

Should we sync? Seascape-level genetic and ecological factors determine seagrass flowering patterns

Marlene Jahnke^{1#}, Jordi F. Pagès^{2†}, Teresa Alcoverro^{2,3}, Paul S. Lavery^{2,4}, Kathryn M. McMahon⁴ and Gabriele Procaccini^{1*}

¹Department of Integrative Marine Ecology, Stazione Zoologica Anton Dohrn, Villa Comunale, 80121 Napoli, Italy;

²Centre d'Estudis Avançats de Blanes (CEAB-CSIC), Accés a la cala Sant Francesc, 14, 17300 Blanes, Spain;

³Nature Conservation Foundation, 3076/5, 4th Cross, Gokulam Park, 570 002 Mysore, India; and ⁴Edith Cowan University, School of Natural Sciences, 270 Joondalup Drive, Joondalup WA 6027, Australia

Summary

1. Spatial and temporal heterogeneity in flowering occur in many plant species with abiotic pollination and may confer fitness advantages through mechanisms such as predator satiation or pollination efficiency. Environmental factors such as light quality or quantity and temperature play an important role in inducing synchronization on wide geographic scales. On a smaller geographic scale, external factors such as resource availability and herbivory are theorized to trigger flowering, while genetic factors may also play an important role.

2. In this study, we assessed the importance of ecological and genetic factors in shaping seascape-level spatial heterogeneity in flowering of the seagrass *Posidonia oceanica*. By investigating spatially close sites (<20 km) with similar seascape configurations and depth, we assume that major environmental drivers (temperature and light) were equivalent.

3. We assessed four ecological factors (productivity, leaf nitrogen and carbon content and herbivory) and three genetic factors (heterozygosity, relatedness and clonality) to assess three hypotheses for synchronized flowering in *P. oceanica*: (i) clone synchronization (internal clock hypothesis), (ii) variation in nutrient availability, potentially caused by spatial heterogeneity in herbivory rates or nutrient translocation *via* clonal integration (resource budget hypothesis) or (iii) kin selection and sibling synchronization.

4. Internal relatedness and heterozygosity had a significant positive effect on the abundance of flowers. Moreover, productivity and genotypic richness (clonality) were negatively associated with flower density, although at a lower level of significance. In addition, we found that clones were almost exclusively shared among mass-flowering patches and patches without mass-flowering, respectively.

5. *Synthesis.* The results shed new light on seagrass flowering patterns and on the mechanisms of flower synchronization at the patch level within a wider spatial scale. We found support for the kin selection hypothesis and indirect evidence for the resource budget hypothesis. Thus, a combination of mainly genetic but also ecological factors causes the observed heterogeneous flowering patterns in *Posidonia oceanica* seascapes. In addition, we found a strong positive relationship between the number of flowers and heterozygosity, adding evidence to the controversial association between heterozygosity and fitness when a limited number of loci are used. To our knowledge, this study is the first to link both ecological and genetic factors with flower abundance in a species with a presumed masting strategy.

Key-words: aquatic plant ecology, genetic diversity, herbivory, heterozygosity, internal clock, kin selection, *Posidonia oceanica*, primary production, relatedness, resource budget hypothesis

*Correspondence author. E-mail: gpro@szn.it

#These authors contributed equally.

† Present address: School of Ocean Sciences, Bangor University, Menai Bridge, Anglesey, LL59 5AB, UK.

Introduction

For many flowering plants with abiotic pollination, the likelihood of successful fertilization depends upon the synchrony of sexual activity and the proximity of compatible mates (Knapp, Goedde & Rice 2001; van Tussenbroek *et al.* 2010). One strategy to address these limitations is mast seeding, which involves strong fluctuations of reproductive output by individual plants as well as synchronization among individuals (Crone, Miller & Sala 2009). This strategy, although not very common, has been described mainly in terrestrial plants, ranging from bamboo to *Dypterocarpaceae* (Janzen 1974, 1976). Some marine plants also present similar synchronized reproductive fluctuations (e.g. Inglis & Smith 1998), as well as abiotic pollination, suggesting they may display a masting reproductive strategy. Mast seeding has important disadvantages, such as the decrease in frequency of reproduction or the likely higher density-dependent seedling mortality in mast years (Hett 1971; Waller 1979). However, evolutionarily, synchronization of flowering and seed production may confer fitness advantages through mechanisms such as predator satiation or pollination efficiency to avoid pollen limitation (Kelly 1994; Kelly & Sork 2002). In some species, predators are satiated during mast years, with minor impact on adult individuals, while predator populations are kept in check during non-mast years. Moreover, pollination efficiency is high in mast years, but pollen becomes limiting in non-mast years. These observations explain why synchronization may increase individual fitness, but do not explain the actual mechanisms of synchronization (Crone, Miller & Sala 2009). Our understanding of population dynamics and species distribution will be improved by determining the factors that trigger synchronization, along with those limiting reproductive effort. In fact, if those cues do not exist locally, populations in a given area may only subsist via asexual reproduction, with important implications for their future (Honnay & Jacquemyn 2008; Hughes & Stachowicz 2009; Oliva *et al.* 2014; Jahnke, Olsen & Procaccini 2015a).

