

Floral neighbourhoods in the sea: how floral density, opportunity for outcrossing and population fragmentation affect seed set in *Zostera marina*

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Summary

1 Almost nothing is known about how the floral neighbourhood affects reproductive output in plants with subaqueous pollination (hydrophily), such as seagrasses, an ecologically important group of some 60 marine angiosperms.

2 I studied how floral density, genetic diversity and population fragmentation affect seed set in *Zostera marina* (eelgrass), a functionally hermaphrodite seagrass with extensive clonal propagation.

3 In a field experiment in the south-western Baltic Sea, I increased or decreased the density of flowering shoots in 6 × 6 m plots. Early seed set was a positive, saturation-type function of density suggesting pollen limitation below approximately 5 flowering shoots m⁻².

4 Using molecular markers, I determined local genotypic diversity and outcrossing rates as indicators of outcrossing opportunities. I found only non-significant effects of these variables on early seed set, suggesting that density of the floral neighbourhood is more important than genetic composition.

5 Early seed set was 22% lower in isolated vegetation patches compared to continuous eelgrass meadow (> 50 m²).

6 Given the spatial scale of the observed pollen limitation, and low natural densities of flowering shoots in the field, pollen limitation may be widespread in *Z. marina* and, possibly, other plant species with subaqueous pollination.

Key-words: clonal plant, fertilization, hydrophily, pollination, seagrass

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Introduction

Because plants cannot move, successful reproduction is contingent upon the movement of pollen to female sexual organs (Faegri & van der Pijl 1979; Barrett 2002). Hence, the probability that an individual will reproduce is profoundly affected by its local floral neighbourhood. For example, seed set is correlated with floral density (Allison 1990; Kunin 1993; Aizen 1997) and fragmentation of the population (Cunningham 2000; Knapp *et al.* 2001). The genetic relatedness of conspecific neighbours also influences seed set in complex ways. Pollination by genetically related plants can reduce the number and quality of progeny either by early inbreeding depression (van Treuren *et al.* 1994) or as a result of interference by self-pollen (Ramsey &

Vaughton 2000). The genetic relatedness of plants in a floral neighbourhood may be particularly important in clonal plant species. In clonal plants, individuals are likely to be surrounded by flowering shoots belong to the same genet (i.e. clone), and thus prone to selfing (geitonogamy, Handel 1985) in species with hermaphrodite flowers and those that are monoecious.

Among the diverse mating systems known (Barrett 2002), true hydrophilous (i.e. subaqueous) pollination is probably the most divergent adaptation in higher plant sexual reproduction (Philbrick & Les 1996). Such pollination, however, has largely been neglected in studies on plant reproductive success (but see Williams 1995). Whilst, on land, floral adaptations to attract animal pollinators and assure fertilization abound (Darwin 1861), no animal mediated transfer of pollen has been reported in the sea. Rather, the spores and pollen of plants are transported passively through water movement (Denny & Shibata 1989; Serrao *et al.* 1996; Ackerman 1997; Engel *et al.* 1999), as is the case in

marine angiosperms, or seagrasses. Because abiotic pollination involves random pollen release and non-directional transport towards conspecific individuals, it is predicted that fertilization efficiency will be lower than with animal mediated pollen transfer (Faegri & van der Pijl 1979; Cox 1983; Barrett *et al.* 1993).

Among the seagrasses, only *Enhalus acoroides* does not exhibit true subaqueous pollination by filamentous pollen and specialized female organs (Les 1988; Ackerman 1997). Theoretical analyses (Cox 1983) suggest that successful subaqueous mating is limited to a range of metres due to pollen showing a markedly leptokurtic dispersal function and its rapid dilution in the three-dimensional medium. Some seagrass species also display relatively low pollen : ovule ratios compared to wind-pollinated terrestrial plants (Ackerman 2002). Reproductive output may thus often be limited by access to pollen, particularly if flowering densities are low (Antonovics & Levin 1980). Such pollen limitation may explain low seed set and seedling recruitment rates in many seagrass populations (Les 1988; Barrett *et al.* 1993).

Eelgrass (*Zostera marina* L.) is a clonal marine angiosperm which forms extensive meadows along sedimentary coastlines throughout the northern hemisphere. It is self-compatible and extensive clonal propagation (Reusch *et al.* 2000) markedly reduces the opportunities to outcross for flowering ramets growing in a neighbourhood with low clonal diversity. The resulting geitonogamy incurs high fitness costs for selfed offspring (Reusch 2001), but it is not known whether it also translates to lower seed set.

