aveiro. Nevertheless, in areas with cooler summers than in Portugal, such as in the UK, the flowering density of *Z. marina* was positively correlated with the temperature (Potouroglou et al., 2014). Despite to the effect of extreme temperatures on the flowering of the species seems to vary locally, it seems to be more intensive in regions where the species is close to its upper thermal tolerance limits (Qin et al., 2020; Thom et al., 2003). Therefore, long-term studies of the species in the lagoon would be needed to determine if global warming could negatively affect the flowering of this seagrass in the future.

Despite the great flowering effort that the eelgrass showed in the Ria de Aveiro, other variables related to the sexual reproduction capacity of seagrasses also could be influenced by the effects of low tide exposure. For instance, in Sweden coasts, the number of spathes and seeds of *Z. marina* were correlated with the length of their sexual shoot (Infantes and Moksnes, 2018). In the western Atlantic, spathe density and size was higher in deeper than in shallow areas (Meling-López and Ibarra-Obando, 1999) and seed size showed a great local variation, being lower at the southern limit of distribution of the species (Combs, 2021). Because patches of *Z. marina* in Ria de Aveiro were in an early state of colonization (Guerrero-Meseguer et al., 2021), we avoid destructive sampling of spathes to check other variables such as spathe size or seed size and production, in order to favour eelgrass recovery. Therefore, the effect of low tide exposure on these variables were not studied and thus, we cannot guarantee that the eelgrass reproductive capacity in the Ria de Aveiro could be at the species level. Seed germination is crucial for the recovering of impacted areas of colonized eelgrass meadows (Kim et al., 2014; Potouroglou et al., 2014). Therefore, future studies should determine the ability of these patches to produce seeds and germinate in the Ria de Aveiro to fully understand the potential of natural recovery of the eelgrass patches.

In conclusion, the colonizing *Z. marina* patches of the intertidal can produce sexual spathes at the same level than other worldwide perennial eelgrass meadows. Moreover, the timing and intensity flowering of the eelgrass patches in this study coincides with what was obtained in areas of similar latitudes, which indicate that at the southern limit of its distribution, *Z. marina* completes sexual reproduction cycle at temperatures below 25 °C. Human activities that occur in the intertidal zone of the Ria de Aveiro do not seem to elicit a stress-induced flowering response in *Z. marina*, suggesting that the newly formed eelgrass patches can establish in the lagoon and could continue growing and recruiting seedlings in the future. However, studying the effects of low tide exposure conditions on the eelgrass seed production, as well as on their germination capacity, is necessary to completely know the reproductive and recruitment capacity of this species in the lagoon of the Ria de Aveiro. Moreover, considering the expected increase in temperature in the coming decades due to global warming (IPCC, 2013) and the vulnerability of the eelgrass flowering under heat stress situations (Qin et al., 2020), further studies and long-term monitoring also are necessary for *Z. marina* in the Ria de Aveiro in order to forecast possible negative effects on its natural colonization and apply conservation measures that help to maintain its recovery in the lagoon.

CRediT authorship contribution statement

Laura Guerrero-Meseguer: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Puri Veiga:** Writing – review & editing, Validation, Supervision, Formal analysis. **Marcos Rubal:** Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ecss.2022.108110>.

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