



Morphological response of *Zostera marina* reproductive shoots to fertilized porewater



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ABSTRACT

Exposing *Zostera marina* to fertilized porewater has been shown to increase vegetative shoot density and leaf growth rates, and affect shoot morphology. However, aside from changes in shoot density, records of morphological changes to reproductive shoots are lacking. To address this, five replicate ambient and nutrient enriched patches, each between 0.5 and 2.0 m², were selected within a developing *Z. marina* meadow in Shinnecock Bay, NY. Fertilizer stakes, with a N:P:K of 15:3:3, were used to amend the sediments of a subset of patches in the late fall and early spring. In June, morphological measurements of reproductive shoots and stages of anthesis were recorded. We found significantly increased reproductive shoot height, number of rhipidia, and number of spathes on each rhipidium in response to fertilization. Nutrient enrichment also advanced the stage of ovary development in the first spathes at the time of sampling, indicating that the rate of development had been accelerated or the reproductive shoot had flowered earlier. Additionally, the number of normally developing ovaries, as determined by the size of the seed embryo, was significantly greater in enriched patches. We estimated that these changes acted to increase seed output per reproductive shoot, and may have enhanced pollen access by elevating receptive stigma above the local vegetative canopy.

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1. Introduction

Nutrient and light availability are considered to be the primary physical factors controlling seagrass growth (Short, 1987; Dennison, 1987). However, nutrients and light tend to be negatively correlated within the nearshore ecosystems where most seagrasses are found. For example, chronic nutrient loading has been shown to increase planktonic and epiphytic algal biomass, reducing light penetration (Orth and Moore, 1983; Short and Neckles, 1999) and, over time, restricting seagrasses to areas with relatively little bulk water nutrient concentrations. In contrast to most algae, however, seagrasses can acquire nutrients directly from the sediments using adventitious roots (Agami and Waisel, 1986), a capacity that often exceeds that of foliar uptake (Short and McRoy, 1984; Vonk et al., 2008). Because of this, altering porewater nutrients has been shown to have profound effects on vegetative shoot morphology. Typically, porewater amendments result in increased vegetative shoot density, number of leaves, leaf length, leaf area, leaf growth rate, and areal coverage of *Zostera marina* (Orth, 1977; Short, 1987; Kenworthy and Fonseca, 1992; Carroll et al., 2008; Peralta et al., 2003). Additionally, Roberts et al. (1984) reported that more shoots had sprouted from seedlings planted in areas of nutrient enrichment.

While the effect of porewater nutrients on *Z. marina* vegetative growth has been extensively studied, the impact of nutrient addition

on reproductive shoots remains unexplored. Short (1983) provided the only description of nutrient condition and *Z. marina* reproductive growth, quantifying their density along natural gradients of interstitial ammonia in the Alaskan archipelago. Although he did not directly manipulate porewater nutrients, Short (1983) found increased ammonia concentrations correlated with greater vegetative leaf area, length and width. He and others have interpreted the short-term, shoot proliferation by *Z. marina* as a positive response to nitrogen availability, while the long-term, negative effects associated with chronic eutrophication, such as reduced shoot density and productivity, are the combined result of water-column, epiphytic, macroalgal and self-shading (Short, 1983; Cabaco et al., 2013).

Given the dramatic and numerous impacts of nutrient availability on vegetative growth, we hypothesized that similar changes might occur among the anatomical structures comprising generative growth. In *Z. marina*, flowers consist of spathes nested within sympodial branching rhipidia (De Cock, 1981, Fig. 1). A spathe is the protective sheath surrounding the spike-like inflorescence or spadix (Churchill and Riner, 1978). In *Zostera*, the spadix consists of both male and female flowers (Fig. 2a). The reproductive shoot develops acropetally, causing the lowest rhipidium to be the oldest and the topmost rhipidium to be the youngest (De Cock, 1981). Spadices on each rhipidium develop acropetally, with the oldest spadix (closest to the stem) undergoing anthesis first and the youngest spadix (farthest from the stem) maturing last (Churchill and Riner, 1978; De Cock, 1981). The stages of anthesis are clearly identifiable with the first being the erection of the style to a

