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SPECIAL ISSUE: SEAGRASSES TRIBUTE TO SUSAN WILLIAMS

Intra-Meadow Variation in Seagrass Flowering Phenology Across Depths

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Abstract

Variation in the timing of sexual reproduction, as well as differences in the relative investment in asexual vs sexual reproduction, commonly occurs among populations. Less is known about such variation within populations, yet small-scale differences in phenology and reproductive investment have the potential to limit gene flow and consequently the adaptive capacity of populations. We examined within-site variation in sexual reproduction in seagrasses, marine angiosperms that can reproduce asexually via clonal propagation or sexually via flowering, and that form the foundation of critical marine ecosystems globally. Many factors, including light availability, temperature, nutrient availability, and genetic identity, influence the rate and timing of seagrass flower development, yet little is known about how phenology varies across common environmental gradients within individual seagrass meadows. Here, we investigate how the density, morphology, and phenology of eelgrass (*Zostera marina*) differs across depths within multiple sites in New England. Despite variation in the proportion of reproductive vs vegetative shoots across sites, reproductive investment did not differ across depths within sites. However, a comparison of developmental stages of flowering shoots revealed delays in flower development for deep plants compared with shallow plants for all sites, demonstrating a consistent offset in reproductive phenology across depths. Our results suggest that differences in the timing of flowering may limit gene flow across seagrass meadows, with the potential for repeated genetic divergence in eelgrass populations spanning a depth gradient.

Keywords *Zostera marina* · Seagrass · Flowering · Phenology · Reproduction · Morphology · Depth gradient

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Introduction

In angiosperms, flowering phenology and relative investment in asexual vs sexual reproduction depend on a variety of environmental factors, such as temperature, precipitation, light/photoperiod, and elevation (Cerdán and Chory 2003; Galloway and Burgess 2012; Ranjitkar et al. 2013; van Kleunen 2007; Zhang et al. 2018). Consequently, angiosperms often display intraspecific variation in phenology and life history across environmental gradients. The scale of these environmental gradients ranges from biogeographic (e.g., latitudes) to regional (e.g., kilometers) to local (e.g., meters), with distinct abiotic factors influencing phenology and reproduction at different scales (Lowry et al. 2014). At small scales, environmental gradients can contribute to divergence in phenotypic traits such as flowering, potentially resulting in microgeographic variation (Richardson et al. 2014). Furthermore, microgeographic divergence in reproductive investment and flowering phenology may determine the capacity of populations to respond to rapidly changing conditions via effects on gene flow, plasticity, and local adaptation. In an era of rapid environmental change, understanding how flowering phenology and reproductive investment vary across environmental gradients within populations is particularly important for dominant and foundation species that play critical roles in community dynamics and ecosystem function.

Seagrasses, a polyphyletic group of marine angiosperms, are critical foundation species that promote biodiversity and support entire ecosystems (Hughes et al. 2009; Orth et al. 1984). *Zostera marina*, or eelgrass, is the most abundant species of seagrass globally (Olsen et al. 2004); found along both coasts of North America, the east coast of Asia, and in Europe, *Z. marina* is restricted to shallow coastal waters (Blok et al. 2018) by light availability (Dennison and Alberte 1985). Like all seagrass species, *Z. marina* has two methods of reproduction: (1) asexually via the production of vegetative or clonal shoots from rhizomes and (2) sexually via the production of flowers or inflorescences, which produce seeds that can grow into new shoots (Marbá et al. 2004). The relative proportion of sexual vs asexual reproduction is highly variable in *Z. marina*, with the majority of shoots flowering in a given year in annual or mixed-annual populations, and clonal propagation dominating in perennial populations (Jarvis et al. 2012; Keddy 1987; Phillips et al. 1983a). Several recent studies highlight the need for a greater understanding of the role of sexual reproduction in *Z. marina* survival and performance (Johnson et al. 2020; Kendrick et al. 2012; Hughes et al. 2016; Hays et al. 2020), particularly with respect to the capacity of populations to adapt to changing environmental conditions.

Variation in flowering phenology and reproductive investment of *Z. marina* has been well documented across latitudinal and regional scales, but relatively less is known about variation across smaller scales. For example, the proportion of flowering shoots in an individual bed ranges from 0 to 100% across the species range, with *Z. marina* often displaying an annual life history strategy at its range limit (Phillips et al. 1983a). Additionally, eelgrass in more northern latitudes typically flowers later in the year than its warmer, southern counterparts (Blok et al. 2018; Phillips et al. 1983b; Silberhorn et al. 1983). Regionally, climatic differences can influence *Z. marina* phenology, with sites that experience milder winters and summers flowering for an extended period of 5–7.5 months, whereas sites with colder winters and warmer summers flower for a shorter period of 3.5 months (Qin et al. 2019). Similarly, variation in photoperiod and temperature is thought to drive differences in flowering time and flower density across sites in *Zostera capricorni* meadows in southeastern Australia (Inglis and Smith 1998). Differences in nutrient availability among sites within a region can also influence flowering intensity (van Lent et al. 1995).

At a local scale, less is known about flowering phenology patterns and reproductive investment across depths within seagrass meadows, and whether they are consistent across sites. The few available studies suggest that within-site variation can be high. Johnson et al. (2017) found differences in the density of vegetative and flowering shoots, the number of spathes per flowering shoot, and the height of flowering shoots across a nutrient gradient within a single site in Virginia. They also found that lower light intensities negatively impacted shoot densities. Kim et al. (2014) compared the shallow and deep parts of an eelgrass bed in Korea and found that the shallow population was perennial, flowering at a rate of 30–45%, while the deep population was annual, with all shoots flowering every year due to intense seasonal light reduction in the fall caused by algal blooms. However, the shallow population had a greater number of inflorescences per flowering shoot and seeds per inflorescence than the deep population (Kim et al. 2014). Other studies have also found reduced flowering shoot density or frequency with depth (Infantes and Moksnes 2018; Kamel et al. 2012). Yet little is known about variation in the duration and timing of flowering in *Z. marina* across depths, a common within-site gradient typically associated with predictable variation in factors such as light and temperature.

Light and temperature have been found to impact the timing and duration of flowering in many terrestrial angiosperms. Fitter and Fitter (2002) determined that 16% of terrestrial British plants flowered earlier in the 1990s than in the previous four decades due to increased temperatures caused by anthropogenic climate change. Photoperiod can also influence flower development: for example, decreased light levels resulted in less ripening in lowbush blueberries. Conversely, higher temperatures can reduce ripening time, such as in sour cherries (Rathcke and Lacey 1985). To what extent the phenology of *Z. marina* is impacted by these factors, particularly at a smaller scale, is less well known. Here, we surveyed multiple eelgrass populations to investigate how *Z. marina* density, morphology, and phenology vary across a depth gradient (shallow vs deep) within sites, and to examine whether this variation is consistent across sites. We hypothesized that the deep zone would have shorter shoots and reduced densities of both flowering and vegetative shoots relative to the shallow zone, as well as delayed seed development due to lower temperatures and irradiance.

Methods

Site Description

In summer 2019, we assessed local-scale differences in flowering shoot density, morphology, and phenology across tidal depths by conducting weekly to biweekly surveys of two *Z. marina* beds (Table A1) in the Gulf of Maine, USA: Curlew Beach in Nahant, MA, and West Beach in Beverly, MA

(Fig. 1). Curlew Beach is a small, southwest facing bay that opens up into Nahant Harbor. It has a 334-acre *Z. marina* bed (Costello and Kenworthy 2010). West Beach is in Salem Sound, faces to the southeast, and is sheltered by the Misery Islands. It has existed since at least 1931 and is part of a 462-acre bed that is one of the deepest beds in Massachusetts, running from Beverly Harbor to West Beach (Carr and Ford 2017).

