Pollen limitation of female reproductive success at fine spatial scale in a gynodioecious and wind-pollinated species, *Beta vulgaris* ssp. *maritima*

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Abstract

In sexually polymorphic plants, the spatial distribution of sexes is usually not random. Local variation in phenotype frequencies is expected to affect individual fitness of the different phenotypes. For gynodioecious species, with co-occurrence of hermaphrodites and females, if sexual phenotypes are structured in space and pollen flow is spatially restricted, local pollen availability should vary among patches. Female fitness may thus be low when hermaphrodites are locally rare. To test this hypothesis, we analysed how the reproductive output of females varied among patches within two natural study sites of the gynodioecious wind-pollinated *Beta vulgaris* ssp. *maritima*. Plants growing in female-biased areas and experiencing pollen limitation were found to have low fruit and seed sets but did not reallocate resources towards better offspring. Our results show that fine-scale processes influence individual fitness and the evolution of sex ratio in sexually polymorphic plants.

Introduction

Sexually polymorphic flowering plants are defined by separate sexes or distinct mating groups that differ in floral morphology (Barrett, 2002). For these species, the reproductive output of a given sexual morph may depend not only on the characteristics of its own sex-category but also on the local frequency of the sexual phenotypes with which it can mate (e.g. Carlsson-Granér *et al.*, 1998; Jesson & Barrett, 2002; Stehlik *et al.*, 2006; Van Rossum *et al.*, 2006). Frequency dependence of reproductive output in sexually polymorphic plant populations plays a major role in the evolution of sexual morph ratios (Fisher, 1930).

Frequency-dependent processes are expected to take place in gynodioecious species, in which females and

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hermaphrodites co-occur. The genetics underlying sex expression in gynodioecious plants commonly involves cytonuclear epistatic interactions: cytoplasmic male sterility (CMS) genes located in the mitochondria confer a female phenotype, unless their action is counteracted by nuclear male fertility restorers (Saumitou-Laprade *et al.*, 1994; Chase, 2007). In gynodioecious species, the frequencies of the genes that determine sexual phenotype and the sex ratios often vary considerably among populations (e.g. Tarayre & Thompson, 1997; Olson & McCauley, 2002; Asikainen & Mutikainen, 2003; Alonso, 2005; Nilsson & Agren, 2006; Cuevas *et al.*, 2008; Dufay *et al.*, 2009). Pollen availability should thus vary among populations and be limited when hermaphroditic plants are locally rare. When the sex ratio shows pronounced variation among populations, each gender clusters with its own gender rather than with the other. Hermaphrodites are thus likely to grow in hermaphrodite-biased populations and to experience less pollen limitation, whereas clustered females are more likely to undergo pollen limitation. The negative effect of pollen limitation on fitness is then expected to mainly affect female

individuals (McCauley & Taylor, 1997; Pannell, 1997). Because any difference in seed production between genders has theoretically been shown to affect the maintenance of females in gynodioecious populations (Charlesworth & Charlesworth, 1978; Gouyon *et al.*, 1991) and because several studies have documented pollen-limited seed production of females in populations showing high female frequencies (e.g. Alonso, 2005; Zhang *et al.*, 2008), pollen limitation, especially in females, should be investigated carefully to understand the evolution of sex ratios in gynodioecious populations.

Moreover, sex ratio in gynodioecious species is also known to vary at very local scales, within populations (Laporte *et al.*, 2001; Olson *et al.*, 2006; De Cauwer *et al.*, 2010). This suggests that pollen-limited seed production may also occur in female-biased neighbourhoods within populations, although this has been shown in a restricted number of species only (see Widen & Widen, 1990; Graff, 1999). Because pollen limitation should be found at small scale only when pollen flow is spatially restricted within populations, one may wonder to what extent such process could occur in wind-pollinated species. Pollen from wind-pollinated plants is usually thought to be abundant (Cruden, 1977) and to travel long distances (Loveless & Hamrick, 1984), and pollen limitation has been found in many animal-pollinated species (Burd, 1994; Larson & Barrett, 2000; Ghazoul, 2005; Knight *et al.*, 2005) but rarely in wind-pollinated plants (Friedman & Barrett, 2009; but see Knapp *et al.*, 2001; Koenig & Ashley, 2003; Davis *et al.*, 2004).

The study of the effect of pollen limitation on female fitness was carried out on wind-pollinated, gynodioecious *Beta vulgaris* ssp. *maritima* (L.) Arcangeli. In this species, to develop as a female, an individual must carry an unrestored CMS gene. To develop as a hermaphrodite, an individual must either carry a CMS gene in combination with the appropriate restoration alleles (restored hermaphrodite) or carry a non-CMS cytoplasm (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Fénart *et al.*, 2006). Previous studies have suggested that the fitness of female plants could be frequency-dependent in *B. vulgaris*, even if it is a wind-pollinated species. Population structure in terms of sexual phenotypes is very pronounced at small spatial scales (Laporte *et al.*, 2001; De Cauwer *et al.*, 2010), probably resulting from limited seed dispersal and random founder events (Fievet *et al.*, 2007). Dufay *et al.* (2008) showed that pollen viability is highly variable among pollen producers and significantly lower in restored (CMS) hermaphrodites compared to non-CMS hermaphrodites. As restored hermaphrodites are often clustered with females in natural populations because of the strong genetic structure observed for cytoplasmic genes (Laporte *et al.*, 2001; De Cauwer *et al.*, 2010), only a restricted proportion of pollen reaching stigmas on females may be viable. Finally, the distribution of pollen dispersal events appears to be leptokurtic, with both a non-negligible proportion of long-distance dispersal

events and more than 40% of mating events occurring at < 15 m (De Cauwer *et al.*, 2010; see also Fénart *et al.*, 2007). Restricted within-population pollen movement in *B. vulgaris* ssp. *maritima* could then induce female reproductive failure when hermaphrodites are locally rare.