I studied whether seed set in *Zostera marina* is affected by two attributes of the floral neighbourhood: density and genetic composition. In a field experiment, I tested whether early seed set varies with pollen availability by decreasing or increasing the density of flowering shoots in large (6 × 6 m) plots relative to ambient levels. I then used molecular markers to determine local genotypic diversity and outcrossing rates in the immediate neighbourhood of target flowering shoots to assess whether early seed set is positively correlated with opportunities for outcrossing. I also compared the proportion of flowers that developed seeds in continuous eelgrass meadows (area > 50 m²) with plants in isolated patches to test whether habitat fragmentation lowers the reproductive output of *Z. marina*.

Materials and methods

STUDY SPECIES AND STUDY SITES

Eelgrass (*Zostera marina* L.) grows from subarctic to subtropical latitudes of the northern hemisphere (Den Hartog 1970). Perennial eelgrass populations reproduce sexually and propagate clonally. A number of morphological adaptations, such as filiform pollen and erect bifurcated stigmata, increase chances of successful subaqueous pollination, and thus, sexual

reproduction (Ackerman 1997, 2002). Flowering shoots carry a number of inflorescences (spadices) each with up to 12 alternating male and female flowers (De Cock 1980). Within each spadix, male flowers only open after female flowers have lost their styles. Although male and female function are separated within spadices (= monoecy), the species is a functional hermaphrodite because protogyny is not synchronized across an entire flowering shoot (De Cock 1980), let alone across ramets of the same genet. As a result, *Z. marina* possesses a mixed mating system in which geitonogamous selfing results in varying outcrossing rates depending on the extent of clonal growth (Reusch 2001).

All observations and experiments were conducted using SCUBA diving equipment at two shallow sites (1.5–3 m water depth) in the atidal and brackish Baltic Sea (Falkenstein, 54°24' N, 10°12' E, Maasholm, 54°41' N, 10°00' E). Further details on study sites can be found in Reusch (2001).

FIELD EXPERIMENT: MANIPULATION OF FLOWERING SHOOT DENSITY

At Maasholm, I changed the density of flowering shoots relative to ambient levels to assess the potential for pollen limitation on seed set and the extent of such limitation. Between 25 and 28 May 2002, I established eight 6 × 6 m plots in a large, uninterrupted eelgrass meadow. Before manipulation, these areas had 6.1 ± 0.74 flowering shoots m⁻² ($n = 12$ quadrats of 1 × 1 m). Two plots were assigned randomly to each of the four density levels which followed a logarithmic scale (i.e. 3⁰ = 1, 3¹ = 3, 3² = 9, and 3³ = 27 flowering shoots m⁻²). For the addition treatments, flowering shoots were excavated nearby in the meadow (= 20 m away), immediately transplanted to the target areas and fixed in the sediment with u-shaped wire staples. One vegetative shoot was removed for every flowering shoot added. Time constraints prevented the replacement of flowering shoots with vegetative shoots in the treatments subjected to decreases in density (1 and 3 flowering shoots m⁻²). However, the total number of shoots (vegetative + flowering) in these treatments was reduced by no more than 4%. One experimental unit of the 9 flowering shoots m⁻²-treatment could not be completed for logistical reasons.

Fourteen days after manipulation, I counted all flowering shoots within three or four central 1 × 1 m areas of each experimental plot to quantify actual densities of flowering shoots. Some plants in the addition treatments (9 and 27 flowering shoots m⁻²) had been lost, while in the low density plots (1 and 3 flowering shoots m⁻²) some flowering shoots had been overlooked during manipulation. I therefore included the actual number of flowering shoots as a covariate rather than as categorical variable when analysing the experiment.

I terminated the experiment on 20 June 2002 (24 days post-manipulation) before seeds had ripened

completely. I sampled 12 flowering shoots from the central 2×2 m of the each experimental plot (four shoots in the 1-flowering shoots m^{-2} treatment). In all spadices containing ripening seeds (stage 4, De Cock 1980) ovules were categorized as developing seeds or non-growing. The latter category included both, non-fertilized ovules and ovules that aborted between fertilization and sampling. The proportion of ovules that were developing seeds was defined as 'early seed set'. On average, 35 ovules were examined per flowering shoot. I observed seed herbivory in 3% of the spadices and these were omitted from further analysis. Proportions of growing ovules in a spadix were averaged for each flowering shoot, and over all shoots growing in the central 2×2 m of each experimental plot to give one observation per plot.

I used least square regression to assess whether early seed set varied with flowering shoot density. Because treatment levels were on a logarithmic scale, the independent variable 'density of flowering shoots' was rescaled by a log-transformation.