To assess the generality of the patterns documented in our surveys of these two sites, we also conducted one mid-season survey (late June/early July) and one late-season survey (August) at two additional sites—Lynch Park in Beverly, MA, and Niles Beach in Gloucester, MA (Fig. 1). Both of these sites have depth ranges comparable with Curlew Beach and West Beach, and they are located in the same region (North Shore, MA). Lynch Park is located in Beverly Harbor and is part of the same contiguous 462-acre bed as West Beach. However, the two sites are still separated by 4.5 km. Niles Beach is located within Gloucester Harbor, a sheltered cove which contains a total of 54 acres of eelgrass. It is one of the few sites in all of Massachusetts that has experienced increases in eelgrass cover over the past few decades, most likely due to the relocation of Gloucester's wastewater outfall (Costello and Kenworthy 2010).

Field Surveys

In summer 2019, we conducted eleven surveys of *Z. marina* at 1–2 week intervals in both the shallow and deep zones (Table A2) of our two focal sites from June 4th to August 27th. Because we were interested in determining the degree of variation across the entire depth range of our sites rather than simply looking at the effects of specific depths, our surveys were conducted at the shallow and deep edges of the

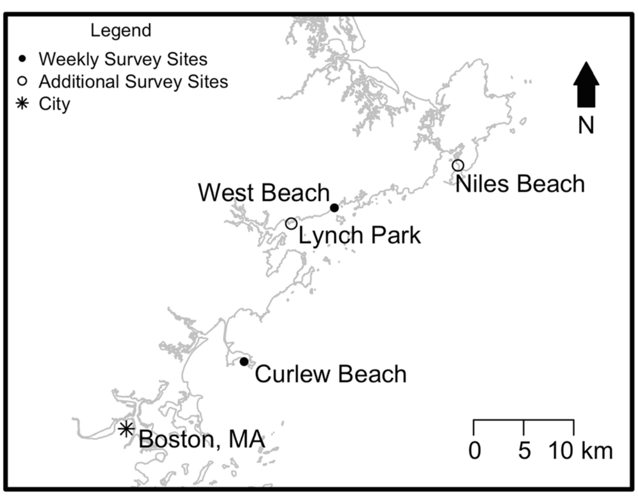

Fig. 1 Map of our study region in Massachusetts, USA, depicting our weekly survey sites (filled circles), additional survey sites for the mid- and late-season surveys (open circles), and the city of Boston (black star)

beds. For each survey, we set one 20-m transect per depth parallel to shore using SCUBA. To capture small-scale variation and account for patchiness within our sites, the exact location of the transect changed across sampling dates. We sampled ten 0.25-m^2 quadrats separated by 2 m and alternating sides along the transect. We placed the quadrats immediately next to the transect unless there was no eelgrass at that point, in which case quadrats were placed in the closest eelgrass patch within 1 m of the transect on that side. If there was no eelgrass within 1 m of the transect at a given point, that quadrat was recorded as a zero, and another quadrat was added 2 m from the end of the transect.

In each quadrat, we counted the total number of vegetative and flowering shoots, and we measured the length of up to five randomly selected vegetative shoots and up to five flowering shoots. For each flowering shoot, we also counted the total number of spathes and recorded the developmental stage of the oldest and youngest spathes. Consistent with acropetal development in *Z. marina* (De Cock 1980; Jackson et al. 2017), we considered the oldest spathe to be the bottommost and the youngest to be the topmost on an individual shoot. We defined flowering stages following De Cock (1980): 1 (styles erect out of spadix); 2 (styles bend back into spadix after pollination); 3 (half-anthers release pollen); 4 (half-anthers have been released, seeds maturing); 5 (seeds have started to release); and 6 (post-seed release when the flowering shoot begins to wither; Fig. 2). We also included two additional stages that occur before the flowering of the pistils based on our observations in the field: pre-spathe (PS; when a spathe is present, but pistils and anthers have not yet formed) and 0 (pistils and anthers have formed, but styles have not yet erected).

For the mid- and late-season surveys of Niles Beach and Lynch Park, we used the same methods as described above to assess flowering shoot density and phenology in the shallow (1 m and 2 m MLLW, respectively) and deep zones (5.6 m and 4.9 m MLLW, respectively). For the late-season survey, we also counted all seeds from 18 to 25 haphazardly collected flowering shoots each from the deep and shallow zones at all four sites to generate a snapshot of seed production across sites and depths ($N = 42$–46 per site).

Environmental Conditions

To determine how environmental factors that could affect *Z. marina* survival, growth, and reproduction differed within and between sites, we monitored water temperature and light availability over the course of the study using three Onset HOBO Pendant® Waterproof Temperature/Light Data Loggers per depth per site for both Curlew Beach and West Beach. Loggers were placed 45 cm above the sediment, 18 m apart, parallel to shore, and in the same depth zones that surveys were conducted. They were changed approximately

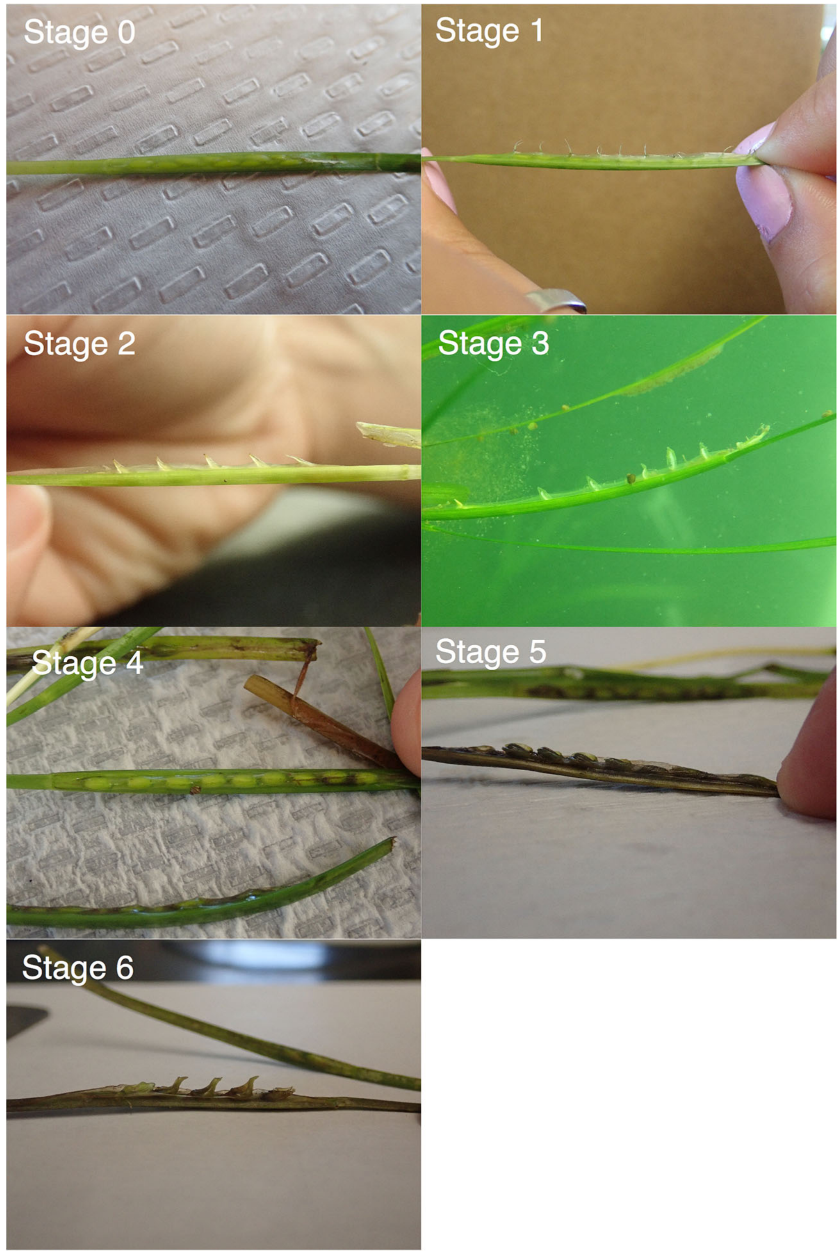

Fig. 2 Flowering stages of *Z. marina*. Stage 0: Spathes have developed, but styles have not yet erected; stage 1: Styles erect out of spadix; stage 2: Styles bend back into spathe after pollination; stage 3: Half-anthers release pollen; stage 4: Half-anthers have been released, seeds maturing; stage 5: Seeds are starting to release; and stage 6: Post-seed release and the flowering shoot begins to wither. Stages 1–6 are described in more detail in De Cock (1980)

