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

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**Journal of Ecology**

DOI:  
[10.1111/1365-2745.12470](https://doi.org/10.1111/1365-2745.12470)

Published: 01/11/2015

Peer reviewed version

[Cyswllt i'r cyhoeddiad / Link to publication](#)

*Dyfyniad o'r fersiwn a gyhoeddwyd / Citation for published version (APA):*

Jahnke, M., Pages Fauria, J., Alcoverra, T., Lavery, P. S., McMahon, K. M., & Procaccini, G. (2015). Should we sync? Seascape-level genetic and ecological factors determine seagrass flowering patterns. *Journal of Ecology*, 103(6), 1464-1474. <https://doi.org/10.1111/1365-2745.12470>

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

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24   Running headline: Flower synchronisation in *P. oceanica*

## 25 **Summary**

- 26 1. Spatial and temporal heterogeneity in flowering occur in many plant species with  
27 abiotic pollination and may confer fitness advantages through mechanisms such as  
28 predator satiation or pollination efficiency. Environmental factors such as light quality  
29 or quantity and temperature play an important role in inducing synchronisation on  
30 wide geographic scales. On a smaller geographic scale, external factors such as  
31 resource availability and herbivory are theorised to trigger flowering, while genetic  
32 factors may also play an important role.
- 33 2. In this study, we assessed the importance of ecological and genetic factors in shaping  
34 seascape-level spatial heterogeneity in flowering of the seagrass *Posidonia oceanica*.  
35 By investigating spatially close sites (<20 km) with similar seascape configurations  
36 and depth, we assume that major environmental drivers (temperature and light) were  
37 equivalent.
- 38 3. We assessed four ecological factors (productivity, leaf nitrogen and carbon content  
39 and herbivory) and three genetic factors (heterozygosity, relatedness and clonality) to  
40 assess three hypotheses for synchronised flowering in *P. oceanica*: (1) clone  
41 synchronisation (internal clock hypothesis), (2) variation in nutrient availability,  
42 potentially caused by spatial heterogeneity in herbivory rates or nutrient translocation  
43 *via* clonal integration (resource budget hypothesis) or (3) kin selection and sibling  
44 synchronisation.
- 45 4. Internal relatedness and heterozygosity had a significant positive effect on the  
46 abundance of flowers. Moreover, productivity and genotypic richness (clonality) were  
47 negatively associated with flower density, although at a lower level of significance. In  
48 addition we found that clones were almost exclusively shared among mass-flowering  
49 patches and patches without mass-flowering, respectively.

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

60

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

64

## 65 **Introduction**

66           For many flowering plants with abiotic pollination the likelihood of successful  
67 fertilisation depends upon the synchrony of sexual activity and the proximity of compatible  
68 mates (Knapp *et al.* 2001; van Tussenbroek *et al.* 2010). One strategy to address these  
69 limitations is mast seeding, which involves strong fluctuations of reproductive output by  
70 individual plants as well as synchronisation among individuals (Crone *et al.* 2009). This  
71 strategy, although not very common, has been described mainly in terrestrial plants, ranging  
72 from bamboo to *Dypterocarpaceae* (Janzen 1974; 1976). Some marine plants also present  
73 similar synchronised reproductive fluctuations (e.g. Inglis & Smith 1998), as well as abiotic  
74 pollination, suggesting they may display a masting reproductive strategy. Mast seeding has  
75 important disadvantages, such as the decrease in frequency of reproduction or the likely  
76 higher density-dependent seedling mortality in mast years (Hett 1971; Waller 1979).  
77 However, evolutionarily, synchronisation of flowering and seed production may confer  
78 fitness advantages through mechanisms such as predator satiation or pollination efficiency to  
79 avoid pollen limitation (Kelly 1994, Kelly & Sork 2002). In some species, predators are  
80 satiated during mast years, with minor impact on adult individuals, while predator  
81 populations are kept in check during non-mast years. Moreover, pollination efficiency is high  
82 in mast years, but pollen becomes limiting in non-mast years. These observations explain  
83 why synchronisation may increase individual fitness, but do not explain the actual  
84 mechanisms of synchronisation (Crone *et al.* 2009). Determining the triggers of  
85 synchronisation can have important implications for understanding population dynamics and  
86 species distribution, as can the factors limiting reproductive effort. In fact, if those cues do  
87 not exist locally, populations in a given area may only subsist via asexual reproduction, with  
88 important implications for the future of that population (Honnay & Jacquemyn, 2008; Hughes  
89 & Stachowicz, 2009; Oliva *et al.* 2014; Jahnke *et al.* 2015a).

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Masting species display several mechanisms of flower synchronisation that include internal and environmental cues (e.g. Taiz & Zeiger 2002). Light and temperature are recognised as the two main environmental signals that can provide a consistent prompt to initiate reproductive growth of plants. However, the exact mechanism of synchronisation may not be easily discernible and may be strictly related to environmental cues or to the plants' ability to gain resources (Crone *et al.* 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 nutrients. 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, synchronisation with neighbours might also be regulated via kin selection and the extent of flowering synchronisation might depend on the relatedness of the community (File *et al.* 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 synchronisation may be mediated by external environmental cues related to resource availability or by internal cues related to clone synchronisation, genetic fitness or kin selection and sibling synchronisation.