Masting species display several mechanisms of flower synchronization that include internal and environmental cues (e.g. Taiz & Zeiger 2002). Light and temperature are recognized as the two main environmental signals that can provide a consistent prompt to initiate reproductive growth of plants. However, the exact mechanism of synchronization may not be easily discernible and may also depend on the plants' ability to gain resources (Crone, Miller & Sala 2009 and references therein). Many plant species require more resources to flower and set seed than they gain in a year and therefore flower only when they exceed some threshold amount of stored resources. In this context, clonal integration and the translocation of nutrients within physically connected clones could accelerate the acquisition of sufficient resources. Herbivory, as an external factor, could also blur these patterns by affecting resource acquisition (Planes *et al.* 2011). In contrast, in bamboo and other semelparous plants, the occurrence of synchronous flowering has been explained by an internal clock (Isagi *et al.* 2004). The duration of the internal clock is believed to be fixed for a given species, but the actual flowering year is dependent on the

genotype (Isagi *et al.* 2004). On the other hand, synchronization with neighbours might also be regulated via kin selection and the extent of flowering synchronization might depend on the relatedness of the community (File, Murphy & Dudley 2012) and the resulting balance between the overlap in niche use *vs.* cooperation between relatives. The different mechanisms and possible interactions can cause plants to have cyclical or chaotic patterns of reproduction over time (Isagi *et al.* 1997; Satake & Iwasa 2000). In summary, flower synchronization may be mediated by external environmental cues related to resource availability or by internal cues related to clone synchronization, genetic fitness or kin selection and sibling synchronization.

In the marine environment, effective pollination presents a serious challenge, similar to terrestrial wind pollination systems. However, marine angiosperms (i.e. seagrasses) have evolved a number of traits suitable for a hydrophilous pollination strategy, such as filamentous pollen dispersed passively through water movement (Ackerman 2000). Perennial seagrasses are often characterized by high clonality, relatively low sexual reproductive output and large variation at different spatial scales in the distribution and abundance of flowers (Inglis & Smith 1998; Arnaud-Haond *et al.* 2012). Indeed, asynchronous flowering at small spatial scales often results in a patchy distribution of flowers (Inglis & Smith 1998; van Tussenbroek *et al.* 2010) and might lead to low reproductive output due to geitonogamous or autogamous selfing. However, much of the pollen is likely to become entrained very locally because of leaf fluttering (Kendrick *et al.* 2012), and synchronization with immediate neighbours might, at the centimetre scale, represent the only strategy to ensure pollination – particularly in monoecious seagrass species. Furthermore, given that also 'long-distance' subaqueous pollen transport is limited mostly to the range of metres (Zipperle *et al.* 2011; McMahon *et al.* 2014; Sinclair *et al.* 2014), synchronization at small to medium spatial scales (i.e. patch, cove) may be crucial to prevent pollen limitation, while ensuring outcrossing where the maximum dispersal distance exceeds clonal range (Sinclair *et al.* 2014).

Posidonia oceanica (L.) Delile is a long-lived and slow-growing Mediterranean endemic seagrass. It is an ecosystem engineer and forms monospecific meadows that provide important ecosystem services, such as sediment stabilization and acting as a nursery for juveniles of multiple commercially important species (Diaz-Almela & Duarte 2008). *Posidonia oceanica* is a monoecious species that can reproduce asexually by lateral elongation of rhizomes and sexually with hermaphrodite flowers (Ackerman 2006). Flowers appear between September and November (Buia & Mazzella 1991; Calvo *et al.* 2010), the hydrophilic pollen being released into the water column and surviving for several hours during which it is dispersed by local currents (Kendrick *et al.* 2012). Seeds ripen 5 months after the initiation of flowering (Buia & Mazzella 1991) and float to the surface, where they can be transported for one to 3 weeks by surface currents and wind-forcing until they sink and germinate (Serra *et al.* 2010). Flowering patterns in this plant exhibit important spatio-temporal

variations: there are high-prevalence years, when 80% of meadows over large geographical areas flower, and other years when only 3% of meadows flower (Diaz-Almela *et al.* 2006). At a smaller spatial scale, even in flowering years the distribution of flowers within meadows is often patchy (Diaz-Almela *et al.* 2006). The episodic synchronization of flower and fruit production in *P. oceanica* can be considered a masting strategy. Masting in *P. oceanica* may be advantageous and increase individual fitness for two main reasons: first, herbivorous fish are known to preferentially feed on flowers (Vergés *et al.* 2007), and thus, predator satiation may be necessary to ensure a high proportion of successful seeds and to lower the impact on the adult plants; and secondly, in the marine realm, where pollinators are absent and pollen dispersal is limited, synchronization of flowering with neighbouring plants might be crucial for successful fertilization. However, there is a lack of studies addressing the proximate mechanisms mediating flowering in *P. oceanica* and assessing whether masting is favoured in this species.

In 2011, we observed a flowering event in several naturally fragmented *P. oceanica* meadows along the Catalan coast, in the NW Mediterranean. While the investigated patches were at a similar depth only tens of metres apart and thus were

exposed to corresponding environmental cues (i.e. temperature and light availability; Inglis & Smith 1998; Diaz-Almela *et al.* 2006; Montefalcone *et al.* 2013), they presented contrasting flower abundances. The main hypotheses considered to explain the observed patterns and the potential mechanisms of flower synchronization within and among patches included: (i) clone identity and clone synchronization (internal clock); (ii) variation in nutrient availability per individual patch, potentially caused by spatial heterogeneity in herbivory rates or nutrient re-location *via* clonal integration (resource budget); or (iii) kin selection and sibling synchronization. Moreover, we also investigated if levels of genetic diversity, specifically heterozygosity as a proxy for individual fitness, differed between patches with high or low flower abundance.