every 2 months (see Fig. 8 for exact dates). Light was measured in photosynthetic photon flux density (PPFD; μmol m^{-2} s^{-1}) and temperature in °C; measurements were recorded every 5–15 min.

Statistical Analyses

To compare the density of *Z. marina* between sites, depths, and over time, we conducted separate analyses on the number of vegetative shoots, the number of flowering shoots, and the total number of all *Z. marina* shoots in each 0.25-m^2 quadrat using generalized linear models (GLMs) with site, depth, and time (survey number) as fixed effects and including all possible interactions. Density was analyzed using GLMs with a negative binomial regression to account for over-dispersion. We also examined the proportion of flowering shoots using a GLM with a quasibinomial distribution and a logit link function to account for over-dispersion to determine if reproductive investment by *Z. marina* changed over time, at either of our sites, and/or across depths. Survey number was treated as a categorical factor with 1 being the first survey that was conducted at a particular site and 11 being the last.

To examine whether shoot morphology differed between sites, across depths, and/or through time, we examined vegetative shoot height, flowering shoot height, and spathe number per flowering shoot using separate linear mixed effects models with site, depth, and time as fixed factors and unique quadrat as a random effect to account for non-independence of measurements within the same quadrat. We also directly compared the height of vegetative and flowering shoots within quadrats by including both types of shoots in the same analysis and adding a fixed effect of reproductive status to the mixed model described above. All models included all possible interactions.

To look at the timing of flowering across sites and depths, we compared the developmental stages of the youngest and oldest spathes separately. We combined stages 1–3 into a single stage since relatively few of these stages were found over the course of our study and because flowers only remain in these stages for a few hours to 1 week at most. Furthermore, the sequence of these stages is not always linear, as stage 3 can begin at the same time as stage 1 or before stage 2 if the flower has not yet been pollinated (De Cock 1980). We used linear regression to assess whether flowering phenology differed among locations, with time (measured in days since June 1st, a few days before we began our surveys) as our response variable, site, depth, and developmental stage (treated as a categorical factor) as our predictors, and including all possible interactions.

We also compared the timing of development between the oldest and youngest spathes across sites and depths. For this analysis we looked at the time it took spathes to reach stage 4, the stage in which seeds begin to develop. If stage 4 is reached, it means (for the most part) that a given spathe can contribute to the proliferation of the *Z. marina* population. We used a linear regression with days since June 1 as our response variable, site, depth, and spathe identity (youngest or oldest) as our predictors, and including all possible interactions.

Using data from the two surveys that were conducted at all four sites, we analyzed the proportion of flowering shoots using GLM with a quasibinomial distribution, with site, depth, and survey month (a categorical factor) as fixed effects, and including all possible interactions. We analyzed the number of seeds per flowering shoot at all four sites using GLM with a negative binomial regression and independent and interactive effects of depth and site.

Light and temperature data from Curlew Beach and West Beach were analyzed separately using a linear regression with site, depth, and time as fixed effects and including all possible interactions. Both data sets were analyzed using daily averages taken from May 13th to August 20th for Curlew Beach, and through August 22nd for West Beach.

Statistical analyses were conducted using the R Statistical Software v. 3.6.0 (R Core Team 2019). Negative binomial regressions were done using the glm.nb function in the MASS package (Venables and Ripley 2002). Linear models and mixed linear models were done using the lme4 and lmerTest packages (Bates et al. 2015; Kuznetsova et al. 2017). We used a significance level of $\alpha = 0.05$ for all of our analyses.

Results

Shoot Density

There was a significant depth*time interaction for vegetative shoot density ($\chi^2_{10,458} = 38.02$, $p < 0.001$; Fig. 3a and b; Table A3a). While eelgrass shoot density varied through time across depths, the shallow zones always had higher vegetative shoot densities than the deep zones at Curlew Beach and West Beach. Similarly, total shoot density (i.e., sum of flowering and vegetative shoots) per 0.25-m^2 quadrat varied across depths and through time ($\chi^2_{10,458} = 49.28$, $p < 0.001$; Fig. A4; Table A3b). It also varied by site and depth ($\chi^2_{1,458} = 4.00$, $p = 0.045$), with the shallow zone at West Beach being the most dense, followed by the shallow zone at Curlew Beach, the deep zone at West Beach, and the deep zone at Curlew Beach.

The number of flowering shoots differed interactively between depths and time ($\chi^2_{10,458} = 32.90$, $p = 0.003$; Fig. 3c and d; Table A3c), as well as between sites and time ($\chi^2_{10,458} = 24.07$, $p = 0.007$). There was a decline in flowering shoot density towards the end of the summer at both sites in the shallow, but no such decline in the deep. This decline was earlier at West Beach than at Curlew Beach. With the exception of the end of the season, after many flowering shoots had likely senesced in the shallow, flowering shoot density was higher in the shallow than in the deep.

The proportion of flowering shoots differed interactively between sites and time ($\chi^2_{1,449} = 38.59$, $p = 0.015$; Fig. 4; Table A3d). At the start of the season, West Beach and Curlew Beach had a similar proportion of flowering shoots, but Curlew Beach quickly surpassed West Beach. Across the entire season, Curlew Beach had a greater proportion of flowering shoots (11.5% ± 0.01; mean ± SE) relative to West Beach (6.2% ± 0.01), flowering at almost twice the rate.

Shoot Morphology

Vegetative shoot height varied interactively by site, depth, and over time ($F_{10,496} = 3.36$, $p < 0.001$; Fig. 5a and b; Table A5a). In general, vegetative shoot height increased over the course of the summer as shoots grew, and vegetative shoots were typically taller in the shallow zone than in the deep zone. In addition, vegetative shoots were taller at West Beach than at Curlew Beach.

Flowering shoot height also varied interactively by site, depth, and over time ($F_{9,333} = 3.81$, $p < 0.001$; Fig. 5c and d; Table A5b). Overall, flowering shoot height was fairly consistent across sites and depths with the exception of the deep zone at West Beach, which tended to be shorter than flowers from the other site*depth combinations.