114 In the marine environment, effective pollination presents a serious challenge, similar  
115 to terrestrial wind pollination systems. However, marine angiosperms (i.e. seagrasses) have  
116 evolved a number of traits suitable for a hydrophilous pollination strategy, such as  
117 filamentous pollen dispersed passively through water movement (Ackerman 2000). Perennial  
118 seagrasses are often characterised by high clonality, relatively low sexual reproductive output  
119 and large variation at different spatial scales in the distribution and abundance of flowers  
120 (Inglis and Smith, 1998; Arnaud-Haond *et al.* 2012). Indeed, asynchronous flowering at small  
121 spatial scales often results in a patchy distribution of flowers (Inglis & Smith 1998; van  
122 Tussenbroek *et al.* 2010), and might lead to low reproductive output due to geitonogamous or  
123 autogamous selfing. However, much of the pollen is likely to become entrained very locally  
124 because of synchronous leaf fluttering (Kendrick *et al.* 2012), and synchronisation with  
125 immediate neighbours might, at the centimetre scale, represent the only strategy to ensure  
126 pollination – particularly in monoecious seagrass species. Furthermore, given that also ‘long-  
127 distance’ subaqueous pollen transport is limited mostly to the range of metres (Zipperle *et al.*  
128 2011; McMahon *et al.*, 2014; Sinclair *et al.* 2014) synchronisation at small to medium spatial  
129 scales (i.e. patch, cove) may be crucial to prevent pollen limitation, while ensuring  
130 outcrossing where the maximum dispersal distance exceeds clonal range (Sinclair *et al.*  
131 2014).

132  
133 *Posidonia oceanica* (L.) Delile is a long-lived and slow growing Mediterranean  
134 endemic seagrass. It is an ecosystem engineer and forms monospecific meadows that provide  
135 important ecosystem services, such as sediment stabilization and acting as a nursery for  
136 juveniles of multiple commercially-important species (Diaz-Almela & Duarte 2008).  
137 *Posidonia oceanica* is a monoecious species that can reproduce asexually by lateral  
138 elongation of rhizomes, and sexually with hermaphrodite flowers (Ackerman, 2006). Flowers

139 appear between September and November (Buia & Mazzella 1991; Calvo *et al.* 2010), the  
140 hydrophilic pollen being released into the water column and surviving for several hours  
141 during which it is dispersed by local currents (Kendrick *et al.* 2012). Seeds ripen five months  
142 after the initiation of flowering (Buia & Mazzella 1991) and float to the surface, where they  
143 can be transported for one to three weeks by surface currents and wind-forcing until they sink  
144 and germinate (Serra *et al.* 2010). Flowering patterns in this plant exhibit important spatio-  
145 temporal variations: there are high-prevalence years, when 80% of meadows over large  
146 geographical areas flower, and other years when only 3% of meadows flower (Diaz-Almela  
147 *et al.* 2006). At a smaller spatial scale, even in flowering years the distribution of flowers  
148 within meadows is often patchy (Diaz-Almela *et al.* 2006). The episodic synchronisation of  
149 flower and fruit production in *P. oceanica* can be considered a masting strategy. Masting in  
150 *P. oceanica* may be advantageous and increase individual fitness for two main reasons: first,  
151 herbivorous fish are known to preferentially feed on flowers (Vergés *et al.* 2007), thus,  
152 predator satiation may be necessary to ensure a high proportion of successful seeds and to  
153 lower the impact on the adult plants; and second, in the marine realm, where pollinators are  
154 absent and pollen dispersal is limited, synchronisation of flowering with neighbouring plants  
155 might be crucial for successful fertilization. However, there is a lack of studies addressing the  
156 proximate mechanisms mediating flowering in *P. oceanica* and assessing whether masting is  
157 favoured in this species.

158

159         In 2011, we observed a flowering event in several naturally fragmented *P. oceanica*  
160 meadows along the Catalan coast, in the NW Mediterranean. While the investigated patches  
161 were at a similar depth only tens of metres apart and thus were exposed to corresponding  
162 environmental cues (i.e. temperature and light availability; Inglis and Smith, 1998; Diaz-  
163 Almela *et al.* 2006; Montefalcone *et al.* 2013), they presented contrasting flower abundances.



164 The main hypotheses considered to explain the observed patterns and the potential  
165 mechanisms of flower synchronisation within and among patches included: (1) clone identity  
166 and clone synchronisation (internal clock), (2) variation in nutrient availability per individual  
167 patch, potentially caused by spatial heterogeneity in herbivory rates or nutrient re-location *via*  
168 clonal integration (resource budget) or (3) kin selection and sibling synchronisation.  
169 Moreover, we also investigated if levels of genetic diversity, specifically heterozygosity as a  
170 proxy for individual fitness, differed between patches with high or low flower abundance.

171

## 172 **Material and Methods**

### 173 *Environmental variables*

174 In October 2011, we noticed a flowering event in several *P. oceanica* seagrass  
175 meadows along the Catalan coast. We selected three shallow (5-8 m depth), naturally  
176 fragmented meadows with a similar seascape configuration that were several kilometres apart  
177 (<20 km) (Fig. 1) and that had been assessed for levels of herbivory and internal resources in  
178 another study three months previously (Pagès *et al.* 2014). For each of these three meadows,  
179 we identified three patches with mass-flowering and three patches without mass-flowering.  
180 Sampling in mass-flowering patches and patches with a low/no density of flowers enabled us  
181 to investigate potential drivers of flowering synchronicity, measured as density of flowers per  
182 patch. Patches were generally small (mean size  $5.6 \pm 0.7 \text{ m}^2$ ) and were all on sandy substrate.  
183 We measured flower abundance and shoot densities in 40 x 40 cm quadrats (4 replicates per  
184 patch; 6 patches - 3 with mass-flowering and 3 without mass-flowering - per site, total n = 24  
185 per site) to control for possible variability in flower or shoot densities among sites. We also  
186 sampled five flowering and five non-flowering shoots in mass-flowering patches, and 10 non-  
187 flowering shoots in patches without mass-flowering for genetic analyses (see below).  
188 Additionally, in each of the patches we collected shoots with long rhizomes (ca. 15 cm; with

189 and without flowers for the mass-flowering patches, and without flowers for patches without  
190 mass-flowering) in order to reconstruct the frequency of inflorescences for the past seven  
191 years at the level of the shoot/patch/site using a lepidochronological approach (e.g. Pergent &  
192 Pergent-Martini 1990, Balestri & Vallerini 2003) (total n = 114 shoots analysed).