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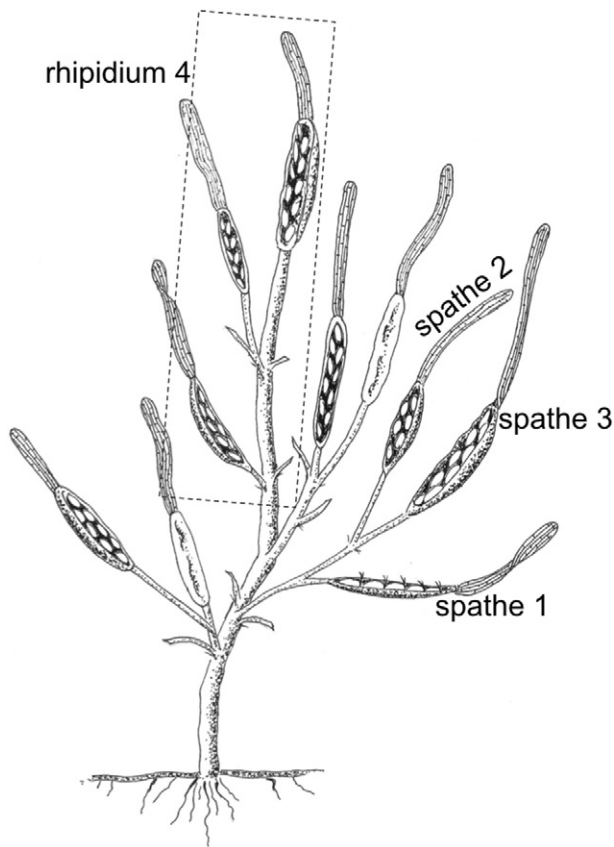


Fig. 1. Stylized representation of a *Zostera marina* reproductive shoot with 4 rhipidia. Spathes on the second rhipidium are numbered sequentially in order of development. The youngest, tallest rhipidium (the 4th) is identified by a dashed box.

90° angle with the ovary (De Cock, 1980). Pollen grains in the water column then contact one of two stigmata. Fertilization of the ovary marks the second stage of anthesis, visible as a dark abscission scar on the style (Fig. 2b). Next, the anthers release their pollen into the water column and dehiscence from the plant, leaving the fertilized ovaries to mature into small seeds (De Cock, 1980, Fig. 2c and d).

Historically, studies of seagrass spatial expansion and persistence have focused primarily on vegetative growth, with attention to seagrass reproductive ecology occurring only for annual species (Orth et al., 2000), those with significant seed banks (Fonseca et al., 2008) or for denuded and disturbed areas (Orth and Moore, 1986; Lee et al., 2007; Peterson et al., 2002). Recently, this clonal-centric view has been challenged (Becheler et al., 2010; Zipperle et al., 2011; Buckel et al., 2012; Peterson et al., 2013), reinvigorating the discussion of sexual recruitment in meadow development and recovery (Macreadie et al., 2014).

In this study, we manipulated porewater nutrient concentrations within five seagrass patches to examine its effect on reproductive growth. We asked the following questions: (1) can fertilization alter the morphology of *Zostera marina* reproductive shoots, and (2) how will these changes affect seed production?

2. Materials and methods

2.1. Study site

The study area (250 m × 225 m) consisted of a patchily distributed *Zostera marina* meadow in Shinnecock Bay, Long Island, NY (40.857237° N, 72.450289° W; Fig. 3). Depth at the site ranged from 0.25–1.25 m (MLLW). Light penetration at the site was $143 \pm 9.2 \mu\text{mol s}^{-1} \text{m}^{-2}$, which was $46 \pm 2\%$ of surface incident light at a depth of 0.89 ± 0.04 m ($n = 5$; LI-COR underwater PAR sensor).

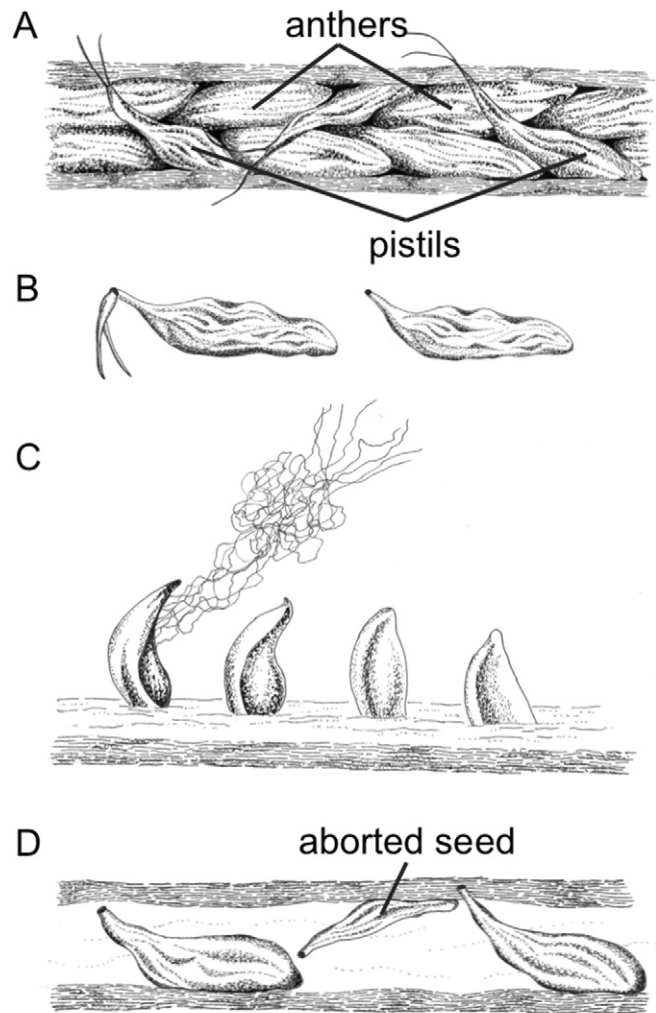


Fig. 2. A *Zostera marina* reproductive shoot spadix (a) contains both male flowering parts (anthers) and female flowering parts (pistils). Stages of anthesis include the erection of the styles to a 90° angle with the ovary, (b) the abscission of the stigmata, (c) the release of pollen by the anthers, and (d) the development of ovules into seeds.

Surficial sediments consisted of siliceous sands uniformly low in organic content (<1% loss on ignition at 500 °C for 5 h). In 2008, Carroll et al. found evidence for nutrient limitation in *Z. marina* growing within this portion of the bay. Flowering phenology in *Z. marina* varies by latitude and is strongly regulated by temperature; at the study site, primordial inflorescences first appear at 0.5–3 °C during winter, anthesis occurs mid-May at 15 °C and fruit maturation is completed by the end of June, after temperatures have reached 21 °C (Churchill and Riner, 1978; Silberhorn et al., 1983).

Seagrass was mapped using available aerial imagery beginning in 2001 and, via a balloon-mounted camera, bi-monthly since 2011 (Furman et al., 2015). These maps allowed for tracking of patch formation, expansion, loss, and the calculation of minimum age. Over 2200 patches were identified via ArcGIS, ranging in size from 0.01 to 20,000 m².

2.2. Experimental nutrient addition

Using a Trimble GeoXT unit with Arcpad 7 software, ten *Z. marina* patches between 0.5 and 2.0 m² were selected and field-located in the shallowest portion of the study site, (0.33–0.39 m MLLW). A 4-m² quadrat, divided into 100 cells (400 cm²), was placed over each patch. Permanent rebar markers secured three of four quadrat corners, ensuring exact replacement for sampling and fertilization. Five patches were

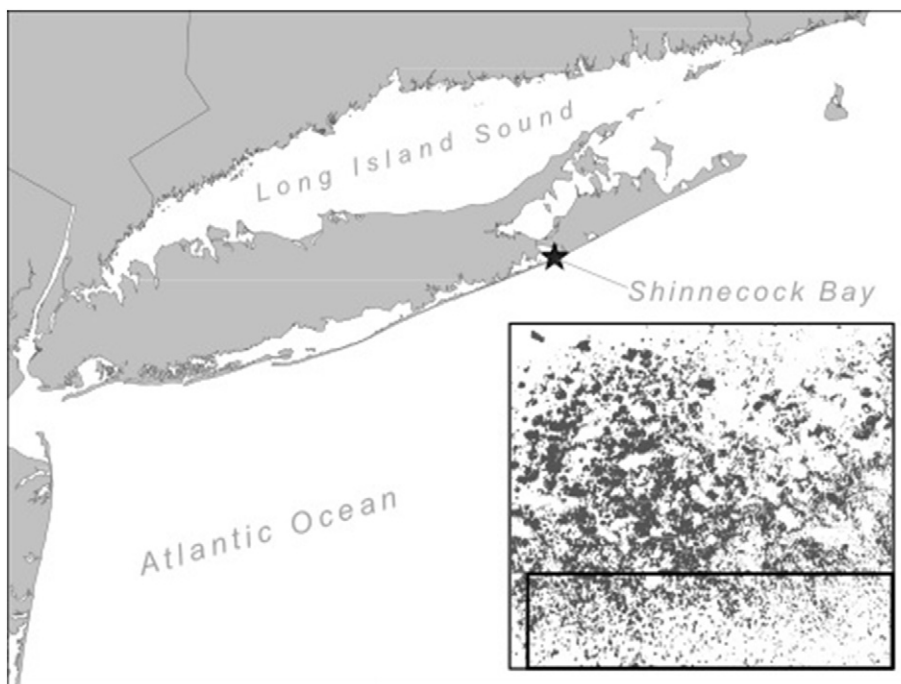


Fig. 3. The star indicates the location of the 250 × 225 m study site in Shinnecock Bay, NY, enlarged at the lower right of the image. The rectangle within the lower portion of the study site designates the area from which the ten patches for this study were selected.

haphazardly selected for nutrient enrichment, while the remaining five served as control patches. In October 2012 and April 2013, Jobe's Tree and Shrub® fertilizer stakes (N:P:K = 15:3:3 M ratio) were driven below the sediment surface of treated patches to increase porewater nutrients. Fertilizer was inserted in October to provide nutrients during the emergence of reproductive shoot primordia in the late-fall, early winter (Churchill and Riner, 1978), and during the fastest reproductive shoot growth in the spring (April, Churchill, *personal communication*). We chose to intersperse stakes at 40-cm intervals following a pilot study conducted in a sub-tidal mesocosm. Results showed elevated total dissolved nitrogen (TDN) within a 20-cm radius after immersion of a single fertilizer stake for 24 h or 2 tidal cycles ($144.8 \pm 130.0 \mu\text{M}$; $n = 15$). Concentrations at 30- to 40-cm radii were nearly half at only $69.7 \pm 48.3 \mu\text{M}$ ($n = 7$). Based on this estimate of nutrient flux, stakes were placed in the center of each group of four quadrat cells containing at least one *Z. marina* shoot, resulting in 53 ± 16 shoots per fertilizer stake ($n = 10$; Fig. 4). Although we acknowledge that our pilot study could not fully replicate the advective processes porewater nutrients would experience in the field (i.e., those driven by wind-waves), we expect that increased lateral advection and sediment-water column exchange would only act to diminish treatment contrasts. Significant treatment effects, then, would indicate that sufficient nutrient retention had taken place.

Eight weeks after fertilization occurred, fifteen vegetative shoots with a minimum separation distance of 20 cm were collected from each patch to confirm nutrient availability and uptake. These fifteen shoots were separated into three replicates of five shoots each. The two youngest leaves of each shoot were selected for analysis, cleaned of epiphytic growth, dried at 60 °C for >72 h, and ground into a homogenous fine powder. Total carbon and nitrogen content were determined for 40 of the samples by oxidation in a Thermo EA1112 elemental analyzer following Fourqurean et al. (1992).

To assess reproductive shoot morphology, a single flowering shoot was selected from each flower-bearing quadrat cell in the beginning of June, yielding 168 reproductive shoots from ambient patches and 193 from fertilized patches. While genetic identity and rhizome connectivity were not surveyed prior to shoot selection, sample independence was likely maintained by the following factors: (1) only the terminal

apical flowers for a connected set of ramets during a given season, (2) genetic diversity of flowering shoots has been shown to be quite high for patches sampled within the same meadow (18 genets m^{-2} ; Furman et al., 2015), and (3) after several years of observing seagrasses in this portion of the bay, rhizome connections of >10 cm have only rarely been found (B. Peterson pers. obs.). Therefore, flowers collected at 20-cm intervals were most likely physiologically independent with a reasonable probability of being derived from unique genets.

The following metrics were recorded in the field without removing the reproductive shoot: shoot height, number of rhipidia, number of spathes occupying each rhipidium, and number of ovaries developing

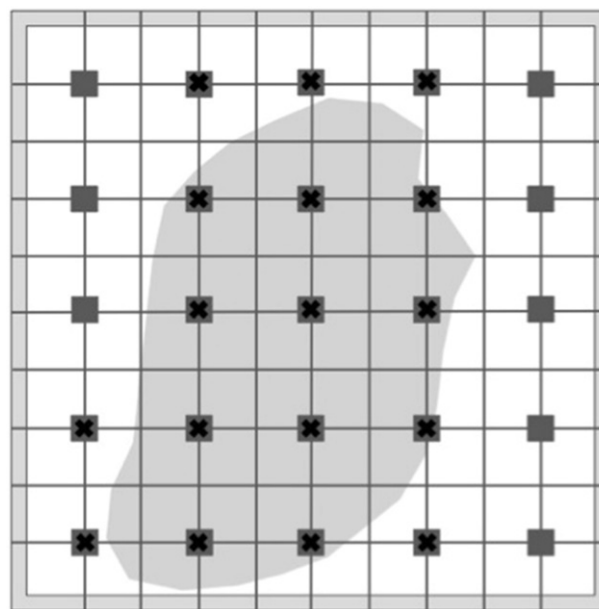


Fig. 4. Diagram of the 2 × 2 m quadrat with 100 (400 cm²) equally sized cells placed in relation to a seagrass patch shown in light gray. Dark gray squares indicate all potential fertilizer stake locations. Black Xs denote locations that would have received a fertilizer stake due to seagrass presence in one of the 4 associated cells.

into seeds, or the stage of ovary development if they were too immature to determine if abortion had occurred, on the first, oldest spadix present on the youngest, topmost rhipidium. For the purpose of enumerating per capita seed output, mature ovaries approaching maximum size were assumed to be potentially viable seeds, hereafter referred to as 'normal' seeds (A.C. Churchill, *personal communication*). We also assumed that any ovary shriveled at the time of sampling had been aborted by the reproductive shoot (Fig. 2d).

2.3. Data analysis

All statistical analyses were run in R 3.0.2 (R Core Team, 2013). The effect of fertilizer on percent nitrogen (N) of new vegetative leaves was determined with a one-way ANOVA ($N_{\text{ambient}} = 20$, $N_{\text{fertilized}} = 20$). Percent N data underwent a 4th-root transformation to meet the assumption of normality. An independent samples *t*-test was conducted to compare reproductive shoot height between shoots exposed to ambient ($N = 168$) and fertilized ($N = 193$) porewater. Height measurements were log-transformed to meet assumptions of normality and homogeneity of variances. Mann-Whitney *U* tests were applied to analyze differences in the number of rhipidia on the shoot ($N_{\text{ambient}} = 168$, $N_{\text{fertilized}} = 193$), the mean number of spathes per rhipidia for each shoot ($N_{\text{ambient}} = 168$, $N_{\text{fertilized}} = 193$), and the number of developing ovaries on the first, most developed spathe of the youngest rhipidium ($N_{\text{ambient}} = 128$, $N_{\text{fertilized}} = 158$) between shoots in ambient and fertilized porewater nutrients. Lastly, a chi-squared test of independence was performed to determine if a relationship existed between development of the ovaries on the first spadix and fertilized porewater ($N_{\text{ambient}} = 164$, $N_{\text{fertilized}} = 179$) using the R package MASS (Venables and Ripley, 2002). Differences in sample sizes occurred if spathes were missing or damaged.

3. Results

Foliar percent N was significantly higher in enriched ($1.45 \pm 0.12\%$; mean ± 1 s.d.) as opposed to ambient patches ($1.29 \pm 0.18\%$; $F_{1,38} = 7.98$, $p = 0.007$), confirming that fertilizer spike-derived nutrients were available to *Z. marina* over sufficient time-scales to impact the stoichiometry of vegetative growth.

The effects of enriched porewater nutrient concentrations on *Z. marina* reproductive shoot morphology manifested in several of the measured variables. Reproductive shoot height was significantly greater (two-tailed *t*-test $t_{358} = -9.81$, $p < 0.001$) in shoots exposed to fertilized (23.1 ± 6.6 cm) rather than ambient porewater (17.4 ± 4.5 cm; Fig. 5). Enrichment resulted in significantly more rhipidia per flowering shoot (fertilized: 3.3 ± 0.8 rhipidia, ambient: 2.9 ± 0.7 rhipidia; $p < 0.001$, two tailed Mann-Whitney *U* test) in addition to a significantly greater mean number of spathes per rhipidia (fertilized: 2.5 ± 0.6 spathes, ambient: 2.0 ± 0.5 spathes; $p < 0.001$, two tailed Mann-Whitney *U* test).

The development of ovaries in the oldest spathe on the youngest rhipidium was examined on reproductive shoots growing in both ambient and fertilized porewater. The null hypothesis that the stage of ovary development within each first spathe was independent of fertilization was rejected by a chi-squared test of independence ($\chi^2(1) = 4.86$, $N = 350$, $p = 0.027$). Reproductive shoots from fertilized patches were more likely to have a first spathe with large, developing ovaries and were less likely to have immature ovaries than shoots drawn from control patches (Fig. 6). Then, examining only the first spathe on the last rhipidium with developed ovaries, we assessed the number of ovaries that were growing into normal seeds (Fig. 2d). In fertilized patches, the reproductive shoots produced significantly greater numbers of such ovaries (5.1 ± 2.1 ovaries) than those in ambient patches (3.6 ± 1.8 ovaries; $p < 0.001$, two tailed Mann-Whitney *U* test). Fertilized plants produced 1.5 more normal seeds per spathe than

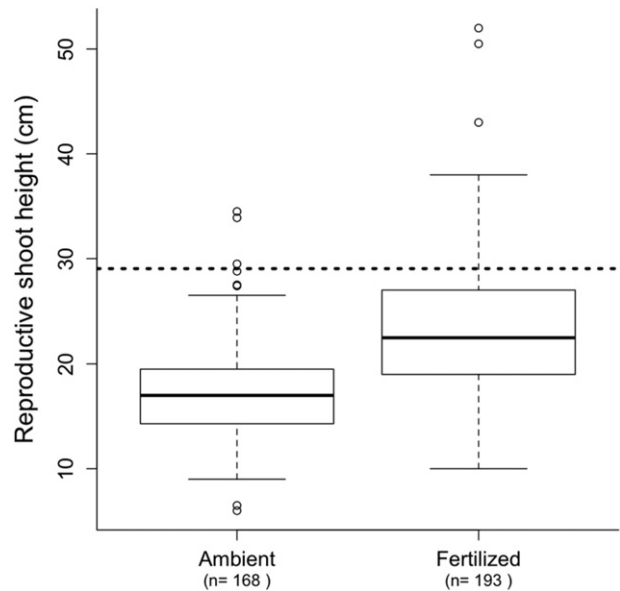


Fig. 5. Differences in reproductive shoot height for shoots grown in fertilized and ambient porewater. Boxplots mark median values with a central bar, the first and third quartiles with a box, the ± 1.5 interquartile ranges with Tukey whiskers, and data points outside of this ± 1.5 interquartile range with a circle. The dashed horizontal line indicates the mean canopy height of the nearby study area.

unfertilized plants, representing an over 40% increase in normal seed output (Fig. 7).

4. Discussion

Experimental manipulation of porewater nutrients resulted in numerous changes to *Z. marina* reproductive shoot morphology. These changes had significant impact on seed quality and production with clear downstream consequences for meadow maintenance and expansion.

Consider the potential impact of increased reproductive shoot height. *Z. marina*, as a hydrophilous plant, depends on the passive transport of slightly negatively buoyant, filamentous pollen strands to deliver

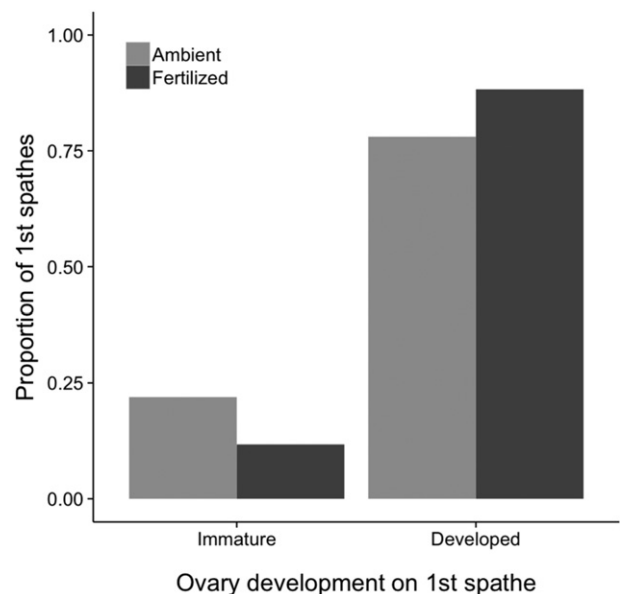


Fig. 6. Proportion of developed and immature ovaries within the first spathe of the last rhipidium at the time of sampling for reproductive shoots grown in fertilized (dark gray) and ambient (light gray) porewater.

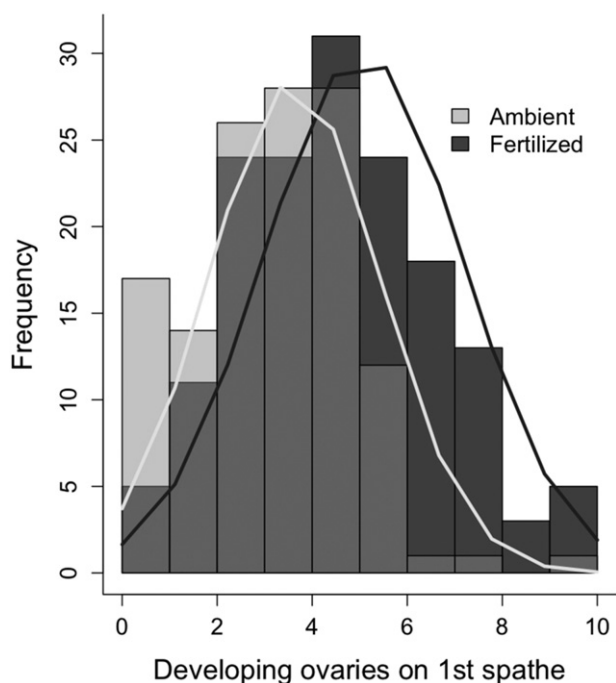


Fig. 7. Histogram of the number of ovaries developing into normal seeds on the first spathe of the last rhipidium in reproductive shoots grown in fertilized (dark gray) and ambient (light gray) porewater.