There was a significant site*depth*time*reproductive status interaction for the heights of all shoots ($F_{9,2727} = 2.78$, $p = 0.003$; Fig. 5; Table A5c). Flowering shoots were taller than

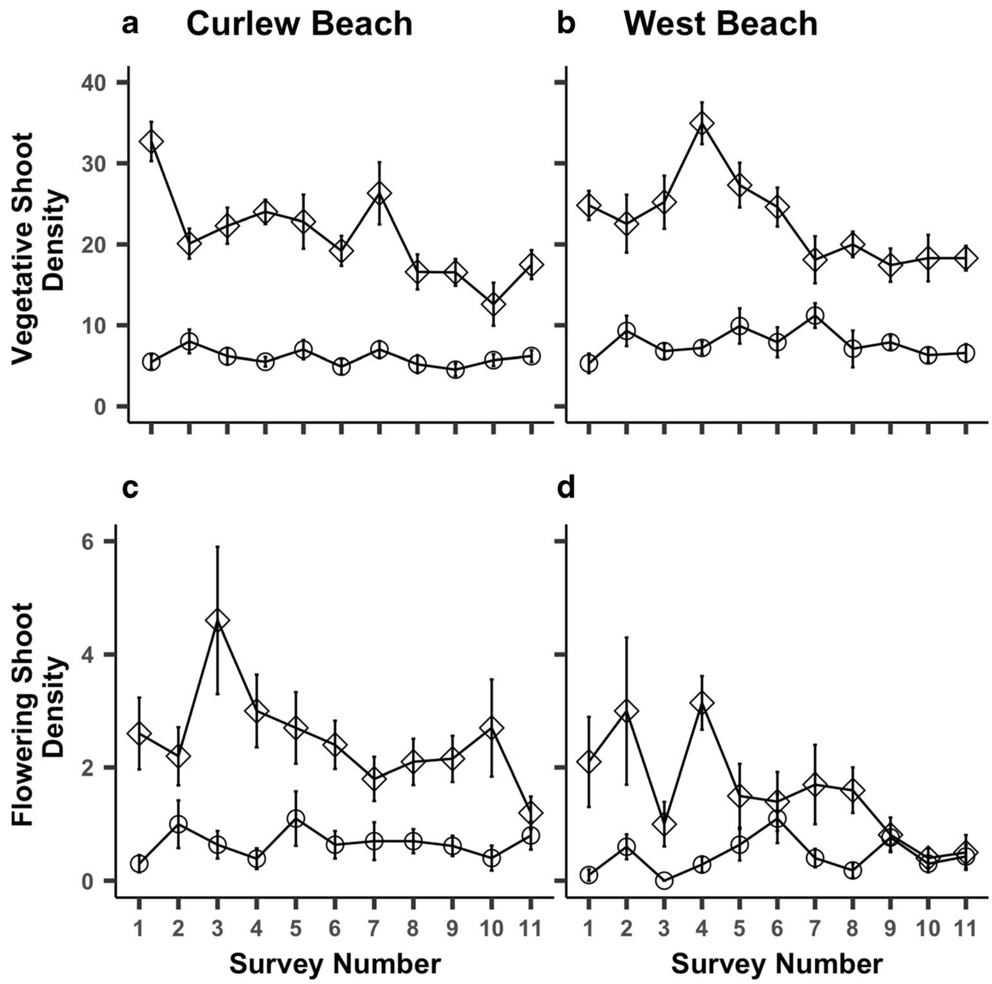

Fig. 3 Average (± SE) densities (per 0.25 m^2 quadrat) of vegetative shoots (**a** and **b**) and flowering shoots (**c** and **d**) in the shallow and deep zones at Curlew Beach (**a** and **c**) and West Beach (**b** and **d**) during the summer (field surveys started early June 2019 and ended late August 2019). Diamonds indicate the shallow zone and circles indicate the deep zone

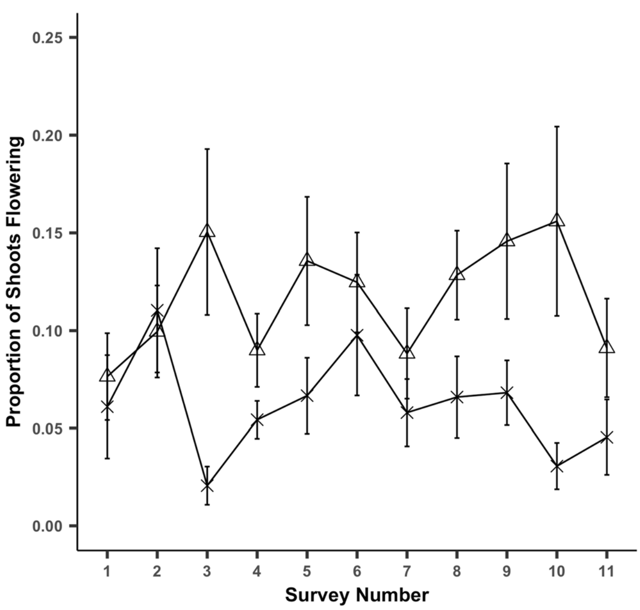

Fig. 4 Average (± SE) proportion of shoots flowering (per 0.25-m^2 quadrat) at Curlew Beach and West Beach during the summer (field surveys started early June 2019 and ended late August 2019). Triangles indicate Curlew Beach and Xs indicate West Beach

vegetative shoots at the beginning of the survey, especially in the deep zones, but by week 5 in the shallow zones and weeks 7–8 in the deep zones (i.e., towards the end of the growing season), vegetative shoots began to equal or even surpass flowering shoots in height at both sites.

There was a significant site*depth*time interaction for the number of spathes per flowering shoot ($F_{8,328} = 3.24$, $p = 0.002$; Fig. 6; Table A5d). The number of spathes per shoot increased to a peak and then leveled off over the course of the survey. Overall, Curlew Beach had more spathes per shoot than West Beach, averaging (± SE) 9.5 ± 0.3 in the shallow zone and 10.5 ± 0.7 in the deep zone, compared with 7.6 ± 0.3 in the shallow zone and 6.5 ± 0.5 in the deep zone at West Beach.

Phenology

The number of days that it took the oldest spathe to reach a given developmental stage varied between the two sites ($F_{5,500} = 2.49$, $p = 0.031$; Fig. 7a and b; Table A6A): oldest spathes at Curlew Beach developed an average of 3.6 days ahead of West Beach. There was also a marginal difference across depths ($F_{5,500} = 2.21$, $p = 0.052$). The oldest spathe