In this study, we investigated whether pollen limitation causes variations in female fitness by estimating differences in seed production among patches that contained either many, few or no hermaphrodites. Only females could be found in all of these cases and our work thus focused on the reproductive output of female plants only. Because processes occurring at a very fine-scale have been suggested to play a major role in the dynamics of gynodioecy (e.g. Olson *et al.*, 2006), we decided to compare the effects of pollen availability among several patches within two sites, rather than among many different sites. Our fine-scale experimental design allowed the survey of reproductive outputs throughout the whole flowering season, thus providing data representative of the global plant reproductive success. To ensure that variable resource levels or differences in plant age among study patches did not influence observed differences in female reproductive output, we also measured female reproductive success of controlled individuals that were transplanted in the studied patches. We address the following questions: (i) Is local hermaphrodite availability correlated with variation in fruit and/or seed production of female plants because of pollen limitation? (ii) If so, do pollen-limited females reallocate resources to produce more ovules and/or better seedlings than females found in more favourable pollen conditions?

Materials and methods

Study species

Sea beet, *B. vulgaris* ssp. *maritima*, is a diploid species ($2n = 18$) widely distributed along the western European coast and around the Mediterranean basin where it colonizes coastal habitats at the upper level of high tides (Laporte *et al.*, 2001; Arnaud *et al.*, 2003; Viard *et al.*, 2004; Fievet *et al.*, 2007). It is a short-lived perennial, wind-pollinated and self-incompatible species (Letschert, 1993). Each individual bears one to several hundred floral stems carrying long, dense racemose inflorescences at their apex. Wild sea beet fruits result from the joint development of several flowers that mature into a single hard and woody fruit aggregate (hereafter fruits). Each cluster of flowers contains 1–8 flowers and, because the flowers are uniovulate, each fruit contains 1–8 seeds. An individual plant bears from a few to several thousand flowers (I. De Cauwer, unpublished data). Only some of the flowers open simultaneously along the floral stems within an individual plant. Plants flower from mid-May through mid-July and are widely synchronous.

Sexual phenotype in *B. vulgaris* is determined by interactions between maternally inherited CMS genes

and biparentally inherited nuclear male fertility restorers. In contrast to some other gynodioecious species, a large part of cytoplasmic diversity in *B. vulgaris* is associated with nonsterilizing factors. Only four of 20 mitochondrial haplotypes described in wild populations are associated with sexual polymorphism, meaning that two types of hermaphrodites co-exist: restored hermaphrodites (carrying CMS genes) and non-CMS hermaphrodites (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Fénart *et al.*, 2006). Populations of *B. vulgaris* are known to be highly structured for nuclear and cytoplasmic genes involved in gender polymorphism (Laporte *et al.*, 2001; De Cauwer *et al.*, 2010), allowing the examination of the effect of population structure and variation in local sex ratio on female reproductive output.

Study sites and local sex ratio

We chose to examine female fitness in sites that show normal variation in hermaphrodite frequency. *Beta vulgaris* individuals are usually found along stretches of beach extending over several hundred metres. Within these sites, the density of individuals is often heterogeneous with plants clustering in discrete patches. Previous studies on *B. vulgaris* showed that, at the site level, hermaphrodite frequencies vary from 57% to 100% (Dufay *et al.*, 2009) and that within-site (*i.e.* among patches) sex ratio variation can be even higher (De Cauwer *et al.*, 2010). In this study, we took advantage of the natural variation in sex ratio in two natural sites: (i) Roscoff site (48°43.268N, 4°00.548E, Brittany – France) comprising several thousand flowering individuals clustered in large patches along the coast, which was surveyed in 2007 and (ii) Audresselles site (50°49.101N, 1°35.676E, Northern France) comprising approximately 400 flowering individuals, which was surveyed in 2008. Within each study site, we defined two types of patches: (i) low hermaphrodite (LH) patches, characterized by LH frequencies (< 15%) and

(ii) high hermaphrodite (HH) patches, characterized by HH frequencies (> 70%). In Roscoff, we studied one LH patch and one HH patch. These two patches were separated by 600 m with a few isolated plants growing in between ($N < 50$). In Audresselles, we chose two LH patches (LH_1 and LH_2) and one HH patch (Fig. 1). As in Roscoff, a few isolated individuals were growing between patches ($N < 10$). LH_1 was closer to the HH patch (140 m) than LH_2 (300 m), which was located at the southern edge of the site. The number of individuals per studied patch varied from 27 to 289 (Fig. 1). All studied patches extended over < 40 m. Sexual phenotype of all flowering individuals in each study patch was determined during the peak flowering period and was used to estimate the local sex ratio (i.e. local hermaphrodite frequency).

Within each study patch, the pollen receipt of a given plant could vary with (i) the local sex ratio, (ii) the distance to pollen producers and (iii) the type of local pollen producers (with restored hermaphrodites producing low quality pollen compared to non-CMS hermaphrodites, see Dufay *et al.*, 2008). While this last aspect was not studied in the current work, our study sites comprised variation of the first two features (see Fig. 1).

Female fitness

Surveyed females

To examine the effect of pollen limitation on female fitness within the two study sites, we randomly selected female plants growing in the patches at the beginning of the flowering survey. Only female individuals were used in this study because of the very low numbers of hermaphrodites in some patches (less than four hermaphrodites in the three LH patches, see Fig. 1). At Roscoff, 23 females growing in the HH patch and 26 females growing in the LH patch were surveyed. At Audresselles, we studied 20 females located in the HH patch, 18 females located in the LH_1 patch and six
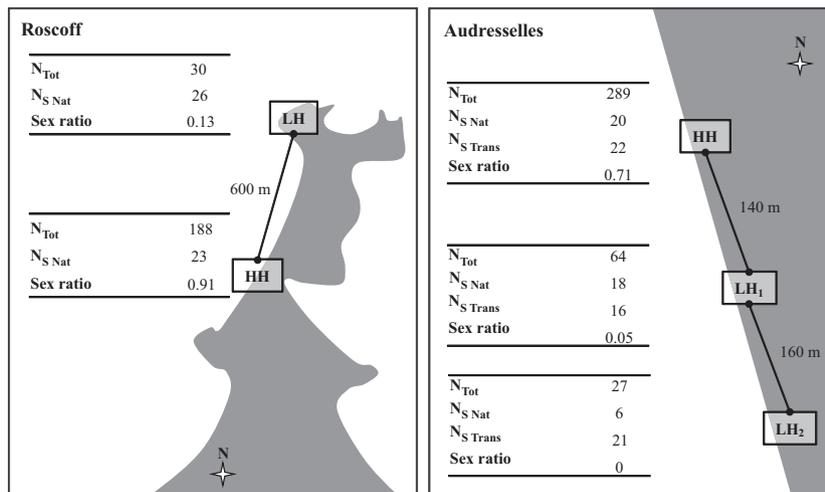

Fig. 1 Schematic diagram of the patches studied within the two study sites along shore lines, showing the total number of individuals per patch (N_{tot}), the number of surveyed individuals (N_S, with N_{SNat} for natural individuals and N_{STrans} for transplanted individuals), local sex ratio (i.e. hermaphrodite frequency within each patch) and distances between patches. Low hermaphrodite (LH) patches contain < 15% of hermaphrodites and high hermaphrodite (HH) patches contain more than 70% of hermaphrodites.

females located in the LH_2 patch (Fig. 1). To prevent confounding effects, we selected plants that were flowering at the same time.

Because any observed variation in female reproductive output could also be caused by some environmental heterogeneity among patches or age differences among plants, we also measured female reproductive outputs in natural conditions using transplanted individuals previously grown in controlled conditions. These individuals were half-sibs sharing the same female mother plant originating from Roscoff. They were sown in autumn 2007 and grown in a greenhouse. In 2008, 90 individuals were transplanted in the Audresselles site before flowering (30 individuals per studied patch). These transplanted individuals were kept in pots during the entire flowering season, allowing us to study the effects of variation of local sex ratio on individuals that (i) had the same age and (ii) were growing in similar conditions. Among the transplanted individuals, three plants expressing a hermaphroditic phenotype and 28 plants that did not bloom were removed. Consequently, 22, 16 and 21 transplanted female plants were surveyed in the HH patch, LH_1 patch and LH_2 patch, respectively (Fig. 1).

Field methods
A large majority of experimental studies estimate pollen limitation in plant populations employing supplemental hand pollination methods. If the reproductive output of an individual plant increases with supplemental hand pollination, the plant is assumed to be pollen-limited. However, this approach has at least two important caveats. Supplemental pollination may involve higher quality pollen than natural pollen (Ashman *et al.*, 2004), and resource reallocation may influence estimates of pollen limitation (Stephenson, 1981; Zimmerman & Pyke, 1988). This latter issue can have important consequences on the estimation of pollen limitation, especially when the supplemental pollination treatment is applied only to a fraction of the flowers of the study individuals (Knight *et al.*, 2006). Circumventing this issue could imply applying pollen supplementation to whole plants (Ashman *et al.*, 2004), but was impossible for *B. vulgaris*, given that each plant produces several hundred to several thousands of flowers in a single flowering season. For all these reasons, we chose to compare open pollinated females located in areas characterized with contrasted sex ratios (LH patches vs. HH patches), without using classical supplementations.

Temporal survey started on 19 May 2007 and lasted 5 weeks in Roscoff and it started on 15 May 2008 and lasted 6 weeks in Audresselles. For each surveyed female, one to five floral stems of similar size were tagged. Starting from the onset of flowering, the number of flowers and the number of flower clusters that opened along the tagged stems were counted each week. Each newly opened stem section was delimited with a mark. This allowed assessing when each ovule was receptive

and potentially fertilized. In Roscoff, a total of 12 924 flowers were observed, forming 4982 flower clusters (mean number of flowers per cluster ± SD: 2.62 ± 0.44). In Audresselles, we observed 24 836 flowers and 10 196 flower clusters (mean number of flowers per cluster ± SD: 2.38 ± 0.58) on natural individuals and 12 081 flowers distributed across 5417 flower clusters (mean number of flowers per cluster ± SD: 2.29 ± 0.42) on transplanted individuals.