Materials and methods

ENVIRONMENTAL VARIABLES

In October 2011, we noticed a flowering event in several *P. oceanica* seagrass meadows along the Catalan coast. We selected three shallow (5–8 m depth), naturally fragmented meadows with a similar seascape configuration that were several kilometres apart (<20 km) (Fig. 1) and

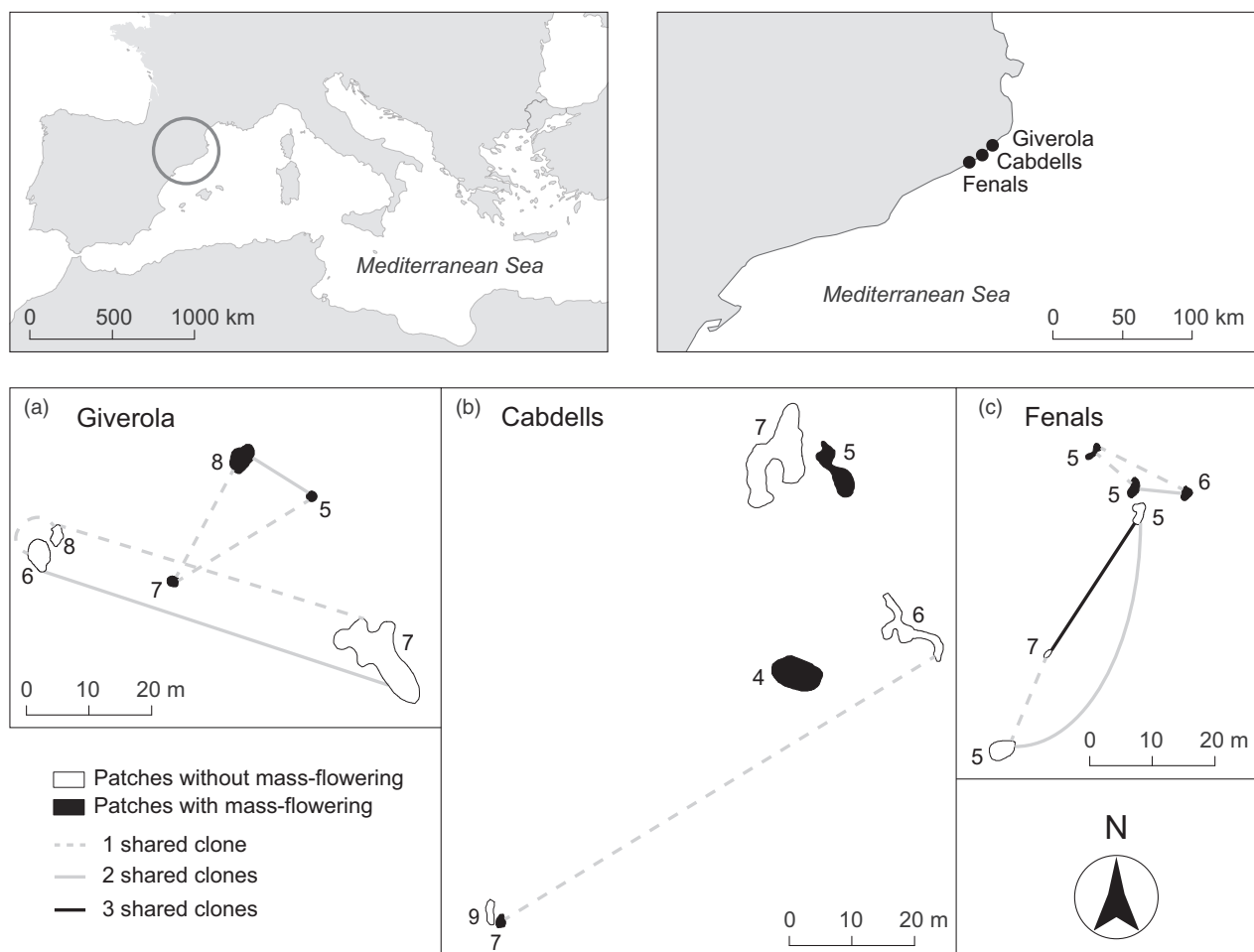


Fig. 1. Map of sampling locations of *Posidonia oceanica* along the Catalan coast and the level of clone sharing between the six patches at each of the three sites: Giverola (a), Cabdells (b) and Fenals (c). We show relative patch size and distance between patches at each location. Numbers at each patch represent the quantity of different genotypes found in each patch. Connecting lines indicate the sharing of clones.

that had been assessed for levels of herbivory and internal resources in another study 3 months previously (Pagès *et al.* 2014). For each of these three meadows, we identified three patches with mass-flowering and three patches without mass-flowering. Sampling in mass-flowering patches and patches with a low/no density of flowers enabled us to investigate potential drivers of flowering synchronicity, measured as density of flowers per patch. Patches were generally small (mean size $5.6 \pm 0.7 \text{ m}^2$) and were all on sandy substrate. We measured flower abundance and shoot densities in $40 \times 40 \text{ cm}$ quadrats (four replicates per patch; six patches – three with mass-flowering and three without mass-flowering – per site, total $n = 24$ per site) to control for possible variability in flower or shoot densities among sites. We also sampled five flowering and five non-flowering shoots in mass-flowering patches, and 10 non-flowering shoots in patches without mass-flowering for genetic analyses (see below). Additionally, in each of the patches, we collected shoots with long rhizomes (ca. 15 cm; with and without flowers for the mass-flowering patches, and without flowers for patches without mass-flowering) in order to reconstruct the frequency of inflorescences for the past 7 years at the level of the shoot/patch/site using a lepidochronological approach (e.g. Pergent & Pergent-Martini 1990; Balestri & Vallerini 2003) (total $n = 114$ shoots analysed).