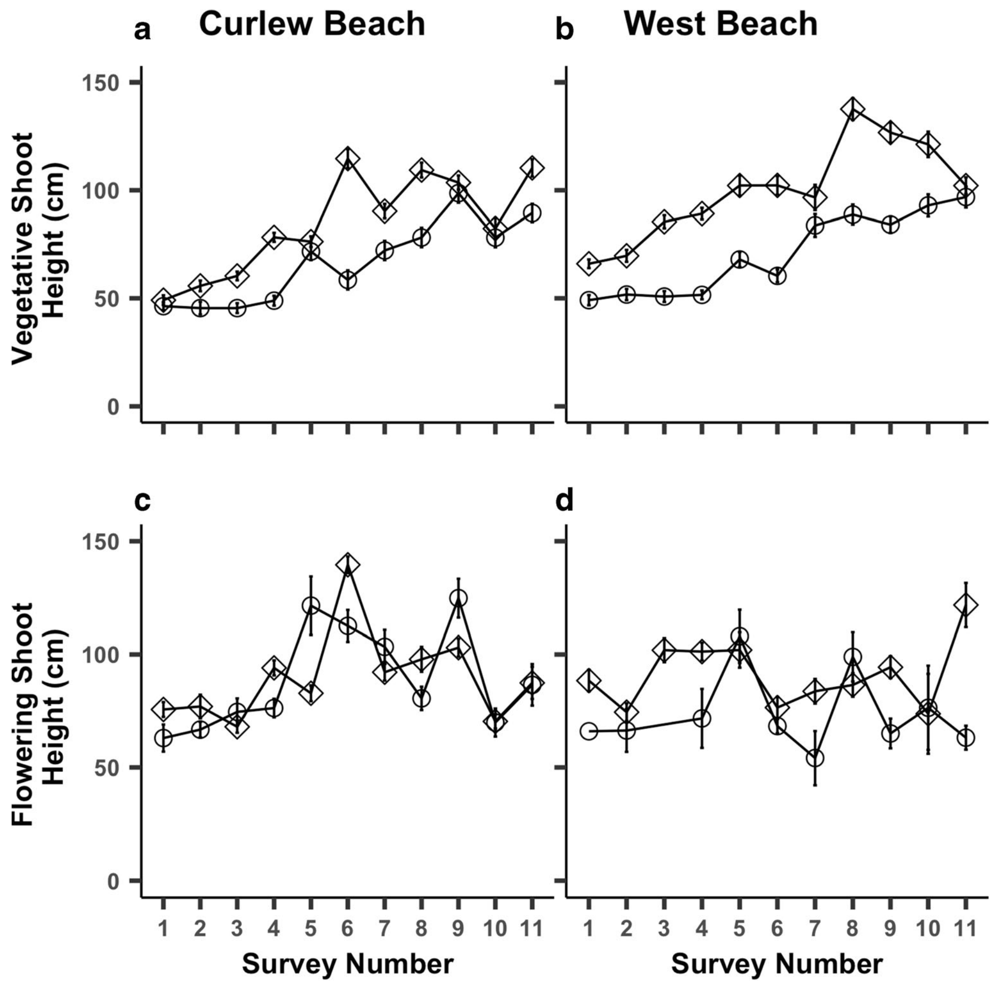

Fig. 5 Average (± SE) heights of vegetative shoots (**a** and **b**) and flowering shoots (**c** and **d**) in the shallow and deep zones at Curlew Beach (**a** and **c**) and West Beach (**b** and **d**) during the summer (field surveys started early June 2019 and ended late August 2019). Diamonds indicate the shallow zone and circles indicate the deep zone

reached each stage an average of 13.2 days earlier in the shallow zone than in the deep zone, regardless of site.

The timing of development of the youngest spathe changed interactively with site and depth ($F_{1,490} = 7.54$, $p = 0.006$; Fig. 7c and d; Table A6b), and depth and flowering stage ($F_{5,490} = 2.63$, $p = 0.023$). The youngest spathe of flowering shoots in the shallow zone typically reached each stage before the deep zone at both sites. The youngest spathe at Curlew Beach reached each stage an average of 14.4 days earlier in the shallow zone than in the deep zone. At West Beach, the youngest spathe reached each stage an average of 6.5 days earlier in the shallow zone than in the deep zone. No spathes in developmental stage 6 (post-seed release) were observed in the deep zone at Curlew Beach by the end of our survey.

The amount of time it took a spathe to reach stage 4 depended on interactions between site and depth ($F_{1,340} = 4.28$, $p = 0.039$; Fig. 7; Table A6c), site and spathe identity (i.e., youngest vs oldest spathe; $F_{1,340} = 6.07$, $p = 0.014$), and depth and spathe identity ($F_{1,340} = 4.03$, $p = 0.046$). The oldest spathe always developed more quickly than the youngest spathe, but the magnitude of the difference was greater in the shallow than the deep zone, particularly at Curlew Beach.

Environmental Data

Temperature differed interactively by depth and over time ($F_{1,392} =$, $p < 0.001$; Fig. 8a and b; Table A7a). The shallow zone averaged (± SE) 14.7 ± 0.02 °C compared with 13.2 ± 0.01 °C for the deep zone over the entire survey, regardless of site. Temperatures were more divergent between depths towards the end of the season.

There were significant site*time ($F_{1,392} = 8.26$, $p = 0.004$; Fig. 8c and d; Table A7b) and depth*time ($F_{1,392} = 8.60$, $p = 0.004$) interactions for light intensity. Over the whole survey period, the shallow zone at Curlew Beach had the highest average (± SE) light intensity of 72.3 ± 0.9 μmol m^{-2} s^{-1}, followed by the shallow zone at West Beach with 65.8 ± 0.7 μmol m^{-2} s^{-1}, the deep zone at West Beach with 23.5 ± 0.3 μmol m^{-2} s^{-1}, and the deep zone at Curlew Beach with 17.8 ± 0.3 μmol m^{-2} s^{-1}.

Additional Site Data

The proportion of flowering shoots depended on depth and sampling month ($\chi^2_{1,243} = 5.70$, $p = 0.017$; Fig. 9a;

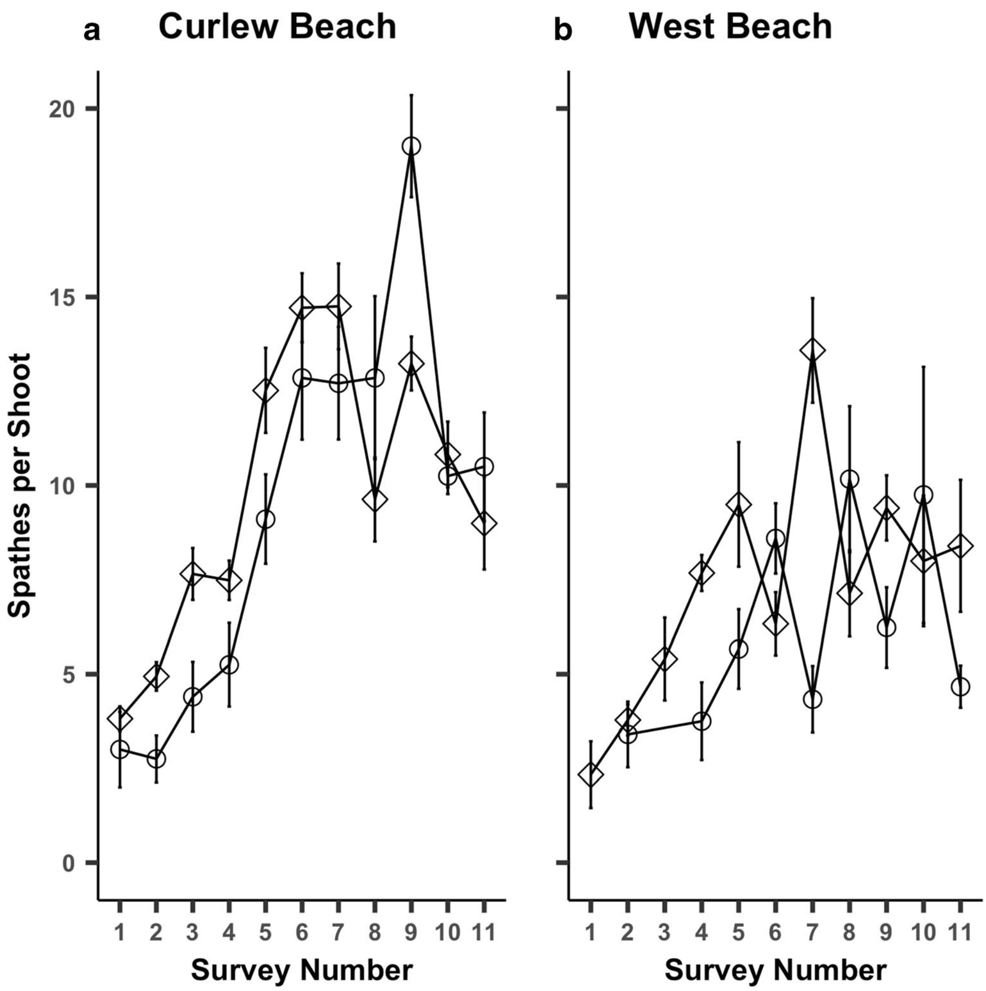

Fig. 6 Average (± SE) number of spathes per flowering shoot in the shallow (diamonds) and deep zones (circles) at (**a**) Curlew Beach and (**b**) West Beach for each survey during the summer (field surveys started early June 2019 and ended late August 2019)

Table A8a) when looking at all four sites. In June, the shallow zone had a slightly higher proportion of flowering shoots than the deep zone across sites, whereas in August, the deep zone had a slightly higher proportion of flowering shoots than the shallow zone. The proportion of flowering shoots also differed across sites and depths ($\chi^2_{3,243} = 17.61$, $p = 0.004$; Fig. 9b). This is driven by a difference in the proportion of shoots that flowered between the shallow and deep at Lynch Park. Our other three sites had a relatively equal proportion of shoots flowering across depths. The number of seeds per shoot also varied interactively by depth and site ($\chi^2_{3,211} = 39.19$, $p < 0.001$; Fig. 9c; Table A8b).