Several weeks after the end of flowering (mid-August), fruits from each surveyed stem were collected between the marks. We then calculated fruit set as the number of fruits divided by the number of flower clusters, for each plant and each week. Given the structure of fruits in *B. vulgaris*, it was not possible to count the number of seeds per fruit and assess seed set (i.e. ratio between the number of seeds and the number of available ovules). Instead, we estimated seed set by counting the number of seedlings that emerged from fruits, which was the only way to estimate the number of viable seeds per fruit. Seed set was thus estimated as the number of seedlings divided by the number of initially available ovules (i.e. the number of flowers, as flowers are uniovulate), for each plant and each week. To do so, all fruits of mother plants (or a random selection of 50 fruits for mother plants producing more) were sown. We sowed 1179 fruits from the Roscoff site, 1635 fruits produced by the natural individuals of the Audresselles site and 927 fruits produced by the transplanted individuals of the Audresselles site. Germination was monitored for 2 months, and each seedling was collected 15 days after emergence. After these 2 months, all fruits from both study sites were stored in dry conditions at room temperature for 4 weeks, to remove dormancy. Final germination rates were determined after a second 2-month germination survey. Because nutrients in potting soil probably decrease with repeated watering, only the seedlings emerging from the first germination study were weighed to estimate offspring quality. They were weighed to the nearest 0.01 mg after being collected and dried at 56 °C for 48 h. Seedlings presenting signs of fungal infection were removed from the weight measurement. In total, we weighed 853 seedlings from the Roscoff site, 1022 seedlings produced by the natural individuals of the Audresselles site and 469 seedlings produced by the transplanted individuals of the Audresselles site.

Data analysis

We tested whether female fertility was limited by pollen availability in a frequency-dependent way, i.e. whether fruit set and seed set were lower in LH patches than that in HH patches. Because flowering duration of the surveyed floral stems was variable within and among studied females, we did not use repeated-measures statistical tests: these tests can only be used to analyse a data set with the same number of temporal measures for

all plants and this would have reduced the sample size. Logistic regressions were used to analyse fruit set (proportion of flower clusters giving a fruit, during a given temporal section) and seed set (proportion of seedlings emerging from fruits relative to the number of flowers produced, during a given temporal section). Two explanatory factors were tested throughout these analyses: flowering date (coded as a quantitative factor: week 1–5, starting on 19 May 2007 in Roscoff and week 1–6, starting on 15 May 2008 in Audresselles) and patch type (coded as a qualitative factor: LH and HH in Roscoff, LH$_1$, LH$_2$ and HH in Audresselles). These regressions were performed using PROC GENMOD (binomial distribution, using a log link function) in SAS (version 9.1, SAS Institute Inc., Cary, NC, USA) with a correction for over-dispersion. All nonsignificant interaction terms between pairs of variables were dropped from the analyses. Differences between patches were explored using contrast analyses.

If fruit set and seed set depend on local sex ratio, flower production and seed quality may also depend on it. With no pollen limitation, energy may be diverted from future flower production towards fruit and seed production, whereas under pollen limitation, plants could allocate resources to increase flower production or seed quality. We thus compared the average number of flowers produced per day and per stem and the average seedling dry mass between LH and HH patches using general linear models (PROC GLM in SAS version 9.1). These analyses tested for an effect of flowering date and patch type. Differences between patches were explored using *post hoc* Tukey's pairwise comparisons.

Results

Characteristics of the study sites

The observed local sex ratios were highly variable within both study sites. In Roscoff, we found 13% of hermaphrodites in the LH patch and 91% of hermaphrodites in

the HH patch. In Audresselles, we observed 0%, 5% and 71% of hermaphrodites in LH$_2$, LH$_1$ and HH patches, respectively (Fig. 1). On average, the surveyed floral stems that were used in this study (one to five for each surveyed female plant, depending on its size) flowered for 18 days (SD ± 6 days) in Roscoff, for 26 days (SD ± 5 days) for natural individuals in Audresselles and 22 days (SD ± 9 days) for transplanted individuals in Audresselles. Flowering duration of the surveyed stems was not significantly affected by patch type (general linear models, $P > 0.05$ in all cases). The size of the surveyed plants, estimated by the total number of flowering stems, did not vary among patches within study sites (general linear models, $P > 0.05$ in all cases).

Female reproductive output and pollination neighbourhood

Local sex ratio and fruit set
Overall, the proportion of flower clusters setting fruit was 20% for plants growing in LH patches (i.e. potentially pollen-limited patches, with < 15% of hermaphrodites) compared to 57% in HH patches (i.e. patches where no pollen limitation was expected, characterized by hermaphrodite frequencies higher than 70%). Patch type appeared to have a highly significant effect on fruit set, independently of study site (Table 1). Individual plants growing in HH patches always set more fruit than individuals growing in LH patches, except for the HH/LH$_1$ comparison involving the natural individuals from Audresselles for which no significant difference was found ($P > 0.05$). For both transplanted and natural individuals of the Audresselles site, fruit set was significantly higher for females growing in the LH$_1$ patch (comprising 5% of hermaphrodites and located at 140 m from the HH patch) compared with females growing in the pure female LH$_2$ patch (located 300 m away from the HH patch and 160 m from the closest hermaphrodites). Flowering date had an overall negative effect on fruit set

Table 1 Effects of patch type and flowering date on fruit set and seed set (both following a binomial distribution) of sea beet (*Beta vulgaris* ssp. *maritima*) for both Roscoff and Audresselles sites. For quantitative factors that were found to have a significant effect, the symbol between brackets shows the sign of the regression slope. All nonsignificant interaction terms between variables were dropped from the statistical analyses.