193

194 Three months before the flowering event, in July 2011, we assessed leaf nitrogen and  
195 carbon content, direct herbivory rates and leaf growth (as a surrogate of primary production)  
196 on the same three sites and seagrass patches with and without mass-flowering (3 + 3 = 6  
197 patches per site) for another study (Pagès *et al.* 2014). Nitrogen and carbon were measured  
198 for each of five randomly chosen shoots per patch (pooled), for which the leaves were  
199 cleaned of epiphytes, dried until constant weight and ground. The samples were then sent to  
200 the Unidade de Técnicas Instrumentais de Análise (Universidade de Coruña) where nitrogen  
201 and carbon concentrations were measured using an elemental analyser EA1108 (Carlo Erba  
202 Instruments). Primary production was estimated using a modified Zieman's method (Zieman  
203 1974; Pérez & Romero 1994), and herbivory was assessed with a tethering technique similar  
204 to that of Prado *et al.* (2007). SCUBA divers marked five shoots per patch ( $5 \times 6 = 30$  shoots  
205 per site). For each shoot, we marked the leaves' base by piercing them with a needle to  
206 measure leaf elongation. We also recorded the initial number of leaves, the initial leaf length  
207 and the state of the apical part of each leaf (broken, eaten by fish, eaten by sea urchin or  
208 intact). Fifteen days later, all marked shoots were collected and transported to the laboratory  
209 for processing. We counted the number of leaves on each shoot and measured the length and  
210 state of the apex of each leaf on the shoot. For each leaf, the new leaf tissue produced  
211 (between the pierced mark and the ligula) was also measured (i.e. leaf elongation). Primary  
212 production ( $\text{cm shoot}^{-1} \text{ day}^{-1}$ ) of pierced shoots was determined by dividing the length of new  
213 tissue produced by the number of days elapsed since marking. Shoot herbivory rates ( $\text{cm}$

214 shoot<sup>-1</sup> day<sup>-1</sup>) were estimated for each of the collected shoots by adding leaf elongation (cm  
215 of new tissues produced) to the initial length and subtracting this total from the final leaf  
216 length, finally divided by the number of days elapsed since marking (Prado *et al.* 2007). Only  
217 leaves that had clear herbivore bite marks were assigned to herbivory and the rest were  
218 discarded to avoid herbivory overestimation.

219

#### 220 *Sampling of genetic material, DNA extraction and microsatellite analyses*

221 In order to test whether the very contrasting abundances of flowers among patches  
222 within each site could be related to the distribution of genotypes or the distribution of  
223 genotypic richness and genetic diversity among patches, we analysed the shoots collected in  
224 mass-flowering patches (n = 5 flowering + 5 non-flowering = 10 shoots) and in patches  
225 without mass-flowering (10 non-flowering shoots) (see above). This resulted in a total of 176  
226 individual samples (60 shoots per site, 3 sites, 4 shoots were discarded), which were cleaned  
227 of epiphytes, dried and stored in silica crystals. For DNA extraction, an approximately 6 cm  
228 long, dry *P. oceanica* leaf fragment was homogenized with a TissueLyser MixerMill  
229 (Qiagen) for 3 min at a frequency of 12 oscillations/second. DNA was extracted from the  
230 pulverized samples with the NucleoSpin® 96 Plant II kit (Macherey-Nagel), following the  
231 procedure described in Tomasello *et al.* 2009.

232

233 Twenty-eight polymorphic microsatellites were used for the analysis, which included  
234 twelve putatively neutral microsatellites (Procaccini & Waycott 1998; Alberto *et al.* 2003), as  
235 well as 16 EST-linked microsatellites (Arranz *et al.* 2013). The previously commonly used  
236 locus Po 5-49 was not used in this analysis and the reverse primer of Po 5-40 (Alberto *et al.*  
237 2003) was replaced with a new primer (Po 5-40M: 5'-  
238 CATGTTATAATCCTTTGTATGGAGGT-3'). Microsatellites were combined in four

239 different multiplexes and all PCRs were run under the following conditions: 95°C for 15 min,  
240 35x (94°C for 30 sec, 60°C for 1min 30 sec, 72°C for 1 min), with a final annealing step of  
241 60°C for 30 min. Scoring was performed following Migliaccio *et al.* (2005) and Tomasello *et*  
242 *al.* (2009).

243

#### 244 *Genetic data analyses: clonal identification, genetic diversity and relatedness*

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

264 standardized expected and observed heterozygosities (Hs\_exp and Hs\_obs) based on PHT.  
265 Finally, to assess whether kinship could regulate flowering synchronisation, internal  
266 relatedness within patches or categories was calculated using the software Storm (Frasier  
267 2008).

268

### 269 *Statistical analyses*

270 We used generalised linear models (GLM) to investigate the patterns and mechanisms  
271 of synchronicity in flowering among seagrass patches. To do that, we tested the effects on the  
272 response variable ‘flower density per patch’ (abundance of flowers per square metre per  
273 patch, n = 6 patches per site, 3 sites) of the fixed continuous variables herbivory, percent  
274 nitrogen content, percent carbon content and primary production, as environmental resource-  
275 related variables to assess the resource-budget hypothesis; genotypic richness to assess the  
276 internal clock hypothesis; shoot relatedness within patches to evaluate the kin/sibling  
277 selection hypothesis; and individual heterozygosity as a surrogate for fitness and a potential  
278 factor further influencing the synchronicity of flowering. All statistical analyses were run  
279 considering the mean of each variable per patch as different shoots were considered for each  
280 of the measured variables (i.e. patch was considered the experimental unit, n = 6 per site). We  
281 considered the possibility of including the random effect ‘site’ into the model to account for  
282 the variance among measurements taken from the same site (three levels, the three sites), but  
283 Akaike Information Criterion (AIC) did not support the inclusion of this random effect. The  
284 final model was thus a GLM with a negative binomial distribution ( $\theta = 1.143$ ) to account  
285 for the existence of extreme counts in the response variable ‘flower density’. We started  
286 model selection with a full model including all explanatory variables. Then, each fixed effect  
287 was dropped one by one in a stepwise backward selection procedure using the Akaike  
288 Information Criterion (AIC) and the likelihood ratio test statistic (Zuur *et al.* 2009). We also