Discussion

We found pronounced differences across tidal depth, a predominant environmental gradient in many seagrass meadows, in *Z. marina* density, morphology, and phenology, but not in the proportion of flowering vs vegetative shoots. The density of both flowering and vegetative shoots was generally higher in the shallow than the deep zones. Despite these differences in shoot density, the proportion of flowering shoots did not differ between the shallow and deep, indicating similar investment in each reproductive strategy across depths. However, flowering phenology differed between depths, with flowers in the shallow zone developing earlier than flowers in the deep zone. Notably, our mid- and late-season surveys of two additional sites confirmed that although the proportion of flowering shoots did not vary across depths within sites (for three out of four sites), flowering phenology was consistently offset between shallow and deep edges. This effect of depth is apparent despite between-site variation in the proportion of flowering shoots and the precise timing/duration of flowering, indicating that differences in flowering phenology across a depth gradient may be a common feature of subtidal seagrass beds that potentially affect gene flow and genetic structure within populations.

Flowering shoot density, like total shoot density in this and earlier seagrass studies (Inglis and Smith 1998; Krause-Jensen et al. 2000), was generally higher at the shallow edge compared with the deep edge (Fig. 3c and d), which may be due to

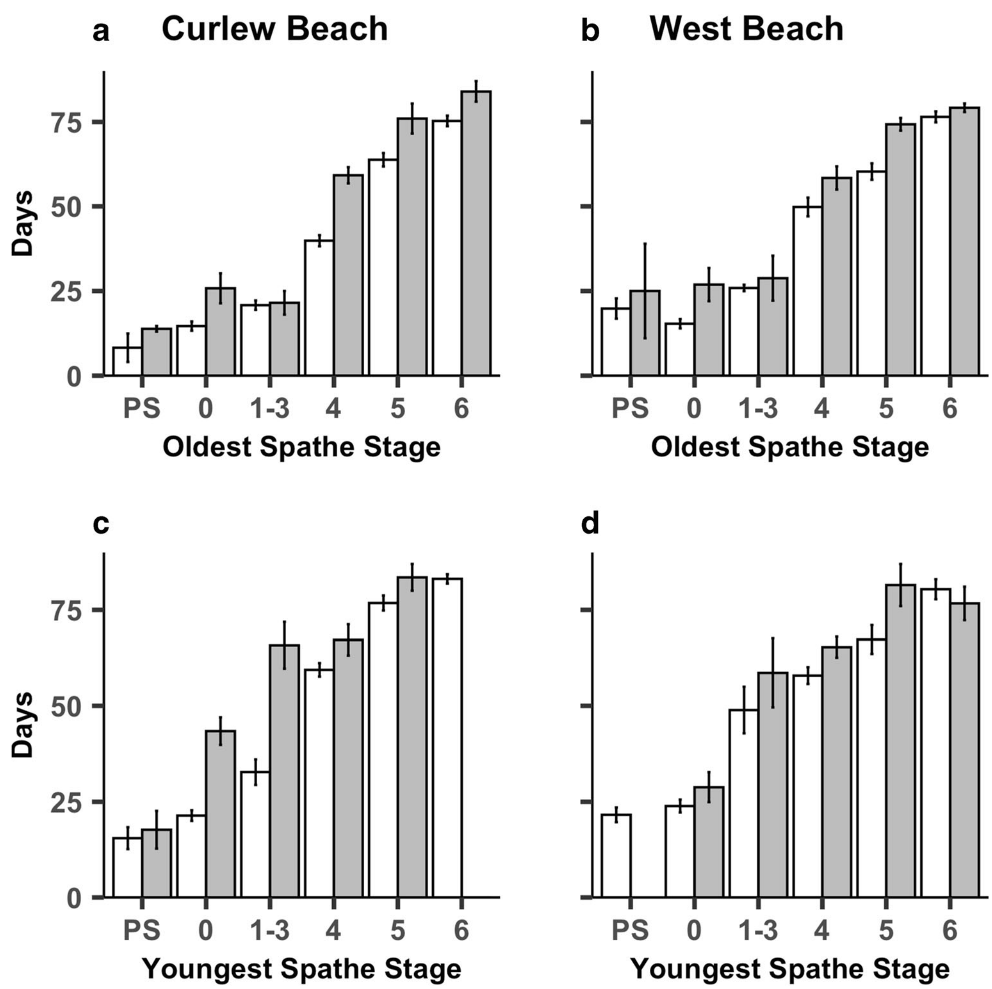

Fig. 7 Mean (± SE) number of days since June 1, 2019, that it took the oldest (**a** and **b**) and youngest (**c** and **d**) spathes of flowering shoots to reach each stage in the shallow (open bars) and deep zones (closed bars) at (**a** and **c**) Curlew Beach and (**b** and **d**) West Beach. For the youngest spathes in the deep zones, no stage 6 spathes were found at Curlew Beach and no pre-spathe (PS) spathes were found at West Beach during our surveys

a variety of abiotic factors, including light availability and temperature. Temperature and light can interact to impact the growth of seagrasses, with higher temperatures slowing growth when light is limiting due to increased respiration (Bulthuis 1987). While temperatures did differ between depths in our study (by 1.5 °C), light variation was more pronounced across depths. The decreased light levels seen in the deep may slow the growth of *Z. marina* and result in less clonal propagation. Consistent with our results, a survey of one site by Johnson et al. (2017) found that *Z. marina* flowering shoot density was positively correlated with light availability. Similarly, a survey of *Posidonia australis* identified equal or greater densities of flowering shoots in the shallow compared with the deep at the majority of sites (Inglis and Smith 1998). In our study, differences in flowering shoot density between the shallow and deep were less pronounced later in the summer, primarily due to decreases in the number of flowering shoots in the shallow, as opposed to increases in the deep. The shallow is more exposed to hydrodynamic disturbances (e.g., wave action) than the deep, likely resulting in more shoots being physically damaged or removed (Krause-Jensen et al. 2000), especially during senescence. Because flowering shoots in the shallow developed earlier in the season, they senesced earlier and were more susceptible to being damaged or dislodged from the sediment, particularly by mid- to late-summer.