Variable and source of variation	Roscoff			Audresselles			Audresselles		
	Natural individuals			Natural individuals			Transplanted individuals		
	d.f.	χ^2	P	d.f.	χ^2	P	d.f.	χ^2	P
Fruit set									
Patch type	1	57.93	< 10^{-4}	2	16.52	0.0003	2	54.58	< 10^{-4}
Flowering date	1	8.27	0.004 (−)	1	0.27	0.6026	1	1.97	0.1602
Patch type × flowering date		−		2	14.79	0.0006	2	16.22	0.0003
Seed set									
Patch type	1	5.83	0.0158	2	6.03	0.049	2	6.13	0.0465
Flowering date	1	1.82	0.1776	1	0.31	0.5765	1	6.74	0.0094 (+)
Patch type × flowering date		−			−		2	5.17	0.0752

in Roscoff, with fruit set decreasing throughout the flowering season. As illustrated by the significant interactions between flowering date and patch type (Table 1), flowering date had an even stronger negative effect on fruit set in HH and LH$_1$ patches for the natural individuals of Audresselles as well as in the HH patch for the transplanted individuals of Audresselles (data not shown).

Local sex ratio and seed set

The results for seed set estimated *via* germination rates mirrored the patterns found for fruit set, with a significant effect of patch type for the three data sets (Table 1). Overall, the effect of patch type on seed set was weaker than what was observed for fruit set: plants growing in HH patches set on average three times more fruits and 1.4 times more seeds than plants growing in LH patches (Fig. 2). Plants growing in HH patches always had significantly higher seed set than plants growing in LH patches, except for the HH/LH$_1$ comparison for the natural individuals of Audresselles, where no difference was observed. Seed set increased through time, but only for individuals transplanted in Audresselles (Table 1). This positive effect of flowering time on seed set was stronger in the two LH patches than in the HH patch (data not shown), as illustrated by the significant interaction between patch type and flowering date (Table 1).

Maternal fitness and offspring quality

Above results show that differences in pollen availability, resulting from spatial variation in sex ratio, affect at least two important components of female reproductive output: fruit set and seed set. To draw clear conclusions on the effect of pollen limitation on fitness, we also assessed whether differences in pollination neighbourhoods could influence the quality of offspring and/or maternal fitness *via* resource reallocations. We thus investigated whether pollen-limited females produced more ovules and/or better seedlings than females growing in more favourable pollen conditions, owing to reallocation of resources not used for setting seeds and fruits. The effects of patch type and flowering date were tested on the average number of flowers produced per day and per stem and on the average seedling weight.

Ovule production

Plants growing under pollen limitation tended to increase their ovule production. Female plants growing in LH patches produced more ovules than those located in HH patch in Roscoff, as did the natural individuals in Audresselles (Table 2 and Fig. 3). In Roscoff, we found no significant effect of flowering date but a significant interaction between patch type and flowering date that can be explained by a positive effect of time on ovule production for LH individuals and a negative effect of the time on ovule production for HH individuals. For the natural individuals in Audresselles, no significant difference was detected between HH and LH$_1$ plants, which all produced significantly less ovules than LH$_2$ plants. For Audresselles plants, ovule production decreased with time. Finally, no flowering date or patch type effects were detected on the ovule production for individuals transplanted in Audresselles (Table 2).

Fig. 2 Fruit set and seed set by patch type for the three data sets (natural individuals in Roscoff, natural individuals in Audresselles and transplanted individuals in Audresselles). Low hermaphrodite patches (LH) are represented by dark grey boxes and high hermaphrodite patches (HH) are represented by light grey boxes. White circles indicate the mean observed values and the horizontal line within the box indicates the median. The boxes contain the observed values from the lower quartile (25%) to the upper quartile (75%) of the distribution and include 50% of the observed values. The box whiskers encompass 98% of the observed values (1–99% of the distribution) and the black circles represent outliers. Different letters indicate significant pairwise comparisons within sites (contrast analyses, $P < 0.05$).
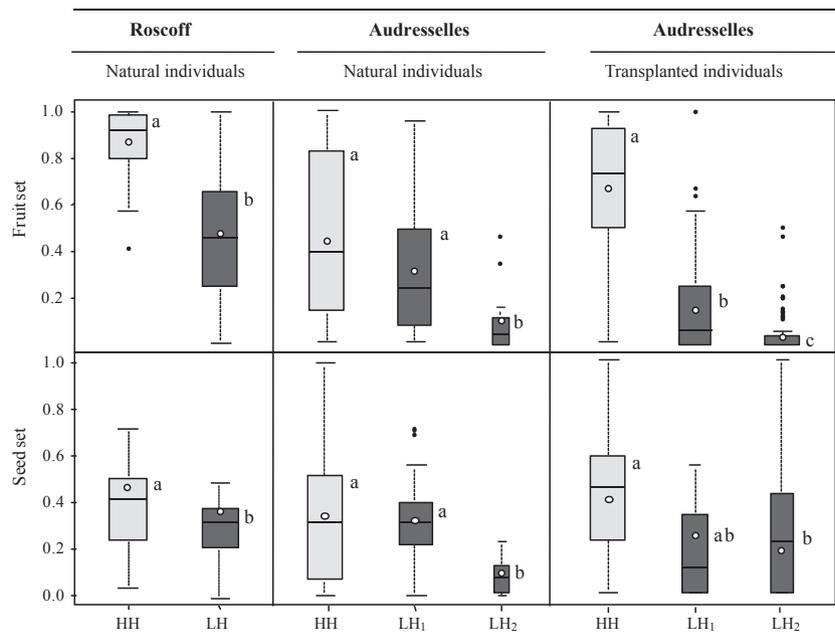
Table 2 Effects of patch type and flowering date on the mean number of flowers produced per day and per stem and the mean seedling weight for the two study sites of sea beet (*Beta vulgaris* ssp. *maritima*): Roscoff and Audresselles. For quantitative factors that were found to have a significant effect, the symbol between brackets shows the sign of the regression slope. All nonsignificant interaction terms between variables were dropped from the statistical analyses.

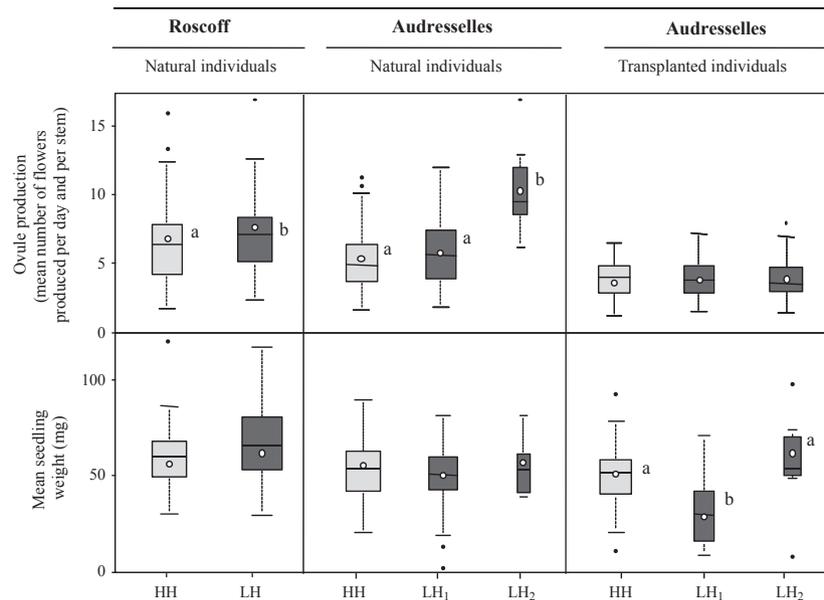| | Roscoff | | | | Audresselles | | | | Audresselles | | | |
| | Natural individuals | | | | Natural individuals | | | | Transplanted individuals | | | |
Variable and source of variation	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*	d.f.	MS	*F*	*P*
Mean ovule number												
Patch type	1	46.61	5.70	0.0186	2	140.53	30.30	$< 10^{-4}$	2	0.39	0.21	0.8145
Flowering date	1	4.72	0.58	0.4489	1	55.83	12.04	0.0007 (−)	1	2.97	1.56	0.2130
Patch type × flowering date	1	76.96	9.42	0.0027				−				−
Error	112	8.17			160	4.63			176	1.90		
Seedling weight												
Patch type	1	9.21E-05	0.69	0.4106	2	8.47E-05	0.29	0.7488	2	3.61E-03	10.93	$< 10^{-4}$
Flowering date	1	2.45E-04	1.83	0.1812	1	1.74E-04	0.6	0.4415	1	5.18E-04	1.57	0.2140
Error	59	7.9E-03			109	3.18E-02			73	3.3E-04		

Fig. 3 Ovule production (mean number of flowers produced per day and per stem) and mean seedling weight by patch type for the three data sets (natural individuals in Roscoff, natural individuals in Audresselles and transplanted individuals in Audresselles). Low hermaphrodite patches (LH) are represented by dark grey boxes and high hermaphrodite patches (HH) are represented by light grey boxes. White circles indicate the mean observed values and the horizontal line within the box indicates the median. The boxes contain the observed values from the lower quartile (25%) to the upper quartile (75%) of the distribution and include 50% of the observed values. The box whiskers encompass 98% of the observed values (1–99% of the distribution) and the black circles represent outliers. Different letters indicate significant pairwise comparisons within sites (contrast analyses, *P* < 0.05).

Offspring quality
Average seedling dry weight 15 days after emergence was 58 mg (SD ± 12 mg) at Roscoff, 52 mg (SD ± 17 mg) and 47 mg (SD ± 20 mg) for natural and transplanted individuals in Audresselles, respectively. We tested whether pollen-limited females reallocate resources that were not used for fruit and/or seed production towards

better offspring. This being the case, females growing in LH environments should produce heavier seedlings compared to females located in HH environments. Overall, there was no clear trend suggesting resource reallocation towards better offspring for females growing in potentially pollen-limited environments. Patch type influenced seedling weight only in individuals

transplanted in Audresselles, with LH_1 individuals producing significantly lighter offspring than HH and LH_2 individuals (Table 2 and Fig. 3). Seedling weight was not affected by flowering date in any of the three data sets (Table 2).

