

# Changes in pollinator fauna cause spatial variation in pollen limitation

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## Summary

1. Pollen limitation may be a consequence of changes in pollinator abundance, diversity and identity. However, no empirical evidence exists concerning the consequences that the spatial variation in pollinator fauna has on pollen limitation intensity and plant reproduction. In this study, we test the effect that changes in flower-visitor abundance, diversity and identity exert on the occurrence and strength of pollen limitation by experimentally quantifying pollen limitation in eight populations of *Erysimum mediohispanicum*, a pollination-generalist plant native to the Iberian Peninsula.
2. Pollen limitation was accounted for by using a comprehensive estimator, the net reproductive rate ( $R_0$ ). Nevertheless, we also determined which components of plant reproduction, from ovule fertilization to seedling survival, were more intensely pollen-limited. Finally, we explored whether the spatial variation in pollen limitation intensity was related to among-population changes in flower-visitor abundance, diversity and identity.
3. The whole reproductive cycle of *E. mediohispanicum* was pollen-limited, although pollen limitation occurred more strongly during the ovule fertilization and seed-production phases than during fruit ripening or seedling emergence and establishment.
4. There was a significant among-population difference in pollen limitation intensity. Pollen limitation strength was associated with variations in flower-visitor diversity, and identity. Populations with lower flower-visitor diversity and with many low-efficiency pollinators (i.e. beetles) showed stronger pollen limitation.
5. *Synthesis.* Our study shows that the intensity of pollen limitation at the population level may depend on several characteristics of the assemblage of flower-visiting insects, such as their abundance, diversity and identity. Our results suggest that any impoverishment of pollinator diversity or any alteration in the specific composition of the pollinator assemblage may exacerbate pollen limitation.

**Key-words:** demography, *Erysimum mediohispanicum*, pollen limitation, pollination generalization, pollinator composition, pollinator diversity, reproductive ecology, spatial variation

## Introduction

Pollen limitation, a decrease in potential plant reproduction due to inadequate pollen receipt, is ubiquitous across Angiosperms (Larson & Barrett 2000; Ashman *et al.* 2004; Knight *et al.* 2005). The frequency and potential consequences of pollen limitation for plant populations and communities have been intensely explored during the last decades, both empirically (see reviews by Burd 1994; Larson & Barrett 2000; Ashman *et al.* 2004; Knight *et al.* 2005; Knight, Steet & Ashman 2006; García-Camacho & Totland 2009) and theoretically

(Haig & Westoby 1988; Ashman *et al.* 2004; Morgan, Wilson & Knight 2005; Harder & Routley 2006; Aizen & Harder 2007; Wesselingh 2007; Burd 2008; Richard, Williams & Harder 2009; Harder & Aizen 2010).

Several non-exclusive factors acting simultaneously or consecutively may cause pollen limitation, such as presence of co-flowering species (Bell, Karron & Mitchell 2005), plant pathogens, nectar robbers, herbivores or seed predators (Vázquez & Simberloff 2004), and habitat fragmentation or degradation (González-Varo, Arroyo & Aparicio 2009; Spliger & Chang 2009). Nevertheless, the proximate direct cause of pollen limitation is always a shortage in pollen quantity or a low-quality supply (Aizen & Harder 2007). Harder & Aizen (2010)

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have proposed five main reasons for pollen limitation: limitation in pollinator visits, limited pollen availability, inefficient pollen transfer, low pollen-tube survival and zygote death. Limitation in pollinator visits can occur because either flower visitors are rare or because they prefer visiting other, more attractive plants (Totland & Sottocornola 2001; Hegland & Totland 2008; Mitchell *et al.* 2009). An impoverishment of the pollinator assemblage may also generate visit limitation when different pollinator species have complementary effects on plant fitness (Klein, Steffan-Dewenter & Tscharrntke 2003; Gómez *et al.* 2007; Perfectti, Gómez & Bosch 2009). In this respect, it is widely assumed that specialized plants will be more prone to experiencing pollen limitation than generalized plants (Knight *et al.* 2005). On the other hand, a shift in the identity of the flower visitors resulting in an overrepresentation of low-quality pollinators can also bring about pollen limitation by causing limited pollen availability, inefficient pollen transfer, low pollen-tube survival and zygote death (Harder & Aizen 2010). In fact, pollen limitation may result not only when pollinators are rare or absent, but also when they are inefficient at pollen transfer, deposit much heterospecific pollen, behave mostly as nectar or pollen thieves, transport mostly autogamous and geitonogamous pollen, etc. (Elle & Carney 2003; Aizen & Harder 2007; McMullen 2009; Harder & Aizen 2010; Vaughton & Ramsey 2010). Unfortunately, the empirical evidence showing how changes in the pollinator fauna can affect the magnitude and intensity of pollen limitation is very scarce (González-Varo, Arroyo & Aparicio 2009), despite this issue being crucial to fully understanding the ability of many plant species to cope with changing environments (Eckert *et al.* 2010).

An accurate estimation of pollen limitation requires an exploration of several reproductive components, since the effect of inadequate pollen transfer can be revealed at different life cycle stages (Knight *et al.* 2005). For a complete picture of its effects on the growth of plant populations, pollen limitation should be determined throughout the plant's lifetime reproductive cycle (Calvo 1993; Ehrlén & Eriksson 1995). Unfortunately, this task is difficult and most studies to date have examined only a very limited portion of the plant reproductive cycle, mostly fruit and seed production (Knight *et al.* 2005; Knight, Steet & Ashman 2006). By contrast, virtually no information has been gathered concerning the consequences of pollen limitation on post-dispersal stages such as seed germination or seedling emergence and survival (Ehrlén & Eriksson 1995; García & Ehrlén 2002; Price *et al.* 2008; Horvitz, Ehrlén & Matlaga 2010). Many self-compatible plants exhibit reproductive assurance, compensating by self-fertilization for a shortage of outcross pollen (Eckert, Samis & Dart 2006). Quantifying pollen limitation during seed production may thereby underestimate its strength in these species, since the consequences of pollen limitation could show up on seed and seedling vigour.

The main goal of this study is testing the hypothesis that variation in pollinator fauna should have a significant effect on the strength of pollen limitation. In this sense, we propose that

(i) pollen limitation decreases in plant populations with higher pollinator abundance, (ii) pollen limitation also decreases in those populations with higher diversity of pollinators and (iii) the relative abundance of high-efficiency pollinators will influence the strength of pollen limitation. To test these hypotheses, we experimentally quantified pollen limitation in eight populations of the pollination-generalist plant *Erysimum mediohispanicum* contrasting in the assemblages of flower-visiting insects. Specifically, we determine (i) the occurrence of pollen limitation across the eight focal populations, quantifying it with a highly inclusive reproductive component, the net reproductive rate ( $R_0$ ), (ii) the occurrence of spatial, inter-population differences in pollen limitation strength, (iii) which component of plant reproduction, from ovule fertilization to seedling survival, is most strongly pollen-limited and (iv) the relationship between the abundance, diversity and identity of flower visitors and the intensity of pollen limitation.

## Materials and methods

### PLANT NATURAL HISTORY

*Erysimum mediohispanicum* is a mostly biennial, monocarpic herb endemic to the Iberian Peninsula. Previous studies have demonstrated that *E. mediohispanicum* reproduction is related to the inter-population variation in its flower visitors (Gómez *et al.* 2007, 2008a). Individual plants grow for 2–3 years as vegetative rosettes and then die after producing 1–8 reproductive stalks that bear up to several hundred hermaphroditic, bright-yellow flowers containing 30–40 ovules (Gómez *et al.* 2009a). *Erysimum mediohispanicum* inflorescences are indeterminate (capable of continuous flower production) and acropetal (developing from the base toward the apex, with basal flowers developing earlier than middle and apical flowers). Consequently, there is a strong position-dependent fruiting pattern, with fruiting probability being highest at medium positions of the infructescences (flowers opening at the flowering peak), and lowest at basal and apical positions (flowers opening too early or too late during the flowering period). *Erysimum mediohispanicum* is partially self-compatible, but requires pollen vectors for full seed set (Gómez 2005).

This study was conducted during 2008 in Sierra Nevada (Granada, south-east Spain), spanning the altitudinal range of *E. mediohispanicum* (1600–2300 m a.s.l.). Plants bloom in the study area from late May to late June, depending on the altitude. For this study, we selected eight populations (see Table S1 in Supporting Information) that have been studied in detail during recent years (Gómez *et al.* 2007, 2008a,b, 2009a,b). Populations were located at least 200-m apart, with a mean inter-population distance of  $818 \pm 82$  m ( $\pm 1$  SE). Despite their relative proximity, populations were clearly differentiated, since genetic divergence among populations is high (Gómez *et al.* 2009a,b).

### EXPERIMENTAL DETERMINATION OF POLLEN LIMITATION

To estimate the degree of pollen limitation, we conducted a pollen-supplementation experiment in each of the eight focal populations (see Table S1). In each population, we labelled 30 plants at the same flowering stage. All experimental plants had one inflorescence and were of similar size. In 20 randomly designated plants, we labelled eight flowers from the central part of the flowering stalk, adding

outcross pollen in the upper four flowers [Pollen added (PA) treatment] and leaving the lower four as control (C treatment). Four flowers were also labelled from the central part of the remaining 10 plants, acting as a procedural control (CC treatment). The flowers used in this experiment, both the pollen-added and the control ones, were chosen from the central part of the flowering stalks in order to avoid any confounding outcome caused by the inherent effect of flower position on reproduction and pollen limitation (Casper & Niesenbaum 1993; Wesselingh 2007). However, we chose C flowers were to be located under PA flowers along the flowering stalk because by doing this, we decreased the potential for resource redistribution, since flowering and fruiting in *E. mediohispanicum* occurs from the bottom up. Procedural control flowers were used to detect any effect of pollen supplementation in the re-allocation of resources from C flowers (Wesselingh 2007). This protocol makes our results conservative and our conclusions robust. Pollen-added flowers were administered pollen from 3 to 4 individuals located at least 1.5 m away. In total, 1920 flowers belonging to 240 plants were used in this experiment.

At the end of the reproductive season, we counted the number of experimental flowers that had produced fruits. These fruits were taken to the laboratory, where we determined under magnifying glasses the total number of ovules produced per flower, the number of ovules fertilized, the number of fertilized ovules that aborted before ripening, and the number of ripe seeds per fruit. We were able to distinguish aborted seeds from unfertilized ovules because in *E. mediohispanicum*, like in many other crucifers (Gómez & Zamora 2003), an aborted seed is invariably dark brown, with shrivelled cotyledons and embryo. By contrast, virgin ovules are consistently the same creamy white colour, lanceolate in shape and of a far smaller size than aborted or ripe seeds. Afterwards, for each plant producing ripe seeds (179 plants), five seeds taken at random were sown in a glasshouse (895 seeds in total). Seeds from each plant were randomly placed in the glasshouse. We recorded seed germination and seedling emergence every 2 weeks from December 2008 to January 2009 and then monthly survival until end of December 2009.

To test pollen limitation, we used the following pre- and post-dispersal components of the plant reproductive output: (i) fruit production, the proportion of flowers setting fruit, (ii) ovule fertilization, the number of ovules within ripe fruits that were effectively fertilized, (iii) seed abortion, the number of fertilized ovules aborting before seed ripening, (iv) seed production, the number of seeds dispersed per flower, (v) seedling emergence, calculated as the proportion of sown seeds germinating and emerging as seedlings and (vi) seedling survival, calculated as the proportion of seedlings surviving until December 2009. Since *E. mediohispanicum* is biennial, seeds used in the experiment would reach adulthood during the spring 2010.

We tested pollen limitation using an inclusive estimate of reproductive success of the experimental plants, the net reproductive rate  $R_0$ . For this, we made a projection matrix for each treatment  $\times$  population combination, considering seven life stages: flowering plant, ovule (number of ovules produced per plant), fertilized ovule (proportion of initial ovules being fertilized), seed (proportion of fertilized ovules setting ripe seeds), dispersed seed (proportion of ripe seeds escaping pre-dispersal seed predation and dispersing), seedling (proportion of dispersed seeds germinating and emerging as seedlings), and juvenile (proportion of seedlings surviving into 2-year juveniles). Since our sampling lasted until December 2009, we assumed no death from this date to May 2010 (the time of flowering). This assumption is not excessively risky, since previous demographic studies have proven that mortality in this plant is extremely low at this stage of the life cycle (Gómez 2005). Afterwards, we calculated the net reproductive

rate of each individual plant ( $R_0$ ) (Caswell 1989). All demographic analyses were performed with the package popbio in R (Stubben & Milligan 2007).

Finally, we determined the pollen limitation index (PL index, a measure of the magnitude of pollen limitation) for each reproductive component. The PL index was calculated as  $1 - RS_C / RS_{PA}$ , where  $RS_C$  is the reproductive success of the control treatment at that given component and  $RS_{PA}$  the reproductive success of the PA treatment (thus, we had two PL indices, one using as control the C plants,  $PL_C$ , and the other using the CC plants,  $PL_{CC}$ ). All PL index values were calculated for each of the individual plants used in the experiment, and thereby they are always expressed at plant level. The PL index ranges from 0, no pollen limitation, to 1, the highest pollen limitation (Larson & Barrett 2000). To obtain a valuable measure of the reliability of our PL estimates, we calculated their 95% confidence intervals by means of bootstrapping with 1000 permutations using package boot in R (Canty & Ripley 2009).

#### DETERMINATION OF ABUNDANCE, DIVERSITY AND COMPOSITION OF FLOWER VISITOR ASSEMBLAGE

We determined the abundance, diversity and composition of the flower visitor assemblage of the focal populations by counting the number of insects visiting the flowers by means of point-centred 60-min surveys, a method successfully used with this species (Gómez *et al.* 2007, 2009a). The survey sites were located around the experimental plants, covering a surface of *c.* 20 m<sup>2</sup> (an area previously proven adequate for sampling and identifying *E. mediohispanicum* flower visitors, Gómez *et al.* 2007, 2009a). Sampling took place during full bloom (10–12 days per population). The number of surveys per population was fitted to the local abundance of insects by means of accumulation curves, using previous information gