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**The effect of patch dynamics and nutrient availability on the production of *Zostera marina*
seeds within Shinnecock Bay, NY**

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Abstract of the Thesis

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Exposing *Zostera marina* to fertilized porewater has been shown to increase vegetative shoot density and leaf growth rates, as well as affect morphological shoot characteristics including increasing leaf length, leaf area and the number of leaves per shoot. However, aside from changes in shoot density, records of morphological changes in reproductive shoots subjected to increased nutrient availability 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 enriched patches in the late fall and early spring. In June, measures of reproductive shoot height, number of rhipidia, number of spathes occupying each rhipidium, and number of developed ovaries on the youngest rhipidium were recorded. Reproductive shoots allocated nutrients to significantly increase reproductive shoot height, the number of rhipidia, and the number of spathes on each rhipidium in response to fertilization. Nutrient enrichment also significantly 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 was capable of flowering earlier. Additionally, the number of ovaries developing into viable seeds, determined by the size of the seed embryo, was significantly greater in reproductive shoots growing in

enriched porewater. Morphological changes following nutrient addition likely increase pollen access by elevating receptive stigma above the canopy and increase seed output per reproductive shoot. Combined, these morphological changes of the reproductive shoots in areas of greater porewater nutrients could hasten patch expansion through hot spots of reproductive success.

With increasing seagrass fragmentation worldwide, understanding the effect of patch dynamics on seagrass reproduction within the seagrass landscape is vital. Patches of *Zostera marina* varying between 2.5m² and 20m² were completely cleared of reproductive shoots in 2011 and 2012 and separated into edge (< 1m from the patch edge) and core (> 1m from the edge) material. Shoots were kept in seawater tables until seeds dehiscence naturally from the plant. Normal seeds, consisting of a firm seed coat filled by the seed embryo, were then enumerated for each patch to quantify the impact of the seascape on seed production. Patch size had no significant effect on the density of normal or nonviable seeds. Location within the patch did have a significant effect on normal seed density in 2012 when reproductive shoot density was controlled for as a covariate. Normal seed density was then found to be significantly greater in younger edge habitat than older, less sexually productive core habitat. *Z. marina* age seems to have an impact on the amount of seeds produced in an area.

Dedication Page

I would like to dedicate all the hard work that went into the completion of this thesis to my amazing family, friends, and advisor.

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Chapter 1

Hot spots of reproductive success morphological response of *Zostera marina* reproductive shoots to fertilized porewater

INTRODUCTION

Nutrient and light availability are considered the two primary physical factors limiting seagrasses (Short 1987; Dennison 1987), as both are necessary to these marine angiosperms for photosynthesis. However, these environmental requirements result in a seeming paradox for seagrasses. As photoautotrophs, seagrasses require light; but they are rooted underwater, a medium that quickly attenuates light. In addition, increases in water column nutrient loading result in rapid planktonic and epiphytic growth, both decreasing light availability to seagrasses (Orth and Moore 1983; Short and Neckles 1999). Therefore, seagrasses are usually limited to areas with relatively low water column nutrient concentrations. However, unlike phytoplankton and epiphytic algae that rely exclusively on water column nutrient sources, seagrasses primarily acquire their nutrients from the sediments using adventitious roots (Agami and Waisel 1986). They are also capable of absorbing nutrients to a lesser extent via foliar uptake (Short and McRoy 1984; Vonk et al. 2008). Altering porewater nutrients has been shown to have profound effects on vegetative shoot morphology. When there are low levels of porewater nutrients, *Zostera marina* allocates greater resources to the development of root biomass. In contrast, as porewater nutrients increase, the below- to above-ground biomass ratio decreases (Peralta et al. 2003). The vegetative shoot density, number of leaves, leaf length, leaf area, leaf growth rate, and areal coverage all increase following porewater fertilization (Orth 1977; Short 1987; Kenworthy and Fonseca 1992; Carroll et al. 2008; Peralta et al. 2003). Additionally, Roberts et al. (1984) reported more shoots sprouting from seedlings planted in areas of nutrient-enriched porewater.

While the effects of exposure to enriched porewater nutrients have been extensively studied in *Zostera marina* vegetative shoots, the impact nutrients have on reproductive shoots remains largely unknown. Short (1983) describes the only observations of the effect of porewater nutrients on *Z. marina* reproductive shoots along natural gradients of interstitial ammonia levels in the Alaskan archipelago. Although he did not manipulate porewater nutrients, Short (1983) found increases in vegetative leaf area, length and width along the natural gradient of ammonia. In contrast to aforementioned studies, both vegetative and reproductive shoot densities had negative logarithmic relationships with ammonia concentrations. It was hypothesized that the short-term response of *Z. marina* to increased nutrients was an increase in shoot density in order to absorb more of the excess nitrogen. As the nutrients persisted, however, there was a negative long-term effect on shoot density as a result of a tradeoff between nutrient uptake and light availability to the larger, self-shading leaves present (Short 1983).

Given the dramatic and numerous impacts of increasing nutrient availability to non-reproductive shoots, it is likely that increasing nutrient allocation to reproductive shoots will impact a number of the unique anatomical structures, which have gone unrecorded when observing only vegetative shoots. One of these features is the rhipidium, which is a branch consisting of several spathes (Figure 1; De Cock 1981). A spathe is the protective sheath surrounding the spadix of the plant (Churchill and Riner 1978; Figure 2a). As a monocious plant, the spadix consists of both male and female flowering parts. Spadices are a second feature that could be affected by an increase in available nutrients. Finally, the rate of seed development may also be influenced by nutrients. The reproductive shoot develops acropetally, making the lowest rhipidium the oldest and the topmost rhipidium the youngest (De Cock 1981). Spadices on each rhipidium also develop acropetally, with the oldest spadix, closest to the stem, undergoing anthesis first and the youngest spadix, farthest from the stem, developing 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 90° angle with the ovary (De Cock 1980; Figure 2b). A pollen grain in the water column comes into contact with the two stigmata, followed by the fertilization of the ovary, resulting in the second stage of anthesis; the style bends backwards and abscission of the stigmata occurs, leaving a dark scar (Figure 2c). Next,

the anthers release their pollen into the water column and dehisce from the plant, leaving only the developing fertilized ovaries behind (De Cock 1980; Figure 2d and 2e).

Historically, studies of seagrass spatial expansion and persistence have focused primarily on clonal vegetative growth (Orth et al. 2000), 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 clonally focused view has been challenged (Becheler et al. 2010; Zipperle et al. 2011; Buchel et al. 2012; Peterson et al. 2013), reinvigorating interest in the role of sexual recruitment in meadow development and recovery (Macreadie et al. 2014). Despite this debate on meadow persistence, reproductive shoots are important in developing meadows since they provide the annual seed set that maintains and expands existing patches. In this study, we fertilized porewater within five seagrass patches to specifically examine the morphological response of reproductive shoots. The following questions were examined: 1) Will fertilization alter the morphology of *Zostera marina* reproductive shoots, and 2) How will these morphological changes affect seed production?