Flowering shoots were also generally taller than vegetative shoots in the first half of the growing season across both of our main sites and depths, though this difference was most pronounced in the deep (Fig. 5). However, flowering shoots were outgrown by vegetative shoots at most site and depth combinations by mid-summer. A relationship between size and flowering frequency has been established in many terrestrial plant species; however, the strength of this relationship varies across sites and species (Aarssen and Taylor 1992). This relationship has also been suggested in *Z. marina*; size may be the driving factor for whether a given shoot will flower (Johnson et al. 2017). In our study, peak shoot height in the shallow was just less than depth relative to MLLW at both sites, so vegetative and flowering shoot height may both be limited by depth in the shallow, decreasing relative differences in shoot height between types. In addition, shoot density in the deep

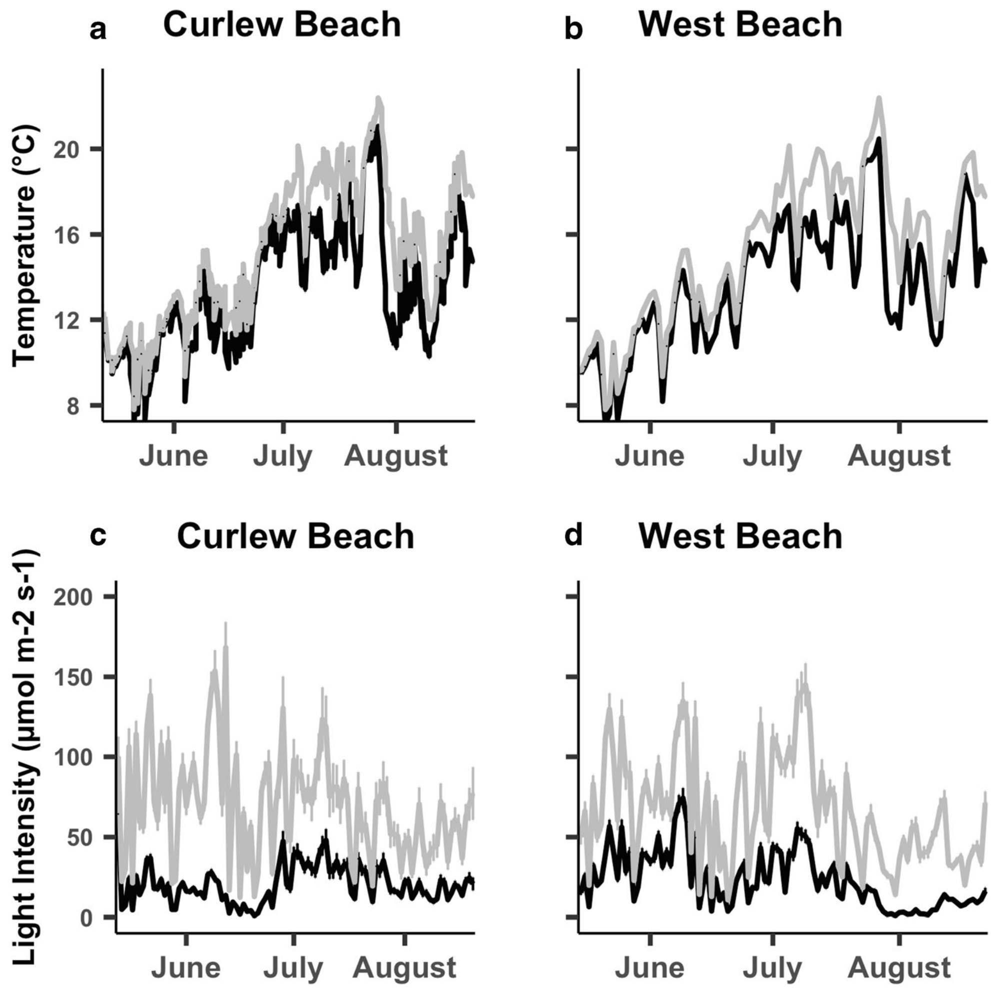

Fig. 8 Average daily water temperature (°C; **a** and **b**) and light availability (photosynthetic photon flux density (PPFD); µmol m^{-2} s^{-1}; **c** and **d**) at the shallow (gray lines) and deep edges (black lines) of (**a** and **c**) Curlew Beach and (**b** and **d**) West Beach. All temperature and light data are presented as daily averages taken from three HOBO loggers per depth per site. Loggers were deployed on 5/14 and swapped out on 6/26 at Curlew Beach and deployed on 5/15 and swapped out on 6/27 at West Beach

was much lower; thus, flowering shoots in the deep may need to grow taller relative to vegetative shoots than in the shallow so that their pollen can spread further and they can catch pollen of other shoots more easily.

The timing of development of flowers and seeds was consistently earlier in the shallow zone than deep zone across sites. The proximal mechanisms for this are unclear, as they may reflect higher light availability or temperature in the shallow and/or differences in genetic composition between these two microhabitats. Many studies have found that increased temperature results in earlier flowering in *Z. marina* (Blok et al. 2018; Silberhorn et al. 1983; Thayer and Fonseca 1984) and terrestrial flowering plants (Fitter and Fitter 2002). A temperature threshold for the initiation of flowering has been proposed (Blok et al. 2018; Churchill and Riner 1978; Setchell 1929, Silberhorn et al. 1983). We cannot confirm or deny this threshold as anthesis had already begun when we began our surveys. However, Blok et al. (2018) found the peak appearance of mature *Z. marina* seeds to be 9.8 days earlier per 1 °C increase in mean annual air temperature across latitudes. In our study, water in the shallow was 1.5 °C warmer than in the deep on average, translating to what should be a 14.7-day difference in flower development—this is comparable with the 13.9 day difference in the time it took flowers to reach stage 4 (seed maturation) between the shallow and deep in our study.

The difference in light availability between depths across the season was much greater than the difference in temperature, indicating that light may be playing a more important role in the morphological and phenological variation we observed. The shallow zones averaged 48.4 more µmol m^{-2} s^{-1} than the deep zones and daily average illuminance ranged ~ 166.5 µmol m^{-2} s^{-1} over the season across sites and depths. Average light availability was above the light saturation point for *Z. marina* in the shallow zones and below the light compensation point for *Z. marina* in the deep zones at both sites (Dennison and Alberte 1985), indicating that deep plants were more stressed. Reduced light levels can result in diminished carbohydrate storage in seagrasses (Ruiz and Romero 2001) and lead to reduced shoot heights and densities (Dennison 1987). It is possible that this reduction in carbohydrates causes seeds to take longer to mature, as has been proposed in some terrestrial systems (Atherton 2013). Based on our data, a carbohydrate requirement may be a better signal for

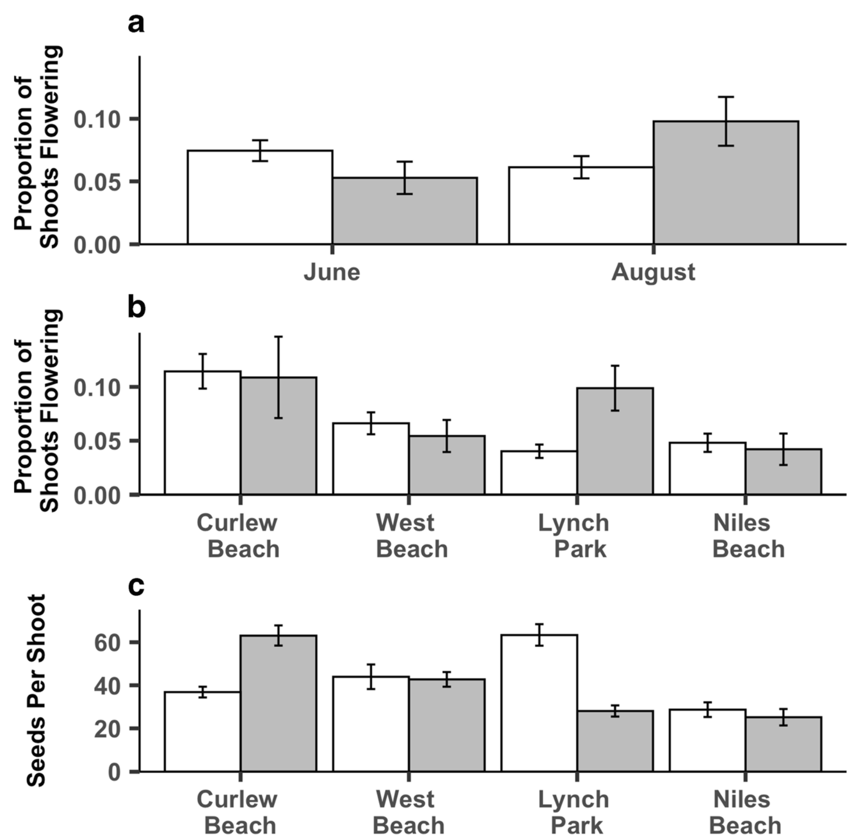

Fig. 9 **a** The proportion of shoots flowering (mean ± SE) across all four sites by month and depth. **b** The proportion of shoots flowering (mean ± SE) across months by depth and site. **c** The number of seeds per flowering shoot (mean ± SE) at all four sites surveyed in August. Open bars indicate the shallow zone and closed bars indicate the deep zone

flower development than crossing a particular temperature threshold, as flowers in each stage were found over the course of the entire season. Reaching this requirement could be related to temperature and thus explain why temperature is often correlated to phenology. In addition to potentially delaying flowering directly, environmental differences between depths may have indirectly delayed flowering in the deep by causing there to be lower densities of flowering shoots. This reduced density may have increased the time it took for flowers to get pollinated, thus lengthening the time it took them to reach stage 4 and begin seed development. Whether light, density, or a combination of these variables is responsible for altering the timing of flowering in *Z. marina* within and across meadows merits further investigation via correlative field surveys measuring carbohydrate content and/or controlled mesocosm studies manipulating light or density.

Regardless of the mechanism, one consequence of this documented phenological difference is clear—shallow and deep plants are more likely to pollinate plants within depths than between depths. Such pre-zygotic barriers to gene flow may help to maintain genetic differences between closely occurring subpopulations in sympatry or parapatry (Gavrilets 2004). In fact, a few studies assessing population genetic structure within meadows found *Z. marina* to be genetically differentiated across depths (e.g., Kamel et al. 2012; Kim et al. 2017), which may be partially attributable to an offset in phenology. Thus, patterns of genetic differentiation in seagrasses may not be limited to larger scales—e.g., low genetic relatedness in eelgrass populations across latitudinal gradients (Olsen et al. 2004; Short et al. 2012) and at regional scales (Kamel et al. 2012; Kim et al. 2017; Novak et al. 2017)—and genetic structure within populations merits further investigation, particularly to inform restoration and management.

Despite similarities in the proportion of flowering shoots across depths within sites, we did find differences in the relative investment in sexual reproduction across our study sites (Fig. 9b), with Curlew Beach flowering at a higher rate than West Beach and Niles Beach. Similar differences in reproductive investment across sites have been documented in *Z. marina* beds in the Chesapeake Bay (Harwell and Rhode 2007). The similarities in flowering investment across depths contrasts with other studies that found increased flowering in *Z. marina* (Kim et al. 2014; Phillips et al. 1983a; Silberhorn et al. 1983; van Lent and Verschuure 1994)—and terrestrial flowering plants more generally (van Kleunen 2007)—when exposed to higher levels of stress, such as reduced light. However, in all of these instances, the stressors that led to increased investment in flowering were either acute or seasonal. In other cases, chronic stressors, such as low levels of light or nutrients, were associated with decreased investment in flowering (Inglis and Smith 1998; Johnson et al. 2017;

Kamel et al. 2012). Despite lower temperatures and light levels in the deep at both of our weekly survey sites, we saw no reduction in the proportion of flowering shoots in the deep when compared with the shallow. Interestingly, Lynch Park, the one site that did have differences in investment across depths, had reduced flowering rates (4.0 ± 0.6%) in the shallow when compared with the deep (9.9 ± 2.1%). This could be related to the proximity of this site to the urban areas of Beverly and Salem; however, more information about the cause of this difference is needed. *Z. marina* has been documented to flower anywhere from 0 to 100% across its range, depending on the population. However, on the Atlantic coast of North America, rates for temperate, subtidal populations are much less variable and consistent with our results—typically 2–13% for perennial subtidal populations (Phillips et al. 1983a)—but can be as high as 28% (Jarvis et al. 2012). Light availability was higher at both depths at West Beach compared with the deep at Curlew Beach, yet the deep at Curlew Beach flowered at almost twice the rate of West Beach. This evinces that light availability is unlikely to be the main cause for the differences in reproductive investment between our sites. It is possible that differences in nutrient availability (Johnson et al. 2017) or genetic variation (Jahnke et al. 2015) contributed to differences in the proportion of flowering shoots between sites. Harwell and Rhode (2007) suggest that the environment may be a more significant factor than genetics; however, further investigations using common garden studies and/or reciprocal transplant experiments are necessary to determine what underlies this trend.

Our results also suggest a possible trade-off between investment in sexual vs asexual reproduction across sites. Curlew Beach flowered at a higher rate and had more spathes per shoot, but this site had shorter and fewer total shoots than West Beach (largely driven by West Beach having a higher number of vegetative shoots). Jahnke et al. (2015) found a similar trade-off between investment in asexual and sexual reproduction in *P. oceanica*. However, Harwell and Rhode (2007) found no trade-off between sexual and asexual reproduction in *Z. marina* in the Chesapeake Bay. van Lent and Verschuure (1994) describe *Z. marina* in the Netherlands as living on a continuum from annual to perennial, with perennial being the preferred life history strategy. All populations surveyed in this study are perennial and we found no deviation in life history strategy across depths, as has been found in other *Z. marina* populations (Kim et al. 2014). In *Sparganium erectum*, an aquatic freshwater plant, younger populations invest more in sexual reproduction than older populations (Piquot et al. 1998). The relative age of our sites is unknown, but it is worth considering how and why reproductive allocation varies across different spatial and temporal scales since this affects genotypic diversity and phenotypic variation, as well as the capacity of populations to respond to changing environmental conditions.

Our study demonstrates that the density, morphology, and phenology of *Z. marina* differ consistently across a depth gradient at multiple sites. The shallow zone has more and taller vegetative and flowering shoots than the deep zone, and its flowers develop earlier than in the deep zone. We found the shallow zone to be anywhere from 5.5–14.4 days ahead of the deep in terms of flower development, depending on the site and identity of the spathe (i.e., youngest or oldest). However, pollen of *Z. marina* is usually viable for only 2 days (De Cock 1980). As a result, between the edges of *Z. marina* beds, gene flow via cross-gradient pollen dispersal and subsequent fertilization may be limited, and seed dispersal may be the primary mechanism of gene flow in seagrass beds. However, negatively buoyant *Z. marina* seeds typically disperse < 5 m, with rafting of detached flowering shoots being the only way by which seeds can travel longer distances (Harwell and Orth 2002; Furman et al. 2015). Studies examining eelgrass genetic structure and relatedness across a depth gradient within beds would help to elucidate whether differences in flowering phenology contribute to limited gene flow between shallow and deep zones. Furthermore, comparisons of shallow and deep *Z. marina* shoots in a common garden experiment would be useful in assessing whether genetic and/or environmental factors primarily underlie the observed differences in density, morphology, and phenology. With temperature and sea level expected to continue to rise (Rahmstorf 2007), it is unclear how genetic differences between depths could affect *Z. marina*'s ability to adapt at a local scale as microgeographic variation shifts and the distribution of low- and high-quality environments changes. Proper management of *Z. marina* beds and associated species requires a better understanding of cross-gradient genetic structure resulting from differences in flowering phenology and reproductive allocation across depths, and how this structure in turn affects the adaptive capacity of seagrass beds facing rapid environmental changes and shifting gradients.

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