LOCAL GENOTYPE DIVERSITY, OUTCROSSING RATES AND SEED SET

Sampling design

A sampling program was conducted in June 1999 to investigate the role of population fragmentation, and of genetic composition of the floral neighbourhood, on seed set. Two categories of stand architecture (isolated patch or continuous meadow) were studied and two variables (the local genotypic diversity and the outcrossing rate measured in a sample of ovules in target shoots) were measured in order to characterize the local, small-scale composition of a plant's neighbourhood. Both variables are indicative of the opportunities for outcrossing in the vicinity of focal shoots.

At both Maasholm and Falkenstein, I randomly selected five areas (hereafter 'plots') of *c.* 1-m diameter each surrounding a cluster of three target shoots within continuous meadow (vegetation area $> 50 \text{ m}^2$). At both sites, I also selected five adjacent isolated patches of $0.3\text{--}1 \text{ m}^2$ in area, situated ≥ 4 m away from any other vegetation. In each plot or patch, the local genotype diversity was determined in the three focal shoots plus an additional seven shoots (i.e. 10 shoots were genotyped per plot). I also measured the outcrossing rates of three to five fertilized ovules in each of the three focal shoots using genetic markers (see below) based on samples collected between 28 and 30 June 1999. In the laboratory, I counted ovules in ripening spadices (identified as stage 4 according to De Cock 1980) and categorized them as above.

Genetic analyses

The local genotypic diversity and the outcrossing rates of focal shoots were determined based on the

polymorphism displayed by eight microsatellite loci (representing 54 alleles in the studied populations) developed specifically for *Zostera marina* as described by Reusch *et al.* (2000) and Reusch (2001). In brief, crude DNA was extracted from leaf tissue or from embryos and subjected to polymerase chain reaction with fluorescently labelled primers. Microsatellite alleles were scored after electrophoresis on an ABI 377 genetic analyser. Ramets with identical multilocus genotypes were assigned as clone members based on criteria given in Parks & Werth (1993). For developing ovules, comparison of the multilocus genotype with that of their maternal shoot allowed the assignment of the pollen source for individual embryos as selfed or outcrossed (Shaw *et al.* 1981; Reusch 2001). Error likelihoods for incorrect clone assignment (Parks & Werth 1993) and for falsely detected selfing (Shaw *et al.* 1981) were small (all $P < 0.01$). Genotypic diversity was calculated as the ratio of the number of genotypes (g) to the number of ramets sampled (n).

Statistical analyses

The effects of local genotypic diversity and of outcrossing rates (both indicative for the genetic relatedness of the floral neighbourhood) on seed set were tested in a least-square, general linear model (GLM). As the response variable, I used the proportion of ovules developing seeds per spadix averaged over all three focal shoots situated in one plot or patch. This analysis also contrasted the two study sites (Maasholm or Falkenstein). 'Site' was included as a fixed factor, because my intention was to make statistical inference only for these two specific sites. The GLM with the best fit was determined based on the total variance it explained, i.e. the highest adjusted r^2 .

Seed set in isolated patches vs. continuous meadow

I also compared early seed set between isolated patches and continuous meadow to assess effects of population fragmentation on reproductive output. As the response variable, I took the proportion of ovules that develop seeds averaged over single flowering shoots (mean of 25 ovules per flowering shoot). The ANOVA model considered 'site' (Maasholm or Falkenstein), 'meadow type' (patch or continuous meadow), and 'plot' nested within 'site' and 'meadow type'.

Results

MANIPULATION OF FLOWERING SHOOT DENSITY

As intended, at the time of sampling, none of the seeds was completely ripe. The average proportion of ovules that were developing seeds varied positively with the local density of flowering shoots in the experimental areas (Fig. 1). A linear regression was fitted after

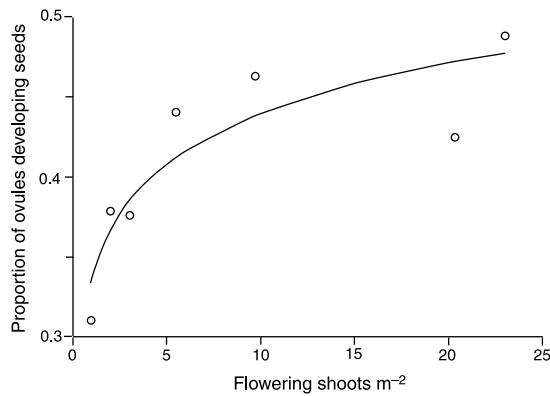


Fig. 1 Early seed set of *Zostera marina* (eelgrass) at Maasholm: proportion of ovules that were developing seeds (averaged over all shoots in one experimental unit) as a function of the experimentally manipulated flowering shoot density at Maasholm.