Discussion

This study showed evidence for pollen limitation of reproduction in *B. vulgaris* spp. *maritima*. Such result was unexpected in a wind-pollinated plant species and at such a small spatial scale (a few hundred metres). As wind pollination is thought to have primarily evolved as a mechanism of reproductive assurance, pollen limitation in wind-pollinated species is probably not as frequent as in animal-pollinated species (Friedman & Barrett, 2009). The results presented here provide a clear account that pollen abundance and high pollen dispersal capabilities – both prime features of wind-pollinated plants – may not always be sufficient to ensure optimal female reproductive output in sexually polymorphic species, even at a small spatial scale. In the next section, we discuss the effect of population sex structure on female fitness, patterns of resource reallocation and the possible outcomes for the dynamics and maintenance of polymorphic mating systems.

Population structure and female reproductive output

In *B. vulgaris*, as in many other gynodioecious species, both nuclear and cytoplasmic genes are involved in sex expression (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Fénart *et al.*, 2006). In this study, sexual phenotypes were strongly structured within sites, which could result from (i) limited seed dispersal, resulting in limited dispersal of the CMS genes and (ii) founding events preventing associations between particular CMS genes and their associated restorers within patches (e.g. Manicacci *et al.*, 1996; Tarayre & Thompson, 1997; De Cauwer *et al.*, 2010). In this study, female reproductive output of *B. vulgaris* (estimated *via* measures of fruit set and seed set) was found to be lower in female-biased patches than in more favourable pollen environments, probably because of insufficient pollen receipt when hermaphrodites are locally rare. Because the same results were found for individuals of same age and grown in the same conditions (transplanted in Audresselles), the observed heterogeneity in female reproductive output among patches cannot be explained by confounding factors such as local environment or age.

Plants growing in the most favourable pollen environments (HH patches) set on average three times more fruits and 1.4 times more seeds than plants growing in pollen-limited areas. The effect of patch type on seed set was thus weaker than what was observed for fruit set. This is a common trend: experimental studies measuring fruit set frequently report a stronger effect of pollen

limitation than those measuring seed set, perhaps because many plants species will not produce fruit unless adequate pollen receipt is reached (Mitchell, 1997; Knight *et al.*, 2006). Our results also suggest that fruit set tends to decrease throughout the flowering season in Roscoff. This trend was also observed in both natural and transplanted individuals in Audresselles, but only for the plants growing in the most favourable pollen environments. This overall negative effect of flowering time can result from (i) a decrease in available pollen during the flowering season (Burd, 1994) and/or (ii) a decrease in individual resources with time (Wesselingh, 2007). Given our experimental design, we cannot quantify the relative impact of these two processes on female reproductive output. However, if the decreased fruit set is partly because of lower pollen availability at the end of the flowering season, this could explain why seed set was not affected by flowering time for the same reasons given earlier (seed set was measured on flower clusters that set fruits and did receive enough pollen).

For both transplanted and natural individuals in Audresselles, fruit set and seed set were higher for female plants growing in the LH_1 patch than those growing in the LH_2 patch. Individuals growing in LH_1 patch showed intermediate levels of fruit and seed production between those of the two extreme situations (HH and LH_2), and, while we found statistical differences between the three patches for transplanted individuals, fruit set and seed set were statistically indistinguishable between HH and LH_1 for natural individuals. This intermediate situation of LH_1 could be explained by its intermediate characteristics in terms of spatial isolation and local sex ratio. The better female reproductive output in LH_1 compared to LH_2 could indeed be explained by (i) the occurrence of a few hermaphrodites in that specific patch (whereas they were completely lacking within the LH_2 patch) and/or (ii) the geographical proximity of the HH patch (140 m). Our results thus suggest that long-distance pollen dispersal in *B. vulgaris* (see Fénart *et al.*, 2007; De Cauwer *et al.*, 2010) does not compensate for the lack of hermaphrodites at short distances in female-biased patches. This is illustrated by the data collected in LH_2 patch in Audresselles: whereas no hermaphrodites were locally available, female plants did set fruits and thus relied on pollen coming from distant patches. However, long-distance pollen dispersal was clearly insufficient to compensate the local absence of hermaphrodites, resulting in low values of fruit set and seed set.

In addition to the effects of spatially restricted pollen dispersal and of variation in local sex ratios, the nature of local pollen producers could also affect female reproductive output. Restored hermaphrodites, producing low quality pollen (Dufay *et al.*, 2008), are often clustered with females in natural populations because of the strong genetic structure observed for cytoplasmic genes (e.g. De Cauwer *et al.*, 2010). The occurrence of restored hermaphrodites could have contributed to pollen limitation

in LH patches. Although such effect was not tested in the current study, it is likely that both limited pollen quantity and limited pollen quality explain the lower female reproductive output observed in female-biased patches.

Pollen limitation and resource reallocation

Our study suggests that sex structure can cause a decrease in the overall female contribution to the next generation compared to a panmictic population, which could ultimately result in a negative frequency-dependent selection against females. However, this trend could be countered if pollen-limited females reallocate resources not used for seed or fruit production towards increased offspring quality (Ashman *et al.*, 2004). Here, we found no clear evidence of such reallocation in pollen-limited environments.

Individuals growing in pollen-limited environments in the two study sites increased their ovule production, excepted individuals transplanted in Audresselles. For these plants, root development, and thus available resource quantity, was probably limited because they were maintained in pots during the flowering survey. A trade-off between ovule production and offspring quality could explain why the plants that produced more flowers to compensate for pollen limitation did not allocate more resources in the few offspring they produced. Reallocating resources towards increased ovule production may actually not increase female reproductive output in *B. vulgaris*. Indeed, flowering was largely synchronous within patches and local sex ratio was thus expected to be quite stable during a given flowering season. In these conditions, increasing ovule production under pollen limitation may not result in higher female fitness.