Three months before the flowering event, in July 2011, we assessed leaf nitrogen and carbon content, direct herbivory rates and leaf growth (as a surrogate of primary production) on the same three sites and seagrass patches with and without mass-flowering ($3 + 3 = 6$ patches per site) for another study (Pagès *et al.* 2014). Nitrogen and carbon were measured for each of five randomly chosen shoots per patch (pooled), for which the leaves were cleaned of epiphytes, dried until constant weight and ground. The samples were then sent to the Unidade de Técnicas Instrumentais de Análise (Universidade de Coruña) where nitrogen and carbon concentrations were measured using an elemental analyser EA1108 (Carlo Erba Instruments, Waltham, MA, USA). Primary production was estimated using a modified Zieman's method (Zieman 1974; Pérez & Romero 1994), and herbivory was assessed with a tethering technique similar to that of Prado *et al.* (2007). SCUBA divers marked five shoots per patch ($5 \times 6 = 30$ shoots per site). For each shoot, we marked the leaves' base by piercing them with a needle to measure leaf elongation. We also recorded the initial number of leaves, the initial leaf length and the state of the apical part of each leaf (broken, eaten by fish, eaten by sea urchin or intact). Fifteen days later, all marked shoots were collected and transported to the laboratory for processing. We counted the number of leaves on each shoot and measured the length and state of the apex of each leaf on the shoot. For each leaf, the new leaf tissue produced (between the pierced mark and the ligula) was also measured (i.e. leaf elongation). Primary production ($\text{cm shoot}^{-1} \text{ day}^{-1}$) of pierced shoots was determined by dividing the length of new tissue produced by the number of days elapsed since marking. Shoot herbivory rates ($\text{cm shoot}^{-1} \text{ day}^{-1}$) were estimated for each of the collected shoots by adding leaf elongation (cm of new tissues produced) to the initial length and subtracting this total from the final leaf length, finally divided by the number of days elapsed since marking (Prado *et al.* 2007). Only leaves that had clear herbivore bite marks were assigned to herbivory, and the rest were discarded to avoid herbivory overestimation.

SAMPLING OF GENETIC MATERIAL, DNA EXTRACTION AND MICROSATELLITE ANALYSES

In order to test whether the very contrasting abundances of flowers among patches within each site could be related to the distribution of genotypes or the distribution of genotypic richness and genetic diversity

among patches, we genotyped the shoots collected in mass-flowering patches ($n = 5$ flowering + 5 non-flowering = 10 shoots) and in patches without mass-flowering (10 non-flowering shoots) (see above). This resulted in a total of 176 individual samples (60 shoots per site, 3 sites, 4 shoots were discarded), which were cleaned of epiphytes, dried and stored in silica crystals. For DNA extraction, an approximately 6 cm long, dry *P. oceanica* leaf fragment was homogenized with a TissueLyser MixerMill (Qiagen, Venlo, Netherlands) for 3 min at a frequency of 12 oscillations/second. DNA was extracted from the pulverized samples with the NucleoSpin® 96 Plant II kit (Macherey-Nagel, Düren, Germany), following the procedure described in Tomasello *et al.* 2009.

Twenty-eight polymorphic microsatellites were used for the analysis, which included twelve putatively neutral microsatellites (Procaccini & Waycott 1998; Alberto *et al.* 2003), as well as 16 EST-linked microsatellites (Arranz *et al.* 2013). The previously commonly used locus Po 5–49 was not used in this analysis, and the reverse primer of Po 5–40 (Alberto *et al.* 2003) was replaced with a new primer (Po 5–40M: 5'-CATGTTATAATCCTTTGTATGGAGGT-3'). Microsatellites were combined in four different multiplexes, and all PCRs were run under the following conditions: 95 °C for 15 min, 35x (94 °C for 30 s, 60 °C for 1 min 30 s, 72 °C for 1 min), with a final annealing step of 60 °C for 30 min. Scoring was performed following Migliaccio *et al.* (2005) and Tomasello *et al.* (2009).

GENETIC DATA ANALYSES: CLONAL IDENTIFICATION, GENETIC DIVERSITY AND RELATEDNESS

Clonal discrimination and identification of multilocus genotypes (MLGs) and multilocus lineages (MLLs) were performed using the software GenClone (Arnaud-Haond & Belkhir 2007) and through the calculation of P_{sex} , the probability that identical MLGs derived by chance from sexual reproduction versus those that are actual clones. After MLG identification, clones were removed so that only unique MLGs were present in each category. However, as somatic mutations and scoring errors could lead to an underestimation of the number of clones, the data set of MLGs was further investigated by removing one locus at a time to identify MLGs that are distinct at one locus, termed MLLs and re-calculating P_{sex} . Calculated P_{sex} probabilities were all lower than 0.01, which is the level that was used to reject the null hypothesis that the ramets belong to individuals derived from distinct sexual events (Serra *et al.* 2007). We used MLGs for all following statistics, but the number of MLLs is also reported. We also pooled samples from all patches within locations to identify MLGs that might be shared among patches. Genotypic richness (clonality) was estimated according to Dorken & Eckert (2001): $R = (G-1)/(N-1)$, with G representing the number of genotypes and N representing the number of sampled shoots. Genomic diversity measurements were calculated using GenAlex 6.5 (Peakall & Smouse 2012). The fixation index and significance were calculated using GENETIX 4.05 (Belkhir *et al.* 2001) with 1000 bootstrap replicates. Individual heterozygosity was calculated using genhet (Coulon 2010), which calculates the proportion of heterozygous loci in an individual (PHt) and the standardized expected and observed heterozygosities ($H_{\text{s_exp}}$ and $H_{\text{s_obs}}$) based on PHt. Finally, to assess whether kinship could regulate flowering synchronization, internal relatedness within patches or categories was calculated using the software Storm (Frasier 2008).

STATISTICAL ANALYSES

We used generalized linear models (GLM) to investigate the patterns and mechanisms of synchronicity in flowering among seagrass

patches. To do that, we tested the effects on the response variable 'flower density per patch' (abundance of flowers per square metre per patch, $n = 6$ patches per site, 3 sites) of the fixed continuous variables herbivory, per cent nitrogen content, per cent carbon content and primary production, as environmental resource-related variables to assess the resource budget hypothesis; genotypic richness to assess the internal clock hypothesis; shoot relatedness within patches to evaluate the kin/sibling selection hypothesis; and individual heterozygosity as a surrogate for fitness and a potential factor further influencing the synchronicity of flowering. All statistical analyses were run considering the mean of each variable per patch as different shoots were considered for each of the measured variables (i.e. patch was considered the experimental unit, $n = 6$ per site). We considered the possibility of including the random effect 'site' into the model to account for the variance among measurements taken from the same site (three levels, the three sites), but Akaike information criterion (AIC) did not support the inclusion of this random effect. The final model was thus a GLM with a negative binomial distribution ($\theta = 1.143$) to account for the existence of extreme counts in the response variable 'flower density'. We started model selection with a full model including all explanatory variables. Then, each fixed effect was dropped one by one in a stepwise backward selection procedure using the Akaike information criterion (AIC) and the likelihood ratio test statistic (Zuur *et al.* 2009). We also conducted a stepwise forward selection procedure that lead to the same best-selected model, adding robustness to the chosen model (see Appendix S1). We also tested the inclusion of some interactions into the best-selected model variable due to the impossibility of the model to converge with more complex designs (only double interactions were tested, see Appendix S1). Normality and homogeneity of variances were checked graphically by inspecting residuals and fitted values. The residuals of the response variable 'flower density' followed the assumption of normality after fitting the model. Even though we used a negative binomial distribution, the final model still displayed a small degree of overdispersion ($\Phi = 1.34$), which should be considered when interpreting marginally significant fixed effects. Data were analysed with the package lme4 and MASS in the statistical software R (Venables & Ripley 2002; R Development Core Team 2012; Bates *et al.* 2014) (see complete model selection procedure in the Appendix S1). We used the package visreg to visualize the effects of each predictor on the response variable with the fit from the multivariate best-selected model and to visualize the combined effects of two predictors to the response variables (Breheny & Burchett 2014).

We used a linear model to analyse the effects of the fixed continuous variables herbivory, per cent nitrogen content, per cent carbon content primary production, heterozygosity and genotypic richness (clonality) on the dependent variable 'relatedness'. Model selection was performed following the same protocol as above (using AIC). The residuals of dependent variable 'relatedness' fulfilled the assumptions of normality and homoscedasticity after model fitting. We also used a linear model to analyse whether shoot density was different between mass-flowering patches and patches without mass-flowering (factor 'patch status', fixed with 2 levels). Normality and homoscedasticity assumptions were again fulfilled.

Results

NUMBER OF FLOWERS IN MASS-FLOWERING PATCHES AND PATCHES WITHOUT MASS-FLOWERING

The average number of flowers was 97 ± 26 flowers m^{-2} in mass-flowering patches and 5 ± 2 flowers m^{-2} in patches without mass-flowering. Differences in flower abundance were not linked to contrasting shoot densities between patches ($P = 0.3$) (mean shoot density 154 ± 8 shoots m^{-2}). The lepidochronological analysis of shoots yielded no signals of flowering events in any shoot for the 7 years before 2011, confirming the rarity of sexual reproduction events in the assessed meadows. This was true both for non-flowering and flowering shoots.

POPULATION GENETIC ANALYSES: HETEROZYGOSITY, RELATEDNESS AND GENOTYPIC RICHNESS

We used a high number of microsatellites on a small spatial scale and 10 out of the 28 microsatellites proved uninformative in this analysis. Despite the small geographic scale, genotypic richness (i.e. the number of clones) (R_{MLG}) – calculated by pooling mass-flowering patches and patches without mass-flowering for each site – was high, ranging from 0.52 to 0.78 (Table 1, see Table S1 in Supporting Information for single-patch values). We did not observe a clear difference in genotypic richness between patches with and without

Table 1. Estimate of population genetics parameters for *Posidonia oceanica* patches with mass-flowering (F) and without mass-flowering (NF) at each location. Allelic richness, heterozygosity and F_{IS} measurements are based on the number of MLGs and therefore not standardized (with the exception of A_{16}). Ten out of 28 loci were uninformative (monomorphic)

Name	N	%Pol	MLG	MLL	R_{MLG}	R_{MLL}	Na (SE)	A_{16}	Ho (SE)	He (SE)	F_{IS}
GIV NF	30	64	21	16	0.69	0.52	2.071 (0.230)	2.02	0.396 (0.075)	0.276 (0.047)	−0.414*
GIV F	30	57	21	11	0.69	0.35	1.929 (0.218)	1.91	0.354 (0.077)	0.249 (0.049)	−0.401*
CAB NF	28	68	22	17	0.78	0.59	2.107 (0.214)	2.01	0.347 (0.074)	0.270 (0.046)	−0.266*
CAB F	30	68	16	12	0.52	0.38	2.107 (0.208)	2.11	0.395 (0.073)	0.311 (0.050)	−0.241*
FEN NF	29	61	17.9	13	0.59	0.41	1.893 (0.173)	1.88	0.385 (0.077)	0.285 (0.050)	−0.323*
FEN F	28	54	16	11	0.56	0.37	1.607 (0.119)	1.61	0.402 (0.087)	0.237 (0.045)	−0.681*

The following estimators are reported: N = number of individuals, %Pol = per cent of polymorphic loci, MLG = multilocus genotype, MLL = multilocus lineage, $R_{MLG} = (MLG - 1) / (N - 1)$, $R_{MLL} = (MLL - 1) / (N - 1)$, Na = No. of Alleles/Locus, A_{16} = standardized allelic richness for the lowest number of samples, Ho = Observed Heterozygosity, He = Expected Heterozygosity, F_{IS} = Fixation Index, * indicates significant F_{IS} values. Site legend: GIV = Giverola, CAB = Cabdells, FEN = Fenals.

Remark: FEN NF has an unequal N, as one locus did not amplify after three trials and its absence was therefore considered informative.

mass-flowering. Flowering shoots belonged to many different genotypes. Up to three genotypes (MLGs) were shared among the different patches within each site, but almost without exception, the shared genotypes among mass-flowering patches were different to those shared among patches without mass-flowering (Table S2, Fig. 1). The exception occurred at Cabdells (Fig. 1b) where one MLG was shared between a mass-flowering patch and a patch without mass-flowering. All patches (with and without mass-flowering, respectively) at Giverola and Fenals shared at least one clone (Fig. 1a,c). The highest number of shared clones ($n = 3$) occurred between two patches without mass-flowering at Fenals (Fig. 1c). Between-sites clone sharing occurred only between Giverola and Cabdells. Two different MLGs were found in a mass-flowering patch at Giverola (both were not flowering) and a patch without mass-flowering at Cabdells. Another MLG was found with three representatives in a mass-flowering patch in Cabdells and one representative each at two patches without mass-flowering at Giverola.

Allelic richness and heterozygosity were similar in patches with and without mass-flowering at each location, ranging from 1.61 to 2.11 (allelic richness standardized to 16 genotypes) and from 0.347 to 0.402 (observed heterozygosity) (Table 1, see Table S1 for single-patch values). The fixation index F_{IS} was negative and differed significantly from expectations under the Hardy–Weinberg equilibrium at all locations, indicating an excess of heterozygosity (Table 1). Average individual heterozygosity was generally higher in the mass-flowering patches (Table S3), and it was not higher in frequently found genotypes compared to genotypes that were only found once (Table S3).

Not all shoots belonging to the same genotype within the same patch flowered at the same time. Conversely, some shoots with identical genotypes did flower at the same time even if they grew in separate patches, where consequently clonal integration or direct communication was not possible (Table S2). Genotype relatedness within patches differed widely, ranging from -0.499 to 0.841 (Table S1).

COMBINED FACTORS TO PREDICT FLOWER SYNCHRONIZATION

Generalized linear model results indicated that both genetic and environmental factors influenced flower density per patch. However, genetic factors appeared to dominate over environmental ones in determining flower density per patch at the assessed scale. There was a significant positive relationship between flower abundance per patch and genetic relatedness as well as between flower abundance and individual heterozygosity (Tables 2 and S4, Fig. 2a,b). These results imply that higher relatedness and heterozygosity within a patch result in higher abundance of flowers (or alternatively that historically high flowering rates in these patches resulted in high heterozygosity and high relatedness). In fact, the best-selected model predicted an additive effect of heterozygosity and relatedness on flower densities per patch, with the highest density of flowers in patches with both high relatedness and high

Table 2. Significance of predictors of the best-selected generalized linear model on the dependent variable ‘Density of flowers per patch’. Change in deviance and corresponding chi-square P -values for each predictor variable are computed by sequentially dropping them one by one. Model coefficients for each of these variables can be found in Table S4

Effect	d.f.	Dev	Residual d.f.	Residual Dev	P
Null			15	40.67	
Heterozygosity	1	9.60	14	31.08	0.00**
Relatedness	1	9.50	13	21.58	0.00**
Genotypic richness	1	3.04	12	18.54	0.08*
Production	1	3.79	11	14.75	0.05*

Significance codes: ** <0.01 , * <0.1 . d.f.: Degrees of freedom. Dev: Deviance.

heterozygosity (Fig. 3). The combined effects of the rest of pairs of selected predictors on flower density per patch can be found in Fig. S1. Moreover, we also found that the abundance of flowers per patch was negatively related to vegetative tissue production (Tables 2 and S4, Fig. 2c) and genotypic richness (clonality) (Tables 2 and S4, Fig. 3d), implying that the higher the production of vegetative tissue, and the more clones/sample in a patch, the lower the abundance of flowering shoots (see Fig. S1). However, care should be taken when interpreting these last two results as the statistical significance was marginal (Table 2), and the model shows some degree of overdispersion (see Materials and Methods). The effect of each selected predictor to the response variable flower density can be inspected in the logarithmic link scale (the one used to fit the GLM) in Fig. S2. The variables herbivory, percentage of nitrogen and carbon in leaves, and all interactions had no effects on flower abundance per patch and were thus dropped from the model (see Appendix S1). We could not test the effects of higher order interactions due to computational restrictions (model convergence impossible). The best-selected model, that is the model including the fixed effects heterozygosity, internal relatedness, genotypic richness and production, explained 63.7% of total deviance (Table 2). Further, we found a positive relationship between relatedness and leaf nitrogen content ($P = 0.04$), suggesting that shoots with similar genotypes had higher leaf nitrogen contents.