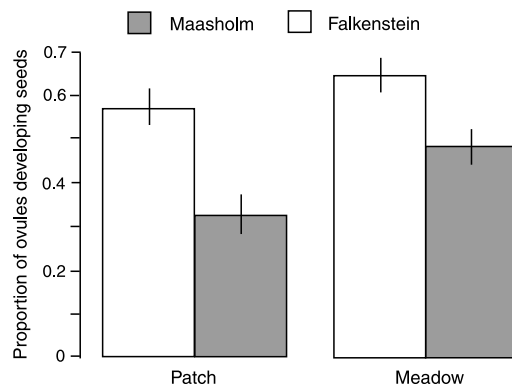


Fig. 2 Comparison of early seed set in *Zostera marina* (average over one focal flowering shoot) between isolated patches (> 4 m away from other vegetation) and eelgrass meadow (vegetated area > 50 m²) at two sites in the southwestern Baltic Sea.

transforming flowering shoot density to logarithmic scale. The equation was $y = 0.333 + 0.106 (\log) x$ and explained 79% of the variance ($F_{1,5} = 19.7$, $P = 0.007$). At the lower density, early seed set was *c.* 0.3. With increasing density, it levelled off above approximately 10 flowering shoots m⁻². The statistical model was also significant if I used the intended experimental densities (1, 3, 9 and 27 flowering shoots m⁻²) as categorical variables instead of the actual densities measured 14 days after manipulation ($F_{1,5} = 8.24$, $P = 0.035$).

Experimental transplantation did not affect early seed set of flowering shoots. The mean proportion (\pm SE) of ovules that were developing seeds in a sample of transplanted vs. non-transplanted flowering shoots was 0.49 ± 0.02 vs. 0.50 ± 0.03 (ANOVA, $F_{1,23} = 0.27$, $P = 0.6$).

LOCAL GENOTYPE DIVERSITY, OUTCROSSING RATES AND SEED SET

The general linear model with the highest adjusted r^2 (0.44) incorporated 'genotypic diversity', 'outcrossing rate' and 'site', but none of the possible interactions. I

Table 1 Early seed set in *Zostera marina* (eelgrass) at Maasholm and Falkenstein: nested ANOVA on the effects of site (Falkenstein and Maasholm) and meadow type (isolated patch or meadow) on the proportion of ovules developing seeds (averaged over focal shoots). The nested factor 'plot' refers to a group of three focal flowering shoots surrounded by an area of *c.* 1 m², or to a single isolated patch

Source of variation	d.f.	MS	F	P
Site	1	0.5118	10.5625	0.0044
Meadow type	1	0.2275	4.6953	0.0439
Site \times meadow type	1	0.0002	0.0033	0.9549
Plot (meadow type, site)	18	0.0485	2.1792	0.0186
Residual	43	0.0222		

found a positive but non-significant relationship between local genotypic diversity (g/n), $n = 10$ sampled ramets, and early seed set (data not shown, standardized partial $r = 0.18$, $F_{1,15} = 0.8$, $P_{\text{genotypic diversity}} = 0.3$). The mean outcrossing rate had no effect on seed set (partial $r = -0.02$, $F_{1,15} = 0.2$, $P_{\text{outcrossing}} = 0.8$). At Falkenstein, average seed set was 70% higher than at Maasholm ($F_{1,15} = 12.5$, $P_{\text{site}} = 0.003$).

POPULATION FRAGMENTATION AND SEED SET

Population fragmentation had an effect on early seed set. I found a 22% lower seed set in isolated eelgrass patches vs. continuous meadow averaged over both sites (Fig. 2, Table 1). Seed set also varied significantly among 'plots', indicating high spatial variation in fertilization success and, possibly, in pollen availability.

Discussion

Effects of density and genetic composition of the floral neighbourhood on seed set are well established among terrestrial plants (e.g. Allison 1990; Kunin 1993; van Treuren *et al.* 1994; Aizen 1997; Cunningham 2000; Knapp *et al.* 2001). The intention of this study was to extend such analyses to seagrasses, a group of ecologically important flowering plants exhibiting marine subaqueous pollination. Early seed set in a marine clonal angiosperm, *Zostera marina*, depended critically on the density of the floral neighbourhood, while effects of genetic composition were less important. Seed set decreased markedly when there were less than five flowering shoots m⁻², while the effects leveled off at higher densities, probably reflecting saturation of stigmas. Although I did not quantify pollen densities in the water column, the most parsimonious explanation is that limiting amounts of available pollen allow fewer female flowers to be fertilized in the low density treatments. The effects of flowering shoot density on early seed set were non-linear unless the independent variable (flowering shoot density) was transformed to a logarithmic scale.

These results are consistent with the fine-scale genetic structure at the experimental site (Maasholm).

In an autocorrelation analysis, Hämmerli & Reusch (2003b) found positive kinship coefficients after excluding identical ramets of the same genet at distances of 1–5 m, suggesting limited dispersal of either seeds or pollen. Seeds of eelgrass sink once released from the spadix and drop close to the parent plant (Orth *et al.* 1994). Results here suggest that limited pollen dispersal distances also contribute to the fine-scale genetic substructuring in *Z. marina* populations.

The genetic composition of the floral neighbourhood (used as a proxy for outcrossing opportunities) was less important than density. The weak positive correlation between local genotypic diversity and early seed set was not statistically significant, nor was there any relationship between local outcrossing rates and seed set. Effects of pollen source and compatibility on seed set were, however, found by Hämmerli & Reusch (2003a) in a laboratory experiment with plants from the Falkenstein site. In this experiment, plants exposed to a mix of self and foreign (outcrossing) pollen showed 14% higher seed set at the end of the seed ripening period than those receiving only pollen from the same genotype. Because this experiment dealt with late seed set, and was conducted in the laboratory under much tighter control of pollination conditions than in the field, the size of this effect is probably a maximum estimate. Although the effects of pollen compatibility on early seed set appear weak, *Z. marina* does show marked effects of inbreeding depression in later stages of its life history (Reusch 2001; Hämmerli & Reusch 2003c).

Interestingly, maximal seed set at Maasholm did not exceed 0.5, and was approximately similar in the years of field observation (1999) and experiment (2002), suggesting that some fundamental limitation of seed set is present at this site. Seed set at Falkenstein was approximately 50% higher in both continuous meadow and isolated patches. Further studies are warranted which identify any underlying differences in resource availability (Charlesworth 1989; Burd 1994) or hydrodynamic regime (Worcester 1995; Ackerman 2002).

SCALE OF POLLEN LIMITATION

The spatial scale at which pollen density effects were observable was surprisingly small as focal plants were no more than 3 m away from unmanipulated meadow. In the only study determining subaqueous pollen dispersal directly, eelgrass pollen was transported over substantially larger distances (Ruckelshaus 1996). While 44% of released pollen could be recaptured 3 m away, a drop in capture was only recorded beyond 10 m from the pollen source. The methods used by Ruckelshaus (1996) may explain the observed discrepancy with the data presented here. Pollen collectors were situated several metres above the vegetation in the freestream water body and this is not representative of hydrodynamic conditions within a vegetation canopy (see for example Worcester 1995).

EFFECTS OF POPULATION FRAGMENTATION ON SEED SET

Fewer seeds developed per spadix in isolated patches than in continuous eelgrass meadow. Most probably, pollen concentrations are low within isolated patches (total vegetation cover only 5–10%) compared to uninterrupted vegetation. These results mirror recent observations in animal pollinated (Cunningham 2000) and wind-pollinated (Knapp *et al.* 2001) trees which show decreased seed set in isolated forest fragments. Population fragmentation may thus lower the reproductive output of eelgrass, a finding which is relevant for seagrass conservation.

POLLEN LIMITATION IN SEAGRASS BEDS

The densities of flowering shoots that were associated with lower seed set in the experiment are within the natural range observed at the experimental site (Maasholm) and elsewhere. One year before this study, mean flowering shoot densities of only 2–4 m⁻² were observed in areas which partly overlapped my experimental plots (Hämmerli 2002). Even lower abundances are reported from *Zostera marina* beds in the central Baltic (Boström 1995), and from the North-east Pacific subtidal (< 1 flowering shoot m⁻², Ruckelshaus 1994). Sexual reproduction seems to be rare in some of these populations which are characterized by extremely large (and possibly old) clones (Reusch *et al.* 1999). The limitation of mating opportunities may thus be a proximate ecological process triggering the loss of sexual reproduction in marginal populations of clonal plants (Dorken & Eckert 2001; Eckert 2002), and specifically, in eelgrass (Reusch *et al.* 1999). In conclusion, low densities of flowering ramets and meadow fragmentation may result in widespread limitation of seed set and reproductive output in seagrasses. More experimental studies of marine and limnic species are clearly warranted to complement the wealth of data on terrestrial plant reproductive ecology, and to evaluate whether reproductive biology needs to be considered in seagrass conservation.

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