MATERIALS AND METHODS

Study Site

The study area (250 m x 225 m) consisted of a patchily distributed *Zostera marina* meadow in Shinnecock Bay, NY, along the southeast coast of Long Island (Figure 3). Depth at the site ranged from 0.25 – 1.25 m (MLLW), and surficial sediments consisted of uniformly low organic sands (<1% loss on ignition at 500°C). Flowering phenology in *Z. marina* varies latitudinally 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 *in review*). These maps allowed for tracking of patch formation, expansion, loss, and minimum age. Over 2,200 patches were identified via ArcGIS with areal coverage ranging in size from 0.01 to

20,000 m². Light limitation was not considered a stressor for the seagrass across the site as the depth of 20% incident light exceeds 1.5 m at this location. However, Carroll et al. (2008) found evidence for nutrient limitation in *Zostera marina* growing near our study site.

Experimental Nutrient Addition

Using a Trimble GeoXT unit with Arcpad 7 software, ten *Zostera marina* patches between 0.5 and 2.0 m² were selected and field-located from the shallowest area of the study site, all at similar depths. A 4 m² quadrat, divided into 100 cells (400 cm²), was placed over each patch. Permanent rebar markers secured three of the four quadrat corners and ensured exact replacement at sampling and fertilization periods. Five patches were 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 molar ratio) were driven below the sediment surface of treated patches to increase porewater nutrients. Fertilizer was inserted in the fall (October) to provide nutrients for the emergence of reproductive shoot primordial known to occur throughout November and during the fastest reproductive shoot growth in the spring (April; Churchill *personal communication*). A pilot study conducted in subtidal mesocosms indicated a stable spike in the nutrient signal at 20 cm radii, so we interspersed stakes at 40 cm intervals. More specifically, stakes were placed in the center of each group of four quadrat cells containing at least one *Z. marina* shoot (Figure 4).

To assess reproductive shoot morphology, a single flowering shoot was selected from each flower-bearing quadrat cell in the beginning of June. This resulted in 168 reproductive shoots being selected from ambient patches and 193 from fertilized patches. Since *Zostera marina* rarely maintains rhizome connections for ramets separated by greater than 11 cm (Jacobs 1979), flowers collected at 20 cm intervals were likely physiologically independent. 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 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 a flower's seed output, an ovary that appeared to be approaching its proper size, similar to a grain of rice, was assumed to complete development into a viable seed. Any ovary that appeared shriveled at the time of sampling was assumed to have already been aborted by the reproductive shoot (Figure 2e).

Data analysis

All statistical analysis was run in R 3.0.2 (R Core Team 2013). 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. Last, 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.

RESULTS

The effect of fertilizing porewater on *Zostera marina* reproductive shoot morphology was manifest in several of the measured variables. Reproductive shoot height was significantly greater (two-tailed t-test (358) = -9.81, $p < 0.001$) in shoots exposed to fertilized (23.1 ± 6.6 cm; mean \pm SD) rather than ambient porewater (17.4 ± 4.5 cm; Figure 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 the ovaries in the first, oldest spathe on the youngest, topmost rhipidium was examined on reproductive shoots growing in both ambient and fertilized porewater. The spathes were classified as having large ovaries developing into seeds, or as having immature ovaries too small to determine if a seed was developing. 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 (Figure 6). Then, examining only the first spathe on the last rhipidium with developed ovaries, we assessed the number of those ovaries that were growing into seeds (Figure 2e). In fertilized patches, the reproductive shoots produced significantly greater numbers of ovaries developing into seeds (5.1 ± 2.1 ovaries; mean \pm SD) 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 seeds per spathe than unfertilized plants, representing an over 40% increase in seed output per spathe (Figure 7).

DISCUSSION

Fertilizing porewater enlarged numerous morphological characteristics of *Zostera marina* reproductive shoots. This study clearly demonstrated that the reproductive shoots respond to fertilization similarly to the vegetative shoots of *Z. marina*. These morphological changes may have significant impacts on the seed production and consequent expansion and/or genetic diversity of seagrass patches experiencing increased sediment nutrients.

Consider the potential impact of increased reproductive shoot height. *Zostera marina*, as a hydrophilous plant, depends upon the passive transport of slightly negatively buoyant, filamentous pollen strands until contact with nearby inflorescences (De Cock 1980). There was nearly a 6 cm increase in reproductive shoot height observed due to sediment fertilization. The mean canopy height across the study site was 29.1 ± 6.4 cm (mean \pm SD), determined by calculating 80% of the plant shoot height from 16 haphazardly distributed $1/16 \text{ m}^2$ quadrats. Of the reproductive shoots growing in ambient

porewater, only 1.8% stood taller than this canopy height; 14.5% of shoots growing in fertilized porewater extended above this canopy (Figure 5). Access to water flow above the local canopy could have profound implications for the genetic diversity of accessible pollen, as well as for pollen dispersal distance of these taller reproductive shoots (Ackerman 2002). Inflorescences below the canopy may be more likely to become entangled with pollen also released below the canopy by nearby flowers of similar genetics. By contrast, inflorescences above the canopy may have access to pollen from genetically different flowers carried from a farther distance, while simultaneously able to release their pollen into the water column above the canopy to be carried, unencumbered, to unrelated distant flowers.

In addition, the flowering shoots not only grew taller in fertilized porewater, but also had significantly more rhipidia, more spathes on each rhipidium, and more developing ovaries on each spathe. These alterations have important effects on per capita seed production. We assumed that each ovary that was approximately the size of a grain of rice at the time of sampling would become a viable seed, while shriveled ovaries had been aborted, and that each spathe of the plant would develop the same number of viable seeds as the examined first spathe on the youngest rhipidium. By multiplying the number of developing ovaries in that first spathe by the number of spathes on the reproductive shoot, we arrived at an estimate of the potential viable seeds produced by each flowering shoot over the entire span of anthesis. The potential seed output was 22 ± 16 seeds per shoot (mean \pm SD) for ambient reproductive shoots and 44 ± 26 seeds per shoot from fertilized reproductive shoots. Reproductive shoots from fertilized patches produced an average of 1.5 more seeds per spathe than control flowers, more than doubling the potential seed output per shoot. Across the site there was a reproductive shoot density of 40 shoots m^{-2} present in ambient porewater. This value was within range of the density recorded by Churchill and Riner (1978) in 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 8.6 – 16 flowers m^{-2} , respectively. Considering only the ambient porewater reproductive shoot density of 40 flowers m^{-2} , this is equivalent to an underestimation of 880 extra seeds m^{-2} produced in areas of

greater porewater nutrients. If locally retained, this could have important downstream effects on eelgrass areal coverage and shoot density.

A logical next step would be to compare the calculated potential seed output to the actual seed output of reproductive shoots. This task is complicated by the acropetal development of the spadices, which results in seeds maturing and dehiscing from the plant over a period of many weeks. Further, we must consider the significant difference in spadix development at our single observational time point (Figure 6). If fertilization caused spadices to develop at a faster rate or to flower earlier, to accurately assess seed production in the field, all spadices must be sampled at multiple time points, as seeds would dehisce from the fertilized treatments earlier than the ambient treatments.

Fertilizing porewater has been used successfully in the past to enhance growth of *Zostera marina* vegetative shoot transplants (Kenworthy and Fonseca 1992). This research demonstrates that continuing to fertilize over the course of a flowering season can result in measurable effects on per capita seed production. Small, patchily distributed restoration plots could be manipulated into producing many more seeds than otherwise possible, thereby contributing to eelgrass expansion, amalgamation of patches, meadow formation, and the complete restoration of a site.

In addition to aiding *Zostera marina* restoration goals, areas of naturally varying porewater nutrients could influence the local timing of anthesis in much the same way as temperature (Churchill and Riner 1978; Silberhorn et al. 1983). Since porewater nutrients vary at a range of spatial scales, they could be a factor in the small-scale desynchronization of pollen release across the landscape. This desynchronization would not only ensure that as style erection (the first stage of anthesis) occurs in one flower, pollen is released by another (the third stage of anthesis), but also it would mitigate the frequency of near-neighbor, and therefore close kin, fertilizations. Furthermore, heterogeneous porewater nutrients could affect the number of viable seeds produced by the reproductive shoots, influencing the rate of infilling during colonization and disturbance recovery.

Previous studies have examined the role that sedentary bivalves can play in changing sediment nutrient availability (Peterson and Heck 1999; Peterson and Heck 2001; Reusch et al. 1994; Reusch and Williams 1998). These studies demonstrated how bivalves that

elevated porewater nutrients elicited the same morphological changes in vegetative shoots as described when seagrass was artificially fertilized. Given the outcome of this experiment, one can envision that bivalves may play a similar role in changing the morphology of seagrass reproductive shoots. Areas of naturally greater porewater nutrients would then likely result in hot spots of reproductive success with important consequences for meadow development, maintenance and stability.

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FIGURES

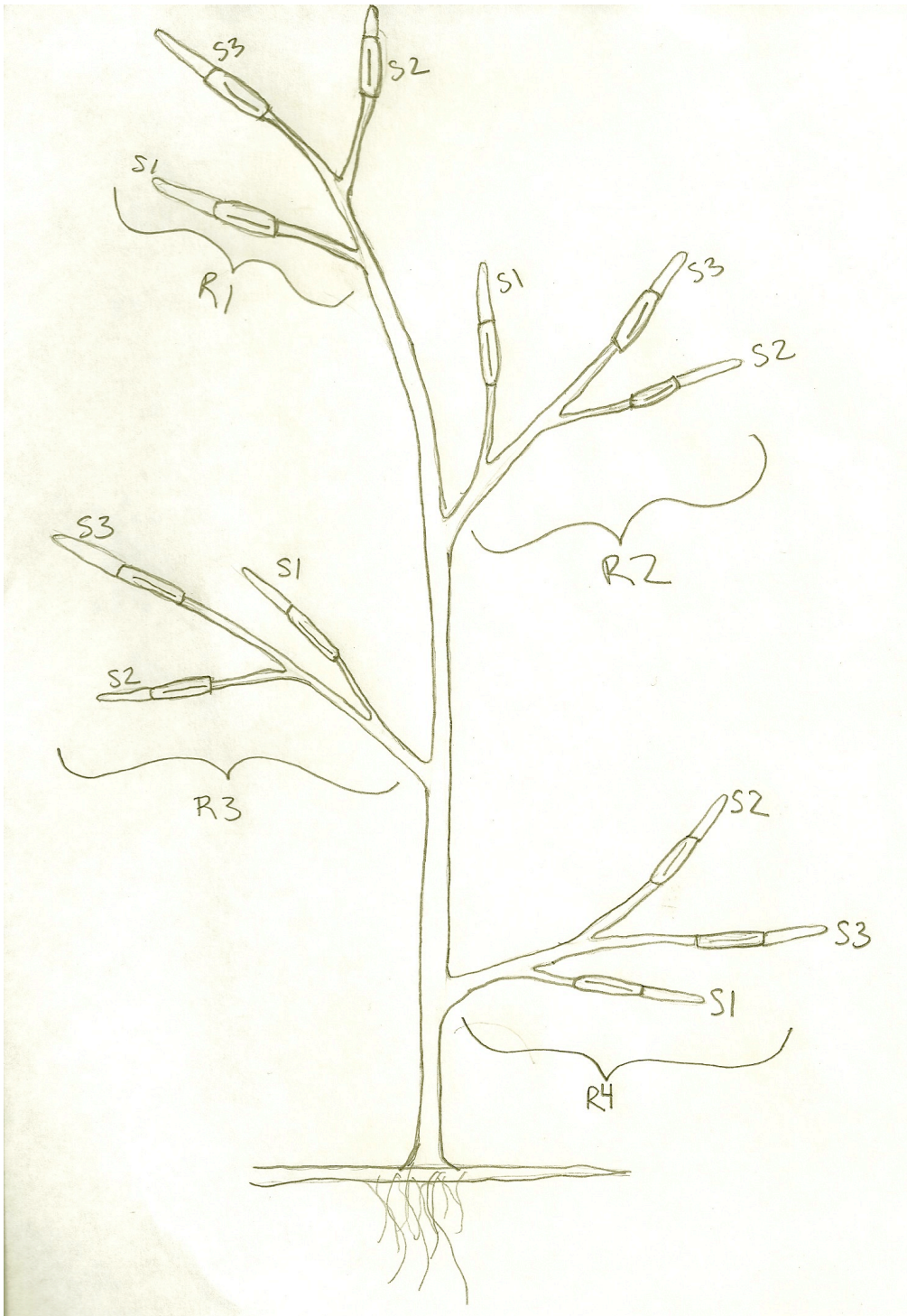


Figure 1 *Zostera marina* reproductive shoot with 4 rhipidia. Spathes are numbered sequentially in order of development.

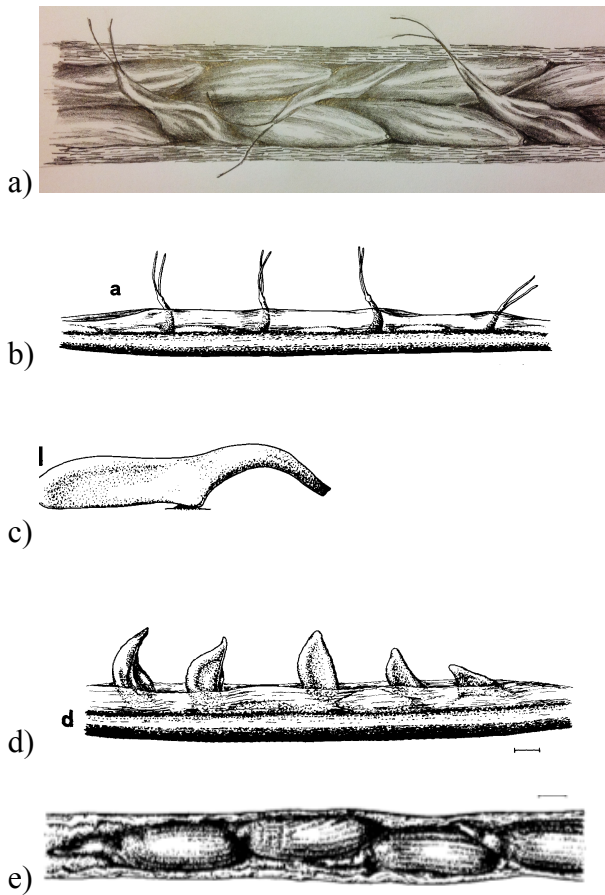


Figure 2 A *Zostera marina* reproductive shoot spadix (a) contains both male flowering parts (anthers) and female flowering parts (pistils). Stages of anthesis include (b) the erection of the styles to a 90° with the ovary (De Cock 1980), (c) the abscission of the stigmata (De Cock 1980), (d) the anthers release pollen (De Cock 1980), and (e) the ovules develop into seeds (De Cock 1980).

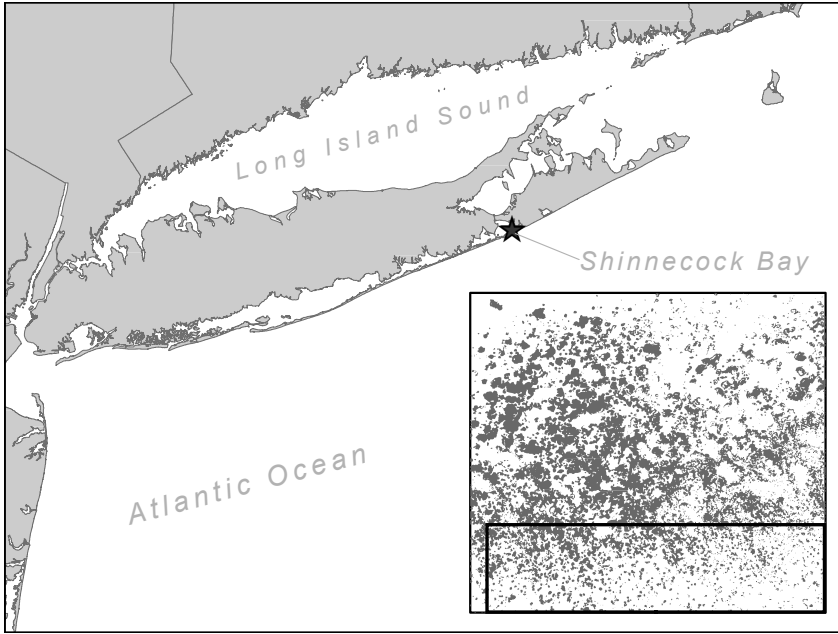


Figure 3 The star indicates the location of the 250 x 225 m study site in Shinnecock Bay, NY, which is enlarged at the lower right of the image. The rectangle within the lower portion of the study site designates the area from which the ten patches for the study were selected.

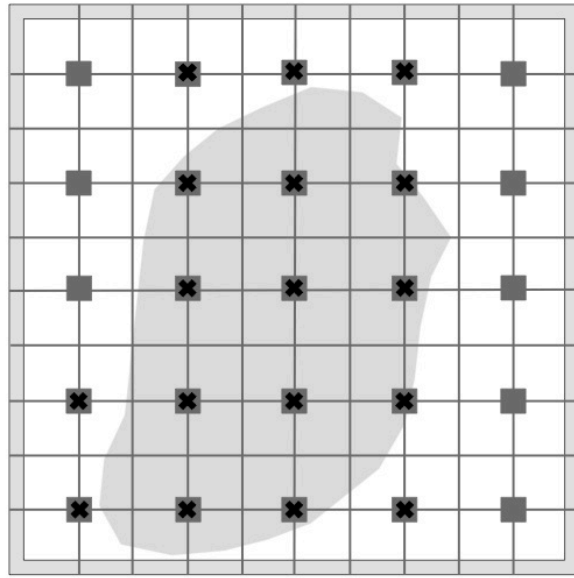


Figure 4 The 2 x 2 m quadrat with 100 (400 cm²) cells placed over a sample seagrass patch in light grey. Dark gray squares indicate possible fertilizer stake locations and black x-shaped crosses indicate locations that would receive a fertilizer stake for this particular sample patch due to *Zostera marina* presence.

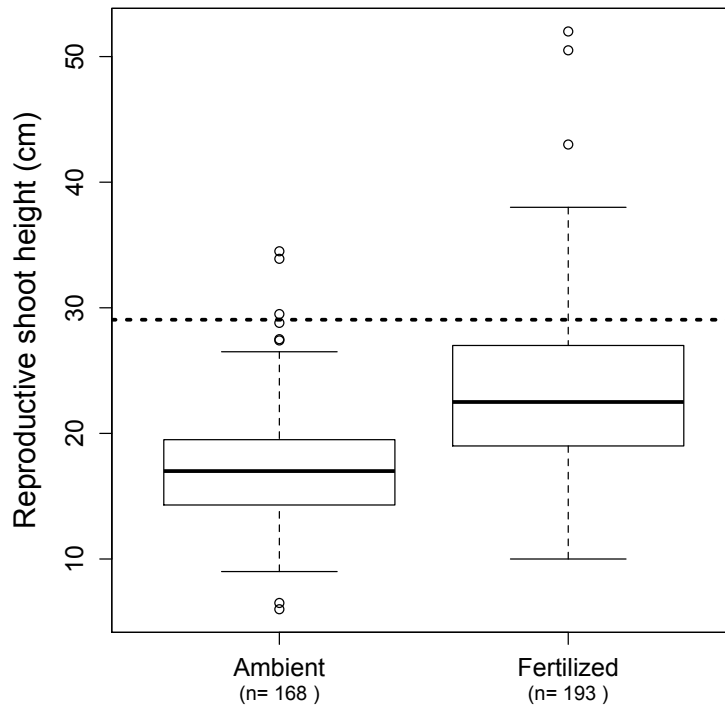


Figure 5 Differences in reproductive shoot height for shoots grown in fertilized and ambient porewater. Boxplots mark median values with a central bar, the first and third quartiles with a box, the ± 1.5 interquartile ranges with Tukey whiskers and data points outside of this ± 1.5 interquartile range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

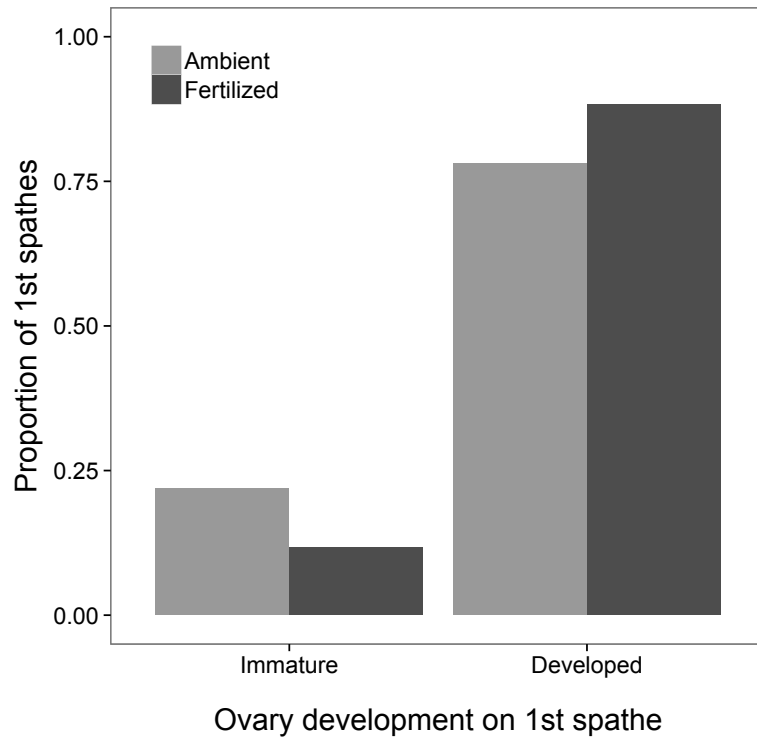


Figure 6 Proportion of developed and immature ovaries within the first spathe of the last rhipidium at the time of sampling for reproductive shoots grown in fertilized and ambient porewater.

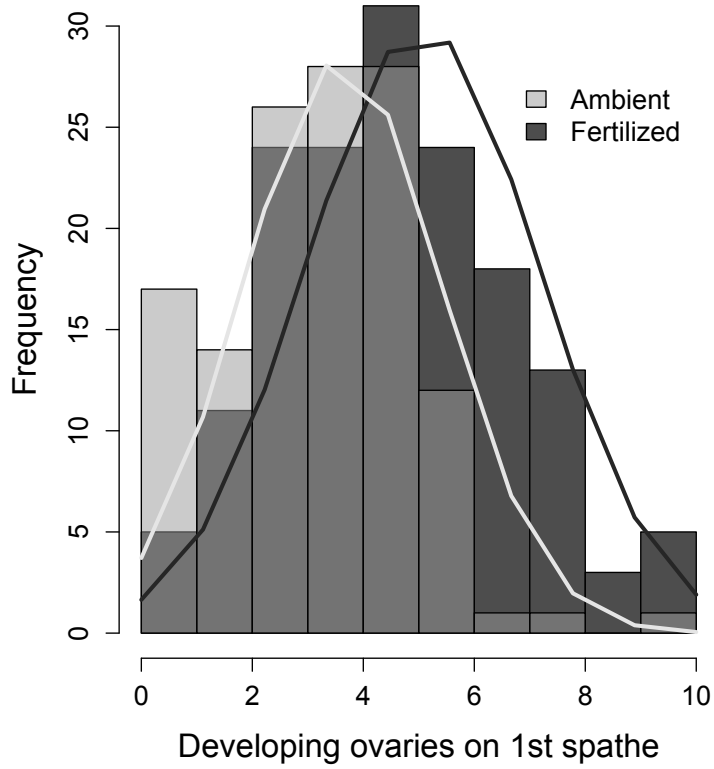


Figure 7 Histogram of the number of ovaries developing into viable seeds on the first spathe of the last rhipidium in reproductive shoots grown in fertilized and ambient porewater.

Chapter 2

The effect of patch size and surrounding seagrass on the production of *Zostera marina* seeds within Shinnecock Bay, New York

INTRODUCTION

Seagrasses are marine angiosperms that expand their areal coverage through sexual and asexual reproduction. Within persistent meadows, clonal growth tends to be more important than sexual reproduction; however, seedling recruitment can be more important during meadow establishment (Jarvis and Moore 2010).

Success of seagrass sexual reproduction is greatly influenced by a flowering shoot's access to pollen. Dioecious seagrasses can experience pollen limitation due to an inadequate number of male flowering shoots in the vicinity or great distance between female and male reproductive shoots (Buckel et al. 2012, Shelton 2008, van Tussenbroek et al 2010, and Reusch 2003). Harwell and Rhode (2007) found a positive relationship between seed production and reproductive shoot density in the monoecious seagrass *Zostera marina*. Ruckelshaus (1996) reported that *Z. marina* pollen drifts only a short distance from the flower in an unencumbered open water column, 15 m away at most. This distance could be even less for pollen released within a structured seagrass canopy. Between 10^3 and 10^4 pollen grains are necessary to fertilize just one *Z. marina* ovule (Ackerman 2002). It is clear that high floral densities are needed to prevent pollen limitation.

Another possible influence on seagrass sexual reproduction is current flow, which controls pollen dispersal. A moderate flow ($> 0 - 0.3 \text{ m s}^{-1}$) is ideal for pollen attachment to flower styles since the negatively buoyant pollen will sink in the absence of current. Conversely, successful attachment rates decrease in extremely turbulent conditions (Ackerman 1997). As current moves through an eelgrass canopy the flow rate decreases, which could alter pollination success within the patch. Fonseca et al. (1982) found that

only 2.07 cm of seagrass for each cm s^{-1} of current velocity was necessary to reach the maximum reduction in current, a value as much as 7.25 cm s^{-1} in only a 50 cm stretch of seagrass. Reusch (2003) found fragmented seagrass meadows to produce fewer seeds per spadix than a continuous meadow, possibly a result of water flow rates being too high and not slowed as much as in the continuous meadow. Increased pollen-style interactions due to decreased current could boost seed productivity in patches greater than a threshold area and possibly create edge effects in areas of fast moving water. Larval settlement shadows have been reported for bivalves associated with seagrass, so greater amounts of larvae settle at the patch edges than in patch centers as less larvae remain in the water column after settling out upon entering the patch (Carroll et al. 2012). It is probable that access to genetically different pollen from outside the patch by flowers within a patch exhibits similar shadows, with high attachment rates to flowers along the edges of the patch that pollen first encounters, leaving less pollen to attach to reproductive shoots in the center of the patch. This would result in a greater seed set from the edge habitat than the core.

The age of seagrass in an area may also influence seed production. In the dioecious seagrass *Thalassia testudinum* the less energetically expensive male flowering shoots were found to be older than female shoots (Durako and Moffler 1985), resulting in the males' central position in a radially, asexually growing seagrass bed. Additionally, the monoecious *Z. marina* produces reproductive shoots only after its second year of growth, at which time it peaks in flower production as areas of older seagrass do not appear to produce as many reproductive shoots (Furman, in review). So as a vegetative patch grows radially from the center, the core area contains older seagrass that may not produce as many flowering shoots. This leads to the prediction that edge habitat will be capable of a more vigorous reproductive effort and greater seed output.

Although pollen limitation is possible, *Z. marina* is capable of self-pollination (Ruckelshaus 1995). Therefore, any ovule could receive pollen from the same plant or a clone (geitonogamy), a close relative (inbreeding), or an unrelated flower (outbreeding). Seeds produced through geitonogamy were found to have lower fitness than outcrossed seeds, indicating inbreeding depression and selection against selfing (Reusch 2001, Ruckelshaus 1995). Further evidence of this selection is observed in the high rates of

outcrossing in *Z. marina* (Reusch 2000). Reusch (2001) found outcrossing rates to be similar between 1 m² monoclonal seagrass patches and genetically diverse meadows. Since these patches consisted of a single clone, if the patch continued to grow vegetatively, larger patches may too consist of a single genet. The large, monoclonal patches will increase the probability of selfing (Hammerli and Reusch 2003), especially for core habitat with less access to pollen from another patch and only clones present in the vicinity to receive pollen from. This geitonogamous fertilization may have a deleterious effect on the offspring from those patches.

The purpose of this study was to test if the seed output of radially growing, monoclonal patches within a patchily distributed *Z. marina* meadow was affected by pollen limitation, age, or inbreeding depression. We selected patches of 2.5, 5, 10, and 20 m² and divided the two largest size classes between edge and center habitat. We assumed that a larger sized patch indicated an older patch since it would take longer for seagrass to grow radially to form the larger patches. We predicted the seagrass in the 5 m² patches would represent the peak in seed density, having time to amp up reproductive shoots production, while not being so large (10 and 20 m²) that pollen access becomes limited to clonal neighboring plants creating an inbreeding depression. We also hypothesized that edge habitat would have a greater density of seeds than core habitat in the 10 and 20 m² patches due to the older age of plants in the center of a radially growing patch and younger age of the edge shoots. In addition, we predicted the core habitat would experience inbreeding depression being surrounded only by clones to supply pollen while edge habitat would have more access to outsourced pollen. Finally, we examined how the amount of seagrass in several buffer radii affected a patch's seed density. We hypothesized that seed density would be positively correlated with the amount of seagrass in the buffer areas due to increased pollen availability from nearby patches.

MATERIALS AND METHODS

Study Site

A 250 x 225 m area of patchy *Zostera marina* meadow in Shinnecock Bay, Long Island, NY was mapped using available aerial imagery from 2001 to 2010 and via

balloon-mounted camera bi-monthly since 2011. These images provide evidence that increases in *Z. marina* (eelgrass) coverage over the past decade have not only reflected clonal growth, but also seed set. In 2010 over 1,000 small, isolated *Z. marina* patches appeared in the spring along the shallow edge of the site in Shinnecock Bay. These patches grew radially and coalesced over the next several years. This initial establishment of isolated patches suggests that the *Z. marina* is highly sexually productive at the site and seed set can be important for the initiation of seagrass meadow formation. Patch growth, amalgamation, and loss were tracked through these images, which established the minimum patch age. In 2011 the site included over 2,200 patches varying in size from 0.01 to 20,000 m². Using ArcGIS these patches were identified, analyzed for areal coverage, and divided between edge (less than 1 m from the seagrass-bare sediment interface) and core habitat (greater than 1 m from the seagrass-bare sediment interface).

2011 Seed Removal

Using a Trimble GeoXT unit with Arcpad 7 software, patches that qualified for one of the four patch area treatments of 2.5, 5, 10, and 20 m² were selected and located. Patches considered to be in the 2.5 and 5 m² treatments included areas ± 0.5 m² while patches ± 1 m² and ± 2 m² were acceptable for the 10 and 20 m² patch treatments, respectively, since there were fewer patches of this size in the study site. Sample size was 11 patches for the 2.5 m² and 5 m² treatments, 10 for 10 m² and 6 for 20 m². In early July, during natural seed dehiscence, every reproductive shoot was removed from each patch and collected in labeled spat bags. Patches 10 (n=6) and 20 m² (n=6) that were large enough to have edge and core habitats had reproductive shoots in the respective zones collected separately. For three weeks the reproductive material was kept in containers with a shallow layer of water and placed under a tarp to enhance humidity. This technique leads to seed dehiscence (Churchill, *personal communication*), after which reproductive shoots were rinsed into a 2 cm sieve sitting atop an 800 μ m sieve. This procedure retained the shoots on the top sieve while seeds were washed through and caught by the bottom sieve. Potentially viable seeds, hereafter referred to as ‘normal’ seeds (Churchill, *personal communication*), which have a firm seed coat filled by the

seed embryo, and nonviable seeds, in which the seed is soft, easily flattened and the embryo does not fill the entire seed coat, were enumerated for each patch. Densities were determined by dividing normal and nonviable patch seed totals by the patch area calculated in ArcGIS.

2012 Seed Removal

Five replicate patches of the same treatment sizes as in 2011 (2.5, 5, 10, and 20 m²) were selected from the study site. During initial patch selection in June, shoot density and canopy height were evaluated. Shoot counts were conducted in three 1/16 m² quadrats haphazardly deployed in the patch. Six quadrats were placed in patches large enough to contain both edge and core area, three haphazardly chosen areas each in the edge and core habitats. The vegetative and reproductive shoots within each quadrat were enumerated separately. Canopy height was evaluated by averaging five vegetative shoot heights haphazardly selected within the patch.

At the end of June, all reproductive shoots were gathered in labeled spat bags. Patches 20 m² (n=6) were large enough to have edge and core areas, from which reproductive shoots were collected separately. None of the 10 m² patches were shaped in such a way as to create core area so all reproductive shoots were collected from edge area. The number of reproductive shoots from each patch was recorded and the spat bags were placed in sea tables with flowing water pumped from Old Fort Pond, off Shinnecock Bay, for one month (Fishman and Orth 1996). This time allowed for seeds to naturally dehisce from the plant into the spat bag. Reproductive shoots were again rinsed into a 2 cm sieve sitting atop an 800 µm sieve to separate other plant material from seeds. Normal and nonviable seeds from each patch were enumerated and densities were determined by dividing the totals by the patch area calculated in ArcGIS.

Statistical Analysis

All statistical analysis was run in R 3.0.2 (R Core Team 2013). A Type I two-way analysis of variance with patch size and year as independent variables was used to establish if there was an interaction effect on normal seed density. Since the interaction effect was not significant ($p > 0.05$), a Type II two-way ANOVA was conducted using

the car package in R (Fox and Weisberg 2011) to determine the effect patch size and year had on the density of normal seeds recovered. A Type II two-way ANOVA with the same independent variables was also used to assess differences in mean nonviable seed densities since there was no significant interaction term for nonviable seeds either. All seed densities were log transformed in the above two-way ANOVAs to meet assumptions of normality and homogeneity of variances. Type II three-way ANOVAs were then used to analyze the influence that the independent variables, location within a patch, year, and patch size (either 10 or 20 m²) had on normal and nonviable seed densities. Normal and nonviable seed densities were square root transformed for the three-way ANOVAs to satisfy assumptions of normality and homogeneity of variances.

A one-way ANOVA was conducted with patch size as the independent variable to determine any effect on 2012 reproductive shoot density, the response variable. Reproductive shoot density was fourth root transformed to satisfy the assumption of normality. A two-tailed t-test was then used to analyze differences in mean reproductive shoot densities between edge and center locations within the 20 m² patches. Finally, a linear regression between reproductive shoot count and normal seed count was examined. Normal seed counts were log transformed to satisfy the assumption of normally distributed residuals.

A one-way ANCOVA was performed on only the 2012 seed data where the dependent variable was normal seed density, the categorical dependent variable was patch size, and the covariate was reproductive shoot density. Another one-way ANCOVA analyzed the effect of patch size on nonviable seed density with the reproductive shoot density covariate. Nonviable seed density was log transformed to satisfy assumptions of homogeneity of variance. One-way ANCOVAs were also conducted on the dependent variables normal and nonviable seed densities with the independent variable location within the patch and covariate of reproductive shoot density.

Buffers with radii of 2, 4, 8, 16, 32, and 64 m were created around the patches with ArcGIS software. Any buffer that crossed over the site boundary was removed from the data analysis. The areal coverage of seagrass in the buffer was computed in ArcGIS and the percent of the buffer area covered by seagrass was calculated. Pearson's

correlations between the independent variables of percent seagrass coverage in the six buffer distances and the dependent variables of reproductive shoot density, normal and nonviable seed densities were calculated in R 3.0.2 (R Core Team 2013). Normal and nonviable seed densities were log transformed and the cube root of reproductive shoot density was used to satisfy the assumption of normality.

Lastly, spatial pattern analysis of the site was conducted with Fragstats 4.2 (McGarigal et al. 2012). The ArcGIS shape file was converted to a raster file with 0.01 m² cell sizes and imported into Fragstats using the 8-neighbor rule for patch definition. Pearson's correlations between reproductive shoot density, normal and nonviable seed densities against patch level shape metrics were calculated individually in R 3.0.2 (R Core Team 2013). The reproductive shoot density was cube root transformed while the normal and nonviable seed densities were log transformed to satisfy the assumption of normality.

RESULTS

The ANOVAs on normal seed density revealed no significant effect of patch size ($F_{3,50} = 1.75$, $p = 0.17$), year ($F_{1,50} = 0.63$, $p = 0.43$), or their interaction ($F_{3,50} = 0.61$, $p = 0.61$; Figure 1). Mean normal seed density for the 2.5, 5, 10 and 20 m² patch treatments was 56 ± 71 , 16 ± 19 , 37 ± 35 , and 21 ± 19 seeds m⁻² (mean \pm SD), respectively. Similarly, there was no significant effect of patch size ($F_{3,48} = 2.21$, $p = 0.10$), year ($F_{1,48} = 3.40$, $p = 0.07$), or the interaction term ($F_{3,48} = 0.57$, $p = 0.64$) on nonviable seed density (Figure 1). Mean nonviable seed density for the 2.5, 5, 10 and 20 m² patch treatments was 52 ± 156 , 7 ± 7 , 31 ± 55 and 7 ± 8 seeds m⁻² (mean \pm SD), respectively.

Patches of the two largest sizes, 10 and 20 m², were separated into edge (seagrass within a 1 m area around the patch) and center habitat (area inside the 1 m area). A type II two-way ANOVA was run on the response variable, density of normal seeds in each location of the patch, after no significant interaction effect between year and location within the patch or patch size and location was found ($F_{1,25} = 0.25$, $p = 0.62$; $F_{1,25} = 2.09$, $p = 0.16$, respectively). There was no significant effect of location on the normal seed density ($F_{1,25} = 0.80$, $p = 0.38$; Figure 2). There was also no significant interaction effect of year and location or patch size and location ($F_{1,25} = 0.03$, $p = 0.87$; $F_{1,25} = 0.87$, $p =$

0.36, respectively) and then no significant effect of location on nonviable seed density ($F_{1,24} = 0.27$, $p = 0.61$; Figure 2).

The one-way ANOVA on the 2012 patches' reproductive shoot densities revealed no significant difference among patch size treatments ($F_{3,16} = 0.68$, $p = 0.58$). Mean reproductive shoot density in the 2.5, 5, 10 and 20 m² patch treatments was 36 ± 65 , 4 ± 2 , 21 ± 16 and 8 ± 8 shoots m⁻² (mean \pm SD), respectively. In addition, reproductive shoot density was not significantly different between edge and center locations of the 20 m² size patches ($t_3 = 0.87$, $p = 0.45$). Reproductive shoot count explained a significant amount of variability in the number of normal and nonviable seeds collected ($R^2 = 0.53$, $F_{1,18} = 20.57$, $p < 0.01$; $R^2 = 0.64$, $F_{1,18} = 31.48$, $p < 0.01$, respectively).

Patch size was still found to have no significant effect on 2012 normal seed density ($F(3,15) = 2.345$, $p = 0.11$) or nonviable seed density ($F(3,15) = 2.618$, $p = 0.09$) after correcting for the reproductive shoot density covariate. Location within the patch did have a significant effect on normal seed density after correcting for reproductive shoot density ($F(1,6) = 23.785$, $p < 0.01$) but this effect was not significant for nonviable seed density ($F(1,6) = 4.277$, $p = 0.08$). Normal seed density was greater in edge (17.3 ± 15.0 seeds m⁻²; mean \pm SD) than core habitat (4.3 ± 4.7 seeds m⁻²; Figure 3).

The density of reproductive shoots was not significantly correlated with amount of seagrass coverage in any of the buffer zones ($p > 0.05$). Nonviable seed density was, however, significantly negatively correlated with percent seagrass coverage in buffers with 2 and 4 m radii ($r_{50} = -0.37$, $p < 0.01$; $r_{50} = -0.38$, $p < 0.01$, respectively; Figure 4). The same effects were found for normal seeds ($r_{52} = -0.39$, $p < 0.01$; $r_{52} = -0.39$, $p < 0.01$, respectively; Figure 4). The seagrass coverage in the larger buffer areas did not correlate significantly with normal or nonviable seed density. In addition, none of the patch-level metrics in the spatial pattern analysis were significantly correlated with reproductive shoot density, normal or nonviable seed densities.

DISCUSSION

Previous reports of pollen limitation (Buckel et al. 2012, Shelton 2008, van Tussenbroek et al. 2010, and Reusch 2003) and radial growth of vegetative, monoclonal seagrass patches (Reusch 2001) led to the conceptual model that edge habitat would

support greater seed production than core habitat. The seagrass developing along the edge was younger, therefore capable of greater reproductive output (Furman, *in review*), and it had greater access to genetically different pollen from outside the patch since the patch edge is the first barrier the pollen encountered as current moved through a patch. This outsourced pollen would also most likely come from genetically different ramets, which would reduce inbreeding depression. In addition, age influence and inbreeding depression supported the prediction that patch size would influence seed production. 5 m² patches would have optimal seed production because most seagrass in them was two years old, and 10 and 20 m² patches would have a greater proportion of older material, leading to a decline in reproductive output, in addition to inbreeding depression.

This model was often not supported by the data reported in this study. Pollen was not a limiting factor at this *Zostera marina* site in Shinnecock Bay. Since pooled 2011 and 2012 data indicated reproductive shoot density and collected seed densities were not statistically different between edge and center habitat (Figure 2), the reproductive shoots must have been exposed to comparable amounts of pollen and had a similar reproductive success rate. The lack of a pollen access shadow at the center of the patch could be a result of pollen from outside the patch floating over the seagrass at higher tides and coming into contact with at least the top rhizodium of more central reproductive shoots. Unlike larval settlement shadows, the density of pollen reaching the center of the patch has not been recorded, only that enough pollen entered to fertilize the center flowers. Additionally, if pollen were limiting we would have expected a positive correlation between the percent of seagrass coverage in the buffer areas surrounding the patches and the amount of seeds the patches produced. Instead, a negative correlation between the amount of surrounding seagrass and normal seeds was observed in the 2 and 4 m radius buffers (Figure 4). The cause of this relationship remains unknown.

There was a difference in normal seed density between edge and center habitats in 2012 with a greater density found at the edge (Figure 3). This supported the conceptual model that radial vegetative growth explained patch expansion at the site. Large patches were comprised of older seagrass at their cores and younger, more sexually productive seagrass at the edge. If age does play such an important role in seed production and

therefore meadow formation, then *Zostera marina* transplants at restoration sites would be benefited by selecting younger edge shoots to transplant.

The only factor capable of predicting seed yield was the number of reproductive shoots, as previously noted by Harwell and Rhode (2007). Patch size, location within the patch, and amount of surrounding seagrass all had no effect on the amount of seed production until the confounding factor of reproductive shoot density was accounted for. Once this occurred 2012 data had a much greater normal seed density at patch edges than centers. Most likely this is caused by differences in age of the plant material and not pollen limitation since there was a negative effect of amount of nearby seagrass on seed production.

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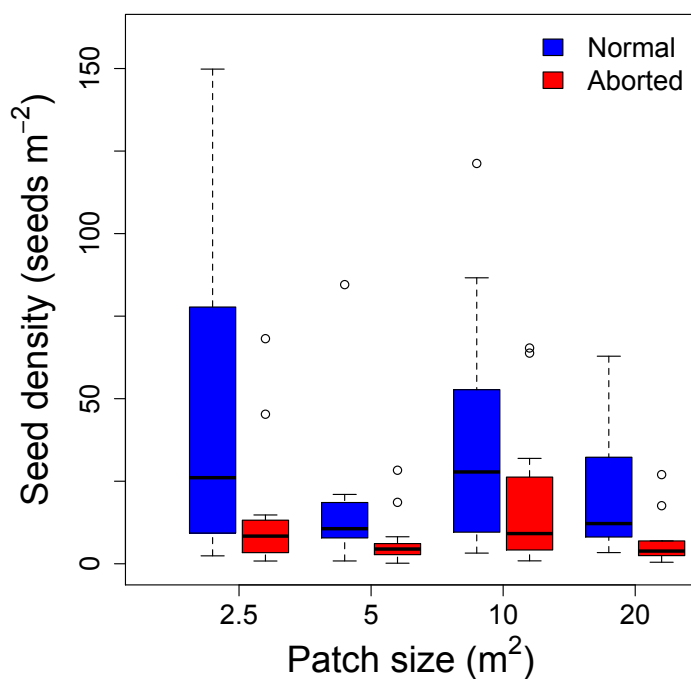


Figure 1 Normal (blue boxplots) and aborted (red boxplots) seed densities at the patch treatment sizes 2.5, 5, 10, and 20 m² in 2011 and 2012. Boxplots mark median values with a central bar, the first and third quartiles with a box, the ± 1.5 interquartile ranges with Tukey whiskers and data points outside of this ± 1.5 interquartile range with a circle.

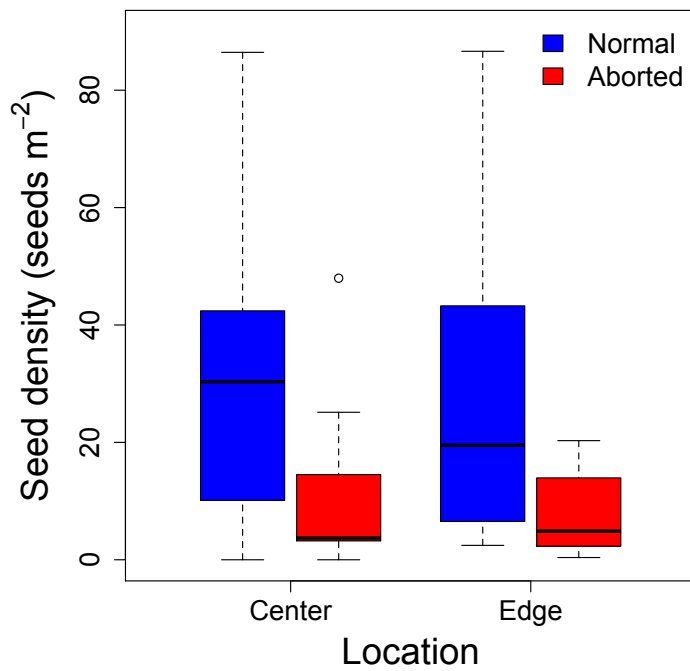


Figure 2 Normal (blue boxplots) and aborted (red boxplots) seed densities at the patch edge and center habitats within the 10 and 20 m² patches in 2011 and 2012. Boxplots mark median values with a central bar, the first and third quartiles with a box, the ± 1.5 interquartile ranges with Tukey whiskers and data points outside of this ± 1.5 interquartile range with a circle.

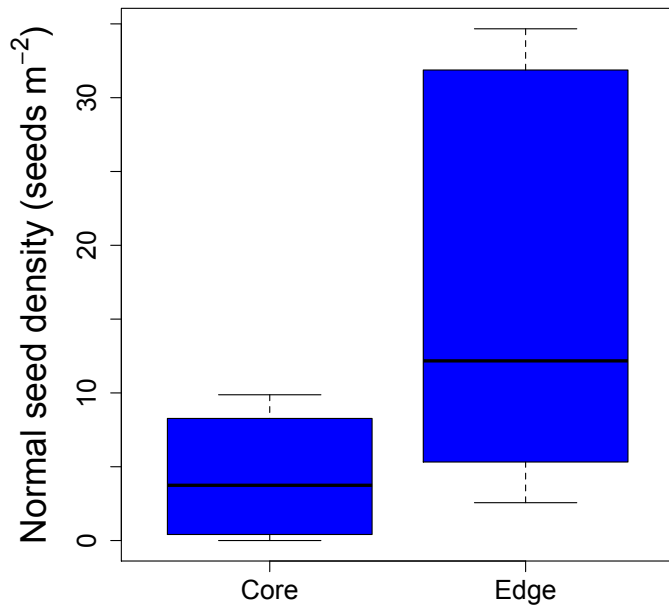


Figure 3 Normal seed density at the edge and core habitat of 20 m² patches in 2012. Boxplots mark median values with a central bar, the first and third quartiles with a box, the ± 1.5 interquartile ranges with Tukey whiskers.

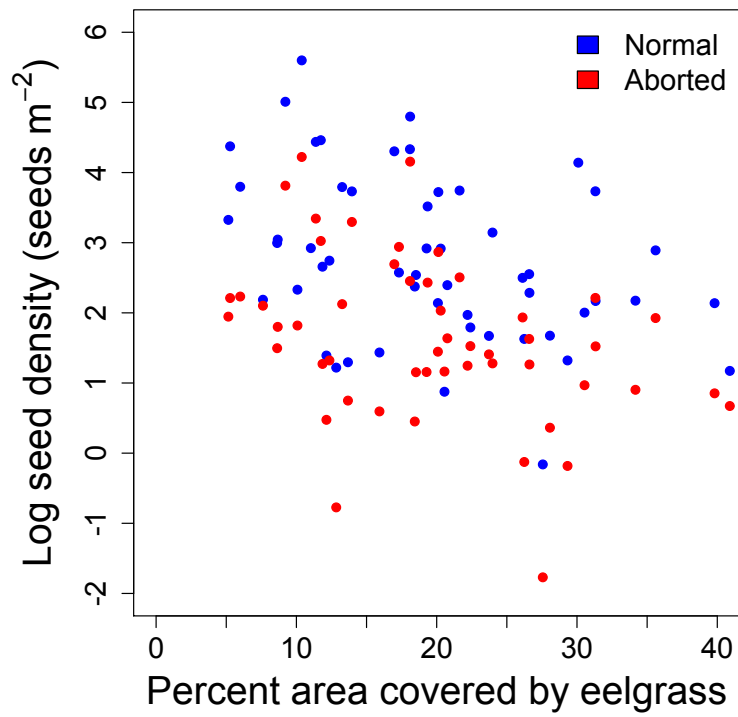


Figure 4 The log of normal (blue points) and aborted (red points) total seed densities from each selected patch within the landscape plotted against the percent of surrounding area 4 m from each patch covered by eelgrass.

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