Finally, as we studied only one flowering season in both study sites, we cannot exclude resource allocation trade-offs between seed or fruit production and other components of maternal fitness in perennial species (such as growth, survival and probability of flowering in the next years, see Ashman *et al.*, 2004; Knight *et al.*, 2006).

Implications for the dynamics of gynodioecy

In gynodioecious species, clustering of sexual phenotypes within populations is likely to benefit to hermaphrodites, which are on average located in favourable pollen environments, and to decrease the reproductive output of females, which are more likely to undergo pollen limitation. As a consequence, pollen limitation may affect more female individuals than hermaphrodites (e.g. Widen & Widen, 1990; Sugawara, 1993; McCauley & Brock, 1998; Graff, 1999; Alonso, 2005; Zhang *et al.*, 2008; but see Shykoff *et al.*, 2003). In that context, pollen limitation is likely to have an effect on the growth of female-biased patches, if growth rates are sensitive to changes in fruit and seed production (Ashman *et al.*,

2004). Overall female frequencies and thus CMS frequencies in structured populations are likely to be lower compared with an ideal panmictic population (McCauley & Taylor, 1997; Pannell, 1997). The perennial life cycle and the long-lived seed bank in *B. vulgaris* can buffer this negative effect of pollen limitation on female reproductive output. However, as gene flow through seed and pollen dispersal is thought to be spatially limited in the study species (Fievet *et al.*, 2007; Arnaud *et al.*, 2009; De Cauwer *et al.*, 2010), seed banks in female-biased patches are likely to be female-biased and pollen limitation should persist, unless non-CMS cytoplasm and/or restorer alleles associated with the local CMS invade the patch. According to Dufay *et al.* (2009), the three main CMSs that occur in *B. vulgaris* are characterized by significantly different average restoration rates. CMSs that are poorly restored may experience more pollen limitation, which could slow down their invasion.

Whereas females are obligate outcrossers and depend entirely upon hermaphrodite pollen for reproduction, hermaphrodites in most gynodioecious species are self-compatible (Charlesworth, 1981). Under pollen limitation, selfing is likely to favour reproduction of hermaphrodites, although this effect can be modified by inbreeding depression (Maurice & Fleming, 1995). However, the compensation effect of selfing probably does not occur in *B. vulgaris*, as it is self-incompatible (Owen, 1942; Larsen, 1977). Hermaphrodites and females may thus undergo similar levels of pollen limitation of fruit and seed production. Fruit and seed set of hermaphrodites are thus expected to be lower in female-biased patches compared to hermaphrodites located in more favourable pollen environments. Our study aimed at comparing extremely contrasted situations in terms of sex ratio, and this particular experimental design did not allow us to investigate pollen limitation in hermaphrodites. However, this constitutes an interesting perspective of this work, which could be carried out through a controlled experimental study. If hermaphrodite fitness is affected by pollen limitation, frequency-dependent selection could act on different levels depending on the mating system: in self-compatible species, selection should act both at the individual level and at the patch level, whereas in self-incompatible species, selection is likely to operate only at the patch level (Dufay & Pannell, 2010).

As in our study sites, *B. vulgaris* populations are frequently a mosaic of patches where CMS genes are frequent and patches where CMS genes are rare (Laporte *et al.*, 2001; De Cauwer *et al.*, 2010). Hermaphrodites bearing a CMS gene and the appropriate restorer allele are thus likely to be located in female-biased patches and should on average suffer more pollen limitation than non-CMS hermaphrodites. In addition to this effect of structure on female fitness, male reproductive output of hermaphrodites in female-biased patches may be

increased because they sire more seedlings than hermaphrodites in hermaphrodite-biased patches, which undergo stronger pollen competition (De Cauwer *et al.*, 2010). Population structure can thus have contrasting and complex effects on different fitness components of hermaphrodites. Sex structure is likely to affect the three sexual phenotypes co-existing in natural population in different ways: (i) female fitness of non-CMS hermaphrodites is favoured because being clustered together provides them reproductive assurance; however, their male fitness is low because of local conspecific pollen competition; (ii) female plants suffer a global fitness reduction because of pollen limitation and (iii) female fitness of restored CMS hermaphrodites is decreased by pollen limitation, but their male fitness is increased because plants are often clustered with females and are the only available local pollen producers. Consequences of the combination of above processes should be investigated by both theoretical and experimental studies of gynodioecy.

Conclusions and perspectives

Our results clearly illustrate how fine-scale population structure of sexual phenotypes affects individual female fitness through inadequate pollen receipt when hermaphrodites are locally rare. Frequency-dependent female reproductive output was unexpected at such local scale, especially in a wind-pollinated species. Our work shows that pollen abundance and long-distance dispersal, which are characteristics of wind-pollinated plants, may thus not be sufficient to ensure optimal female reproductive output in sexually polymorphic species. Fine-scale population structure of sexual phenotypes is likely to result in lower female frequencies and lower CMS frequencies compared with a panmictic population. Empirical studies on the maintenance of gynodioecy, the evolution of sex ratios or, more generally, the dynamics of any polymorphic trait under frequency-dependent selection should probably not consider gene or morph frequencies at the scale of the whole population, but rather investigate how these frequencies vary at small spatial scales.

Acknowledgments

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