289 conducted a stepwise forward selection procedure that lead to the same best-selected model,  
290 adding robustness to the chosen model (see supplementary PosiFlower\_Rmarkdown.html  
291 file). We also tested the inclusion of some interactions into the best-selected model variable  
292 due to the impossibility of the model to converge with more complex designs (only double  
293 interactions were tested, see supplementary PosiFlower\_Rmarkdown.html file). Normality  
294 and homogeneity of variances were checked graphically by inspecting residuals and fitted  
295 values. The residuals of the response variable ‘flower density’ followed the assumption of  
296 normality after fitting the model. Even though we used a negative binomial distribution, the  
297 final model still displayed a small degree of overdispersion ( $\Phi = 1.34$ ), which should be  
298 considered when interpreting marginally significant fixed effects. Data were analysed with  
299 the package lme4 and MASS in the statistical software R (Venables & Ripley 2002; R  
300 Development Core Team 2012; Bates *et al.* 2014) (see complete model selection procedure in  
301 the supplementary file PosiFlower\_Rmarkdown.html). We used the package visreg to  
302 visualise the effects of each predictor on the response variable with the fit from the  
303 multivariate best-selected model and to visualize the combined effects of 2 predictors to the  
304 response variables (Breheny & Burchett 2014).

305

306 We used a linear model to analyse the effects of the fixed continuous variables  
307 herbivory, percent nitrogen content, percent carbon content primary production,  
308 heterozygosity and genotypic richness (clonality) on the dependent variable ‘relatedness’.  
309 Model selection was performed following the same protocol as above (using AIC). The  
310 residuals of dependent variable ‘relatedness’ fulfilled the assumptions of normality and  
311 homoscedasticity after model fitting. We also used a linear model to analyse whether shoot  
312 density was different between mass-flowering patches and patches without mass-flowering

313 (factor ‘patch status’, fixed with 2 levels). Normality and homoscedasticity assumptions were  
314 again fulfilled.

315

## 316 **Results**

### 317 *Number of flowers in mass-flowering patches and patches without mass-flowering*

318 The average number of flowers was  $97 \pm 26$  flowers  $\text{m}^{-2}$  in mass-flowering patches  
319 and  $5 \pm 2$  flowers  $\text{m}^{-2}$  in patches without mass-flowering. Differences in flower abundance  
320 were not linked to contrasting shoot densities between patches ( $P = 0.3$ ) (mean shoot density  
321  $154 \pm 8$  shoots  $\text{m}^{-2}$ ). The lepidochronological analysis of shoots yielded no signals of  
322 flowering events in any shoot for the seven years before 2011, confirming the rarity of sexual  
323 reproduction events in the assessed meadows. This was true both for non-flowering and  
324 flowering shoots.

325

### 326 *Population genetic analyses: Heterozygosity, relatedness and genotypic richness*

327 We used a high number of microsatellites on a small spatial scale and 10 out of the 28  
328 microsatellites proved uninformative in this analysis. Despite the small geographic scale,  
329 genotypic richness (i.e. the number of clones) ( $R_{\text{MLG}}$ ) – calculated by pooling mass-flowering  
330 patches and patches without mass-flowering for each site – was high, ranging from 0.52 to  
331 0.78 (Table 1, see Table S1 for single-patch values). We did not observe a clear difference in  
332 genotypic richness between patches with and without mass-flowering. Flowering shoots  
333 belonged to many different genotypes. Up to three genotypes (MLGs) were shared among the  
334 different patches within each site, but almost without exception, the shared genotypes among  
335 mass-flowering patches were different to those shared among patches without mass-flowering  
336 (Table S2, Fig. 1). The exception occurred at Cabdells (Fig. 1b) where one MLG was shared  
337 between a mass-flowering patch and a patch without mass-flowering. All patches (with and

338 without mass-flowering, respectively) at Giverola and Fenals shared at least one clone (Fig.  
339 1a,c). The highest number of shared clones ( $n = 3$ ) occurred between two patches without  
340 mass-flowering at Fenals (Fig. 1c). Between-sites clone sharing occurred only between  
341 Giverola and Cabdells. Two different MLGs were found in a mass-flowering patch at  
342 Giverola (both were not flowering) and a patch without mass-flowering at Cabdells. Another  
343 MLG was found with three representatives in a mass-flowering patch in Cabdells and one  
344 representative each at two patches without mass-flowering at Giverola.

345

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

354

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

360

361 *Combined factors to predict flower synchronisation*



362 GLM results indicated that both genetic and environmental factors influenced flower  
363 density per patch. However, genetic factors appeared to dominate over environmental ones in  
364 determining flower density per patch at the assessed scale. There was a significant positive  
365 relationship between flower abundance per patch and genetic relatedness as well as between  
366 flower abundance and individual heterozygosity (Table 2, Table S4, Fig. 2a,b). These results  
367 imply that higher relatedness and heterozygosity within a patch result in higher abundance of  
368 flowers (or alternatively that historically high flowering rates in these patches resulted in high  
369 heterozygosity and high relatedness). In fact, the best-selected model predicted an additive  
370 effect of heterozygosity and relatedness on flower densities per patch, with the highest  
371 density of flowers in patches with both high relatedness and high heterozygosity (Fig. 3). The  
372 combined effects of the rest of pairs of selected predictors on flower density per patch can be  
373 found in the supplementary (Fig. S1). Moreover, we also found that the abundance of flowers  
374 per patch was negatively related to vegetative tissue production (Table 2, Table S4, Fig. 2c)  
375 and genotypic richness (clonality) (Table 2, Table S4, Fig. 3d), implying that the higher the  
376 production of vegetative tissue, and the more clones/sample in a patch, the lower the  
377 abundance of flowering shoots (see Fig. S1). However, care should be taken when  
378 interpreting these last two results as the statistical significance was marginal (Table 2) and the  
379 model shows some degree of overdispersion (see Materials and Methods). The effect of each  
380 selected predictor to the response variable flower density can be inspected in the logarithmic  
381 link scale (the one used to fit the GLM) in the supplementary (Fig. S2). The variables  
382 herbivory, percentage of nitrogen and carbon in leaves, and all interactions had no effects on  
383 flower abundance per patch and were thus dropped from the model (see supplementary  
384 PosiFlower\_Rmarkdown.html file). We could not test the effects of higher order interactions  
385 due to computational restrictions (model convergence impossible). The best-selected model,  
386 i.e. the model including the fixed effects heterozygosity, internal relatedness, genotypic

387 richness and production, explained 63.7% of total deviance (Table 2). Further, we found a  
388 positive relationship between relatedness and leaf nitrogen content ( $P = 0.04$ ), suggesting that  
389 shoots with similar genotypes had higher leaf nitrogen contents.

390

## 391 **Discussion**

392 Flowering events are rare in *P. oceanica* meadows. The 2011 event was clearly  
393 unusual, with no prior flowering detected with reconstructive techniques (lepidochronology)  
394 in these meadows in the previous seven years. The spatial heterogeneity in this mast  
395 flowering event gave us a unique opportunity to identify mechanisms of flower  
396 synchronisation between patches with mass-flowering and patches without mass-flowering.  
397 Our results indicate that genetic factors played a major role in driving flowering  
398 synchronicity within and between mass-flowering patches: both relatedness among genotypes  
399 and heterozygosity were clearly associated with flower abundance. The former indicates that  
400 kin selection is a potential mechanism of spatial synchronisation, while the latter indicates  
401 increased fitness of mass-flowering patches. The negative correlation of vegetative tissue  
402 production with flower abundance per patch suggests that patch-level resource availability  
403 may also be a factor in mediating mast strategies in *P. oceanica*. Moreover, genotypic  
404 richness correlated negatively with flower abundance suggesting that – taken together with  
405 the findings on resource availability – clonal integration might also play a role. The strategy  
406 of kin selection as a mechanism of synchronisation, together with the observed increase in  
407 heterozygosity indicate that fitness, cooperation and decreased competition between closely  
408 related individuals may account for an increased ability to invest in sexual reproduction.

409

410 A mechanism frequently advocated to explain how flowering in different individual  
411 plants – mainly semelparous species – may get entrained, is the assumption of an ‘**internal**

412 **clock**', which would synchronise flowering in identical or closely related genotypes (John &  
413 Nadgouda 1999). However, in our study we did not find evidence for such an 'internal clock'.  
414 Indeed, we found that a high number of different genotypes flowered in different patches at  
415 the same time and that not all shoots of identical genotypes flowered simultaneously.  
416 Nevertheless, although different genotypes flowered together, clone identity still played an  
417 important role: we found that identical genotypes were only shared within/among mass-  
418 flowering patches or within/among patches without mass-flowering respectively, but not  
419 between each group of patches (except for one case out of 18, where an identical MLG  
420 occurred both in patches with and without mass-flowering, see Fig. 1b).

421

422 Many plant species require a minimum amount of resources to flower and set seed,  
423 and therefore flower only above some threshold of stored resources (Crone & Rapp 2014).  
424 The '**resource budget hypothesis**' has been observed to be the main mechanism of  
425 synchronisation in some abiotically pollinated perennial grasses (Crone *et al.* 2009; Crone &  
426 Rapp 2014). Indeed, we found a negative relationship between vegetative tissue production in  
427 summer and flower abundance in autumn (albeit at a low level of significance). These results  
428 highlight the inherent trade-off associated with allocating resources to reproductive or  
429 vegetative organs. Previous studies with the same species have suggested that flowering has a  
430 negative correlation with leaf biometry, rhizome elongation and production (Gobert *et al.*  
431 2001; Gobert *et al.* 2005; Calvo *et al.* 2010) and that recovery from the stress induced by  
432 sexual reproduction may take two years (Calvo *et al.* 2006). This evidence for a noticeable  
433 impact of flowering events on shoot performance adds to the argument that flowering in *P.*  
434 *oceanica* is expensive in terms of resources. As such, it is plausible that the negative  
435 association between flowering and vegetative production in the preceding summer reflects  
436 the conservation of resources to sustain the subsequent resource-intensive flowering. In

437 contrast, leaf nitrogen and leaf carbon content collected three months before the mast  
438 flowering did not have a significant effect on flower abundance. Despite the fact that  
439 herbivory may further affect individual nutrient levels, and has been shown to negatively  
440 affect *P. oceanica* flower abundance in highly grazed meadows (Piazzi *et al.* 2000; Planes *et*  
441 *al.* 2011), we did not detect significant effects of herbivory on the abundance of flowers in  
442 this study either. Clonal integration and resource translocation between physically connected  
443 clones may further complicate the resource budget of an individual plant within a patch  
444 (Prado *et al.* 2008), but we did not directly assess this complex process in the present study.  
445 The lack of correlation between flower abundance, herbivory, nitrogen and carbon content  
446 could be partly due to a mismatch between our sampling time of these variables (three  
447 months before flowering) and flower induction (up to seven months before flowering)  
448 (Gobert *et al.* 2001). Moreover, we only measured nitrogen and carbon content of leaves;  
449 while in seagrasses most carbon storage takes place in the rhizome (Alcoverro *et al.* 2001,  
450 Roca *et al.* 2014). Finally, whereas the genetic make-up is a permanent characteristic of a  
451 plant, and patch genetic structure may require years to decades to change (see for instance  
452 Zupo *et al.* 2006; Jahnke *et al.* 2015a), nutrient levels and the amount of herbivory may have  
453 changed between the time of flower induction and when ecological data were collected.

454

455 **Kin selection and sibling synchronisation** could also help to explain flower  
456 synchronisation in *P. oceanica*, although this is a process that has been much less studied. We  
457 assessed internal relatedness based on the occurrence and frequency of alleles at all 28 loci  
458 and found a significant positive relationship with the abundance of flowers per patch. Thus,  
459 the higher the relatedness of unique genotypes in a patch, the higher the abundance of flowers  
460 in that patch. A recent study in *Z. marina* found that increased relatedness of experimental  
461 and natural meadows resulted in higher shoot densities (Stachowicz *et al.* 2013). In the

462 absence of inbreeding, the expected value for unrelated individuals is 0, while parent-  
463 offspring or fullsib relatedness values have an expected value of 0.5 (Queller & Goodnight  
464 1989). Relatedness values in our study can be as high as 0.841 and are comparable to those  
465 from a study with *Z. marina* also at a seascape level (Kamel *et al.* 2012). The generally very  
466 high relatedness values in both seagrass species (often higher than expected from parent-  
467 offspring relationships) can be explained by potential inbreeding (parent-offspring) and by  
468 the fact that reproduction is predominantly asexual, with possibly common somatic  
469 mutations, which may also be transferred to offspring in plants. Kin selection might increase  
470 the competitive ability of more related patches when considering the trait sexual  
471 reproduction.

472

473         The observed heterogeneity in flower densities is also associated with spatial  
474 heterogeneity in heterozygosity, which has been linked to components of fitness in numerous  
475 studies across a wide range of taxa (Di Fonzo *et al.* 2011). Specifically, high mean patch  
476 individual heterozygosity was associated with high flower abundances per patch (see Fig.  
477 2b). Although it is widely accepted that genome-wide heterozygosity is linked with overall  
478 fitness, the debate remains whether a low number of molecular markers is able to reflect  
479 genome-wide heterozygosity (reviewed in Hansson & Westerberg 2002). Our study supports  
480 the link between heterozygosity and fitness (when fitness is defined as sexual reproductive  
481 output, *sensu* Darwin 1872) even using a limited number of loci (28), since we found a strong  
482 positive relationship between the number of flowers and heterozygosity (see Fig. 2b). In  
483 contrast to several studies in seagrasses that associated high heterozygosity with big clones in  
484 so called “general-purpose-genotypes” (Lynch 1984), in this study heterozygosity did not  
485 differ significantly between genotypes that were only observed once and common genotypes  
486 (Table S3).

487

488 All in all, our results shed new light on seagrass flowering patterns and on the  
489 mechanisms of flower synchronisation at the patch level within a wider seascape. We found  
490 support for the kin selection hypothesis and indirect evidence for the resource budget  
491 hypothesis. Our results support that an interaction between genetic factors (relatedness,  
492 heterozygosity and genotypic richness) and ecological factors (leaf production) cause the  
493 observed heterogeneous flowering patterns in *P. oceanica* seascapes. In addition, we found a  
494 strong positive relationship between the number of flowers and heterozygosity, adding  
495 evidence to the controversial association between heterozygosity and fitness when a limited  
496 number of loci are used. While there is a body of literature associating heterozygosity with  
497 fitness, research on implications of neighbourhood and kinship in seagrasses has only  
498 recently been initiated (Kamel *et al.* 2012; Stachowicz *et al.* 2013) and still deserves further  
499 research. Results presented here and results for the seagrass *Z. marina* (Stachowicz *et al.*  
500 2013) indicate that cooperation and decreased competition between closely related  
501 individuals may account for fitness advantages, apparent in either higher levels of sexual  
502 reproduction (our study) or increased biomass accumulation (Stachowicz *et al.* 2013).  
503 Considering only our results, the opposite explanation is, however, also possible. A shoot  
504 growing among closely related individuals might be exposed to increased competition,  
505 because of higher niche overlap (Rautiainen *et al.* 2004). In evolutionary terms, it may  
506 therefore be more beneficial for an individual to invest in sexual reproduction, instead of  
507 asexual propagation. While asexually produced plants will encounter high levels of kin  
508 competition, sexually produced seeds, in contrast, may disperse further aided by currents and  
509 may establish in meadows where their genotype and phenotype are dissimilar to the  
510 neighbouring plants, decreasing niche overlap. Indeed, kin competition has been shown to  
511 play a role in determining flowering intervals in bamboo (Tachiki *et al.* 2015). Both scenarios

512 (kin cooperation and sibling competition) assume that kin recognition is possible in  
513 seagrasses. Although, to our knowledge, it has not been investigated for any seagrass species,  
514 results from terrestrial plants indicate that kin recognition is most likely mediated via root  
515 exudates (Biedrzycki *et al.* 2010), a form of intra-specific communication that should equally  
516 be possible in the marine environment. Another study on a terrestrial plant moreover  
517 confirmed that soil leachates might play an important role in flowering synchronization  
518 among neighbours (Falik *et al.* 2014).

519

520         To our knowledge, this study is the first to link both ecological and genetic factors  
521 with flower abundance in a species with a presumed masting strategy. These findings help to  
522 understand seascape-level synchronisation of individual but spatially close plants during mast  
523 flowering events and open new doors for exploring the role of relatedness in ecosystem  
524 functioning.

525

## 526 **Acknowledgements**

527         We are very grateful to Alessandro Gera and Oriol Mascaró for helping in the field, to  
528 Oriana Carvajal for her help with the lepidochronological analysis and to Frederic Bartumeus  
529 and Josep Anton Sanchez for statistical assistance. We would also like to thank the editors  
530 and two anonymous reviewers who greatly improved the manuscript with constructive  
531 suggestions. Carbon–nitrogen analyses were performed at the Unidade de Técnicas  
532 Instrumentais de Análise (Universidade de Coruña), we thank María Lema for her assistance;  
533 genetic analyses were conducted at the SZN. This research was partially supported by the  
534 European Community's 7th Framework Programme (FP7/2007–2013) CoCoNet, the MIUR  
535 Italian Flagship project RITMARE, the Spanish Ministry of Science and Innovation  
536 (CTM2010-22273-C02-02) and the Spanish National Research Council (PIE201330EO62).

537 M.J. is supported by a SZN PhD fellowship via the Open University and the Spanish Ministry  
538 of Education supported J.P. (scholarship AP2008- 01601).

539

#### 540 **Data accessibility**

541 Data on herbivory, carbon and nitrogen leaf content and production on the patch level  
542 are deposited in Dryad repository (doi:10.5061/dryad.sj6dv; Jahnke *et al.* 2015b). An  
543 Rmarkdown html file with the R scripts used for model selection is available online as  
544 supporting information (PosiFlower\_Rmarkdown.html).

545



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771 **Supporting information**

772 Additional supporting information may be found in the online version of this article:

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774 **Table S1.** Estimate of population genetic parameters per patch of *Posidonia oceanica*.

775 **Table S2.** Number and frequency of sampled genotypes of *Posidonia oceanica*.

776 **Table S3.** Average proportion of heterozygous loci in an individual (PHt) and standardized  
777 observed and expected individual heterozygosity measurements (Hs obs and Hs exp).

778 **Table S4.** Coefficient estimation obtained by fitting a generalised linear model with the  
779 variables heterozygosity, relatedness, genotypic richness and production.

780 **Figure S1.** Combined effects of selected pairs of predictors on flower density per patch.

781 **Figure S2.** Effects of each of the selected predictors to the response variable flower density  
782 per patch in the logarithmic link scale.

783

784 **Tables**

785 **Table 1.** Estimate of population genetics parameters for *Posidonia oceanica* patches with  
 786 mass-flowering (F) and without mass-flowering (NF) at each location. Allelic richness,  
 787 heterozygosity and  $F_{is}$  measurements are based on the number of MLGs and therefore not  
 788 standardized (with the exception of  $A_{16}$ ). Ten out of 28 loci were uninformative  
 789 (monomorphic). The following estimators are reported: N = number of individuals, %Pol =  
 790 percent of polymorphic loci, MLG = multilocus genotype, MLL = multilocus lineage,  $R_{MLG}$   
 791  $= (MLG-1)/(N-1)$ ,  $R_{MLL} = (MLL-1)/(N-1)$ , Na = No. of Alleles/Locus,  $A_{16}$  = standardized  
 792 allelic richness for the lowest number of samples, Ho = Observed Heterozygosity, He =  
 793 Expected Heterozygosity,  $F_{is}$  = Fixation Index, \* indicates significant  $F_{is}$  values. Site legend:  
 794 GIV = Giverola, CAB = Cabdels, FEN = Fenals

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

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

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

<b>Effect</b>	<b>Df</b>	<b>Dev</b>	<b>Residual Df</b>	<b>Residual Dev</b>	<b><i>P</i></b>
<b>Null</b>			15	40.67	
<b>Heterozygosity</b>	1	9.60	14	31.08	0.00**
<b>Relatedness</b>	1	9.50	13	21.58	0.00**
<b>Genotypic richness</b>	1	3.04	12	18.54	0.08·
<b>Production</b>	1	3.79	11	14.75	0.05·

801 Significance codes: <0.001 '\*\*\*' <0.01 '\*\*' <0.05 '\*' <0.1 '.' Df: Degrees of freedom. Dev: Deviance.

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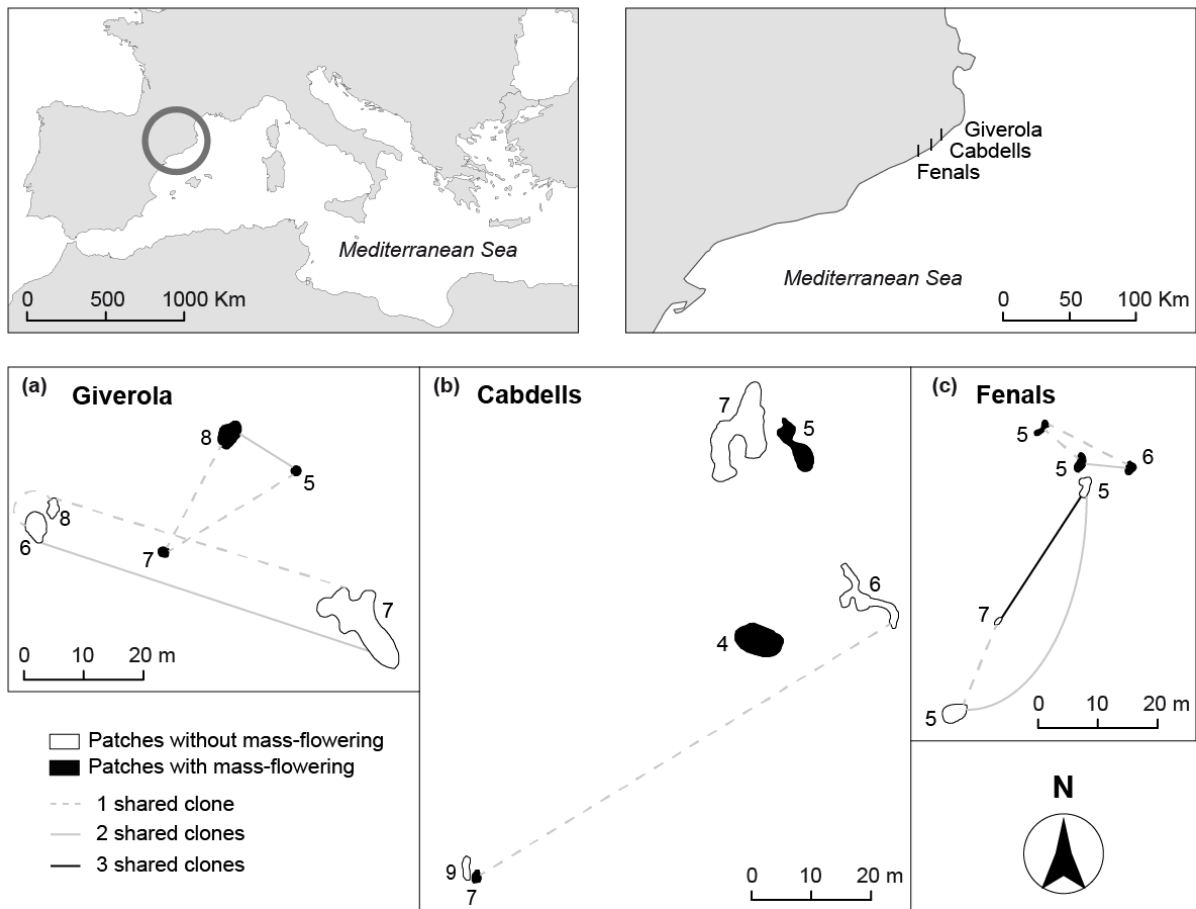
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810 **Figures**