Discussion

Flowering events are rare in *P. oceanica* meadows. The 2011 event was clearly unusual, with no prior flowering detected with reconstructive techniques (lepidochronology) in these meadows in the previous 7 years. The spatial heterogeneity in this mass-flowering event gave us a unique opportunity to identify mechanisms of flower synchronization between patches with mass-flowering and patches without mass-flowering. Our results indicate that genetic factors played a major role in driving flowering synchronicity within and between mass-flowering patches: both relatedness among genotypes

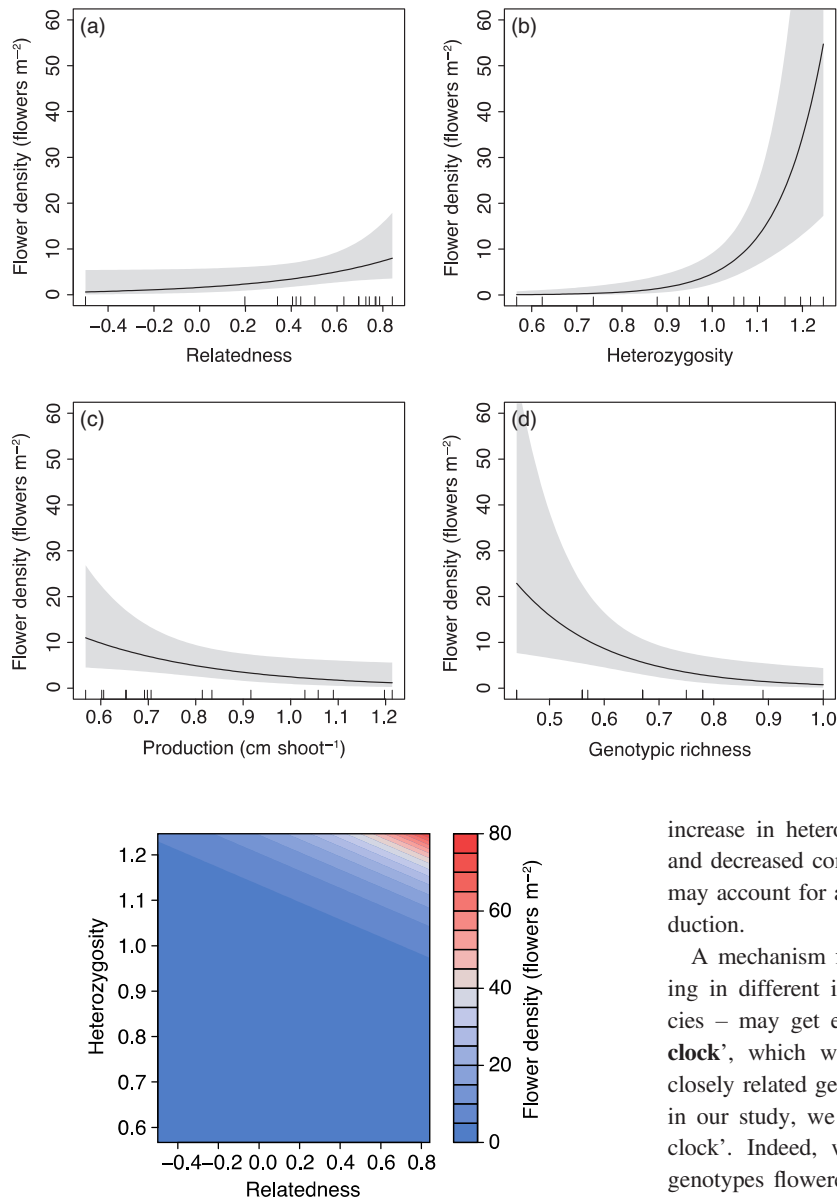


Fig. 3. Combined effects of the two most significant predictors of flower density per patch. The highest predicted flower density is for patches with high relatedness and high heterozygosity (red colours), which highlights the additive effects of these two explanatory variables.

and heterozygosity were clearly associated with flower abundance. The former indicates that kin selection is a potential mechanism of spatial synchronization, while the latter indicates increased fitness of mass-flowering patches. The negative correlation of vegetative tissue production with flower abundance per patch suggests that patch-level resource availability may also be a factor in mediating mast strategies in *P. oceanica*. Moreover, genotypic richness correlated negatively with flower abundance suggesting that – taken together with the findings on resource availability – clonal integration might also play a role. The strategy of kin selection as a mechanism of synchronization, together with the observed

Fig. 2. Relationship between the response variable ‘flower density per patch’ and the fixed effects of the best-selected generalized linear model in the 18 *Posidonia oceanica* patches analysed. (a) Genetic relatedness and (b) heterozygosity had a positive effect on flower density per patch (Table 2). In contrast, the effects of (c) production and (d) genotypic richness were negative (Table 2). Solid lines correspond to the predictions of the best-selected model, shaded areas define the 95% confidence intervals around fitted values, and short lines in the bottom of each panel indicate the position of actual observations.

increase in heterozygosity, indicates that fitness, cooperation and decreased competition between closely related individuals may account for an increased ability to invest in sexual reproduction.

A mechanism frequently advocated to explain how flowering in different individual plants – mainly semelparous species – may get entrained, is the assumption of an ‘**internal clock**’, which would synchronize flowering in identical or closely related genotypes (John & Nadgouda 1999). However, in our study, we did not find evidence for such an ‘internal clock’. Indeed, we found that a high number of different genotypes flowered in different patches at the same time and that not all shoots of identical genotypes flowered simultaneously. Nevertheless, although different genotypes flowered together, clone identity still played an important role: we found that identical genotypes were only shared within/among mass-flowering patches or within/among patches without mass-flowering, respectively, but not between each group of patches (except for one case out of 18, where an identical MLG occurred both in patches with and without mass-flowering, see Fig. 1b).