male gametes to nearby inflorescences (De Cock, 1980). There was nearly a 6-cm increase in reproductive shoot height observed due to sediment fertilization. At the time of anthesis, the mean canopy height across the study site, as determined by calculating 80% of the plant shoot height from 16 haphazardly distributed 0.062-m² quadrats, was 29.1 ± 6.4 cm (mean ± 1 s.d.). Of the reproductive shoots growing in ambient patches, only 1.8% stood taller than the canopy height, while 14.5% of shoots growing in enriched patches were able to access water flow above the vegetative canopy (Fig. 5). The capability to place receptive flowers within the bulk water flow has clear implications for the genetic diversity of resultant offspring. Taller reproductive plants likely gain access to pollen sourced from further afield, increasing the probability that paternal genotypes differ from that of the female flower (Ackerman, 2002). Conversely, inflorescences below the canopy height might be more likely to receive pollen from proximate and either identical or closely related clones.

Further, flowering shoots that grew in fertilized porewater also had significantly greater numbers of rhipidia, more spathes on each rhipidium, and more developing ovaries on each spathe. These responses altered per capita seed production. By assuming that each normal ovary became a viable seed, each shriveled ovary was aborted, and each spathe developed the same number of normal seeds as the first spathe on the youngest rhipidium, we were able to estimate the number of normal seeds produced by each flowering shoot over the entire flowering event by multiplying the number of developing ovaries in that first spathe by the number of spathes on the reproductive shoot. Calculated in this way, we projected seed output to have been 22 ± 16 normal seeds per shoot (mean ± 1 s.d.) for ambient reproductive shoots and 44 ± 26 normal seeds per shoot for fertilized flowering shoots. Reproductive shoots from fertilized patches yielded an average of 1.5 more seeds per spathe than control flowers, more than doubling the potential seed output per shoot. Reproductive shoot densities were found to be 40 shoots m⁻² within similar un-amended patches in the same portion of the meadow (L.J. Jackson, unpublished data). This value was within range of the density recorded by Churchill and Riner (1978) for nearby Great South Bay, NY (53 ± 24 flowers m⁻²). However, the density was much greater than that found in the Baltic

Sea (Reusch, 2003), in Great Harbor, Massachusetts (Ackerman, 2002), and in False Bay, Washington (Ruckelshaus, 1996) at densities of 6.1 ± 0.74, 5, and 13 flowers m⁻², respectively, indicating *Z. marina* at the site allocated proportionally more resources towards flowering. If 40 flowers m⁻² is assumed to be representative of enriched patches as well (flowering densities were not measured as part of this study), then 880 extra seeds m⁻² were produced as a result of nutrient addition. If locally retained, such production could have important effects on meadow maintenance and expansion by influencing the rate of infilling during colonization and recovery following disturbance.

Nutrient enrichment also advanced the stage of ovary development in the first spathes at the time of sampling, indicating that either the rate of development had been accelerated or the reproductive shoot had flowered earlier. If so, natural heterogeneity in porewater nutrients could influence the local timing of anthesis on even smaller spatial scales than seasonal temperatures have already been shown to do (Churchill and Riner, 1978; Silberhorn et al., 1983). Because porewater nutrients in seagrass systems vary as a function of seagrass biomass, meadow age and infaunal densities across a range of spatiotemporal scales (Williams, 1990; Peterson and Heck, 1999; Peterson and Heck, 2001), nutrient condition could be a factor in the desynchronization of pollen release throughout the seagrass landscape, acting to ensure that style erection (the first stage of anthesis) occurs in one flower while pollen is being released by another (the third stage of anthesis), as well as mitigating the frequency of near-neighbor, and presumably close kin, fertilizations. Previous studies examining the effect of infaunal nutrient sources have shown that naturally occurring bivalve densities can elicit the same morphological changes in vegetative shoots as described artificial fertilization. In this experiment, seagrasses were exposed to 1.5 ± 0.3 g N m⁻² d⁻¹ (mean ± s.d.), which falls within the range of nitrogen biodeposition reported by Norkko et al. (2001) and Jaramillo et al. (1992; 1.4 and 3.54 g N m⁻² d⁻¹, respectively). Therefore, it is conceivable that areas of naturally higher porewater nutrient concentrations could result in hot spots of reproductive success within seagrass communities, with important consequences for meadow development, maintenance and stability.

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