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

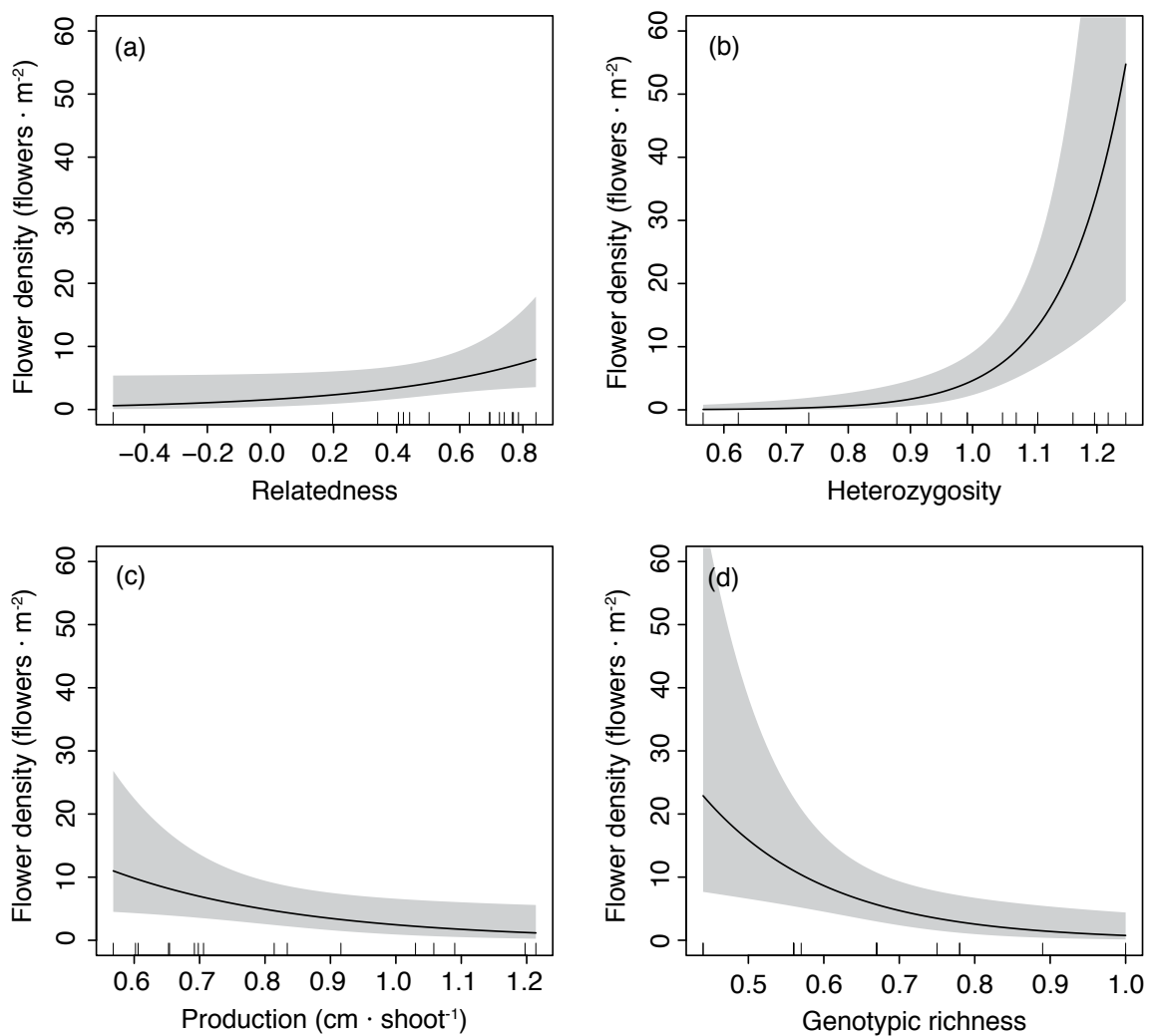


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819 **Fig. 2.** Relationship between the response variable ‘flower density per patch’ and the fixed  
820 effects of the best-selected generalised linear model in the 18 *Posidonia oceanica* patches  
821 analyzed. (a) Genetic relatedness and (b) heterozygosity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



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

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