Many plant species require a minimum amount of resources to flower and set seed and therefore flower only above some threshold of stored resources (Crone & Rapp 2014). The ‘**resource budget hypothesis**’ has been observed to be the main mechanism of synchronization in some abiotically pollinated perennial grasses (Crone, Miller & Sala 2009; Crone & Rapp 2014). Indeed, we found a negative relationship between vegetative tissue production in summer and flower abundance in autumn (albeit at a low level of significance). These results highlight the inherent trade-off associated with allocating

resources to reproductive or vegetative organs. Previous studies with the same species have suggested that flowering has a negative correlation with leaf biometry, rhizome elongation and production (Gobert *et al.* 2001, 2005; Calvo *et al.* 2010) and that recovery from the stress induced by sexual reproduction may take 2 years (Calvo *et al.* 2006). This evidence for a noticeable impact of flowering events on shoot performance adds to the argument that flowering in *P. oceanica* is expensive in terms of resources. As such, it is plausible that the negative association between flowering and vegetative production in the preceding summer reflects the conservation of resources to sustain the subsequent resource-intensive flowering. In contrast, leaf nitrogen and leaf carbon content collected 3 months before the mast flowering did not have a significant effect on flower abundance. Despite the fact that herbivory may further affect individual nutrient levels and has been shown to negatively affect *P. oceanica* flower abundance in highly grazed meadows (Piazzi, Balestri & Cinelli 2000; Planes *et al.* 2011), we did not detect significant effects of herbivory on the abundance of flowers in this study either. Clonal integration and resource translocation between physically connected clones may further complicate the resource budget of an individual plant within a patch (Prado, Collier & Lavery 2008), but we did not directly assess this complex process in the present study. The lack of correlation between flower abundance, herbivory, nitrogen and carbon content could be partly due to a mismatch between our sampling time of these variables (3 months before flowering) and flower induction (up to 7 months before flowering) (Gobert *et al.* 2001). Moreover, we only measured nitrogen and carbon content of leaves, while in seagrasses most carbon storage takes place in the rhizome (Alcoverro, Manzanera & Romero 2001; Roca *et al.* 2014). Finally, whereas the genetic make-up is a permanent characteristic of a plant, and patch genetic structure may require years to decades to change (see for instance Zupo *et al.* 2006; Jahnke, Olsen & Procaccini 2015a), nutrient levels and the amount of herbivory may have changed between the time of flower induction and when ecological data were collected.

Kin selection and sibling synchronization could also help to explain flower synchronization in *P. oceanica*, although this is a process that has been much less studied. We assessed internal relatedness based on the occurrence and frequency of alleles at all 28 loci and found a significant positive relationship with the abundance of flowers per patch. Thus, the higher the relatedness of unique genotypes in a patch, the higher the abundance of flowers in that patch. A recent study in *Z. marina* found that increased relatedness of experimental and natural meadows resulted in higher shoot densities (Stachowicz *et al.* 2013). In the absence of inbreeding, the expected value for unrelated individuals is 0, while parent–offspring or fullsib relatedness values have an expected value of 0.5 (Queller & Goodnight 1989). Relatedness values in our study can be as high as 0.841 and are comparable to those from a study with *Z. marina* also at a seascape level (Kamel *et al.* 2012). The generally very high relatedness values in both seagrass species (often higher than expected from par-

ent–offspring relationships) can be explained by potential inbreeding (parent–offspring) and by the fact that reproduction is predominantly asexual, with possibly common somatic mutations, which may also be transferred to offspring in plants. Kin selection might increase the competitive ability of more related patches when considering the trait sexual reproduction.

The observed heterogeneity in flower densities is also associated with spatial heterogeneity in heterozygosity, which has been linked to components of fitness in numerous studies across a wide range of taxa (Di Fonzo *et al.* 2011). Specifically, high mean patch individual heterozygosity was associated with high flower abundances per patch (see Fig. 2b). Although it is widely accepted that genomewide heterozygosity is linked with overall fitness, the debate remains whether a low number of molecular markers is able to reflect genomewide heterozygosity (reviewed in Hansson & Westerberg 2002). Our study supports the link between heterozygosity and fitness (when fitness is defined as sexual reproductive output, *sensu* Darwin 1872) even using a limited number of loci (28), since we found a strong positive relationship between the number of flowers and heterozygosity (see Fig. 2b). In contrast to several studies in seagrasses that associated high heterozygosity with big clones in so called general-purpose genotypes (Lynch 1984), in this study heterozygosity did not differ significantly between genotypes that were only observed once and common genotypes (Table S3).

All in all, our results shed new light on seagrass flowering patterns and on the mechanisms of flower synchronization at the patch level within a wider seascape. We found support for the kin selection hypothesis and indirect evidence for the resource budget hypothesis. Our results support that an interaction between genetic factors (relatedness, heterozygosity and genotypic richness) and ecological factors (leaf production) cause the observed heterogeneous flowering patterns in *P. oceanica* seascapes. In addition, we found a strong positive relationship between the number of flowers and heterozygosity, adding evidence to the controversial association between heterozygosity and fitness when a limited number of loci are used. While there is a body of literature associating heterozygosity with fitness, research on implications of neighbourhood and kinship in seagrasses has only recently been initiated (Kamel *et al.* 2012; Stachowicz *et al.* 2013) and still deserves further research. Results presented here and results for the seagrass *Z. marina* (Stachowicz *et al.* 2013) indicate that cooperation and decreased competition between closely related individuals may account for fitness advantages, apparent in either higher levels of sexual reproduction (our study) or increased biomass accumulation (Stachowicz *et al.* 2013). Considering only our results, the opposite explanation is, however, also possible. A shoot growing among closely related individuals might be exposed to increased competition, because of higher niche overlap (Rautiainen, Koivula & Hyvärinen 2004). In evolutionary terms, it may therefore be more beneficial for an individual to invest in sexual reproduction, instead of asexual propagation. While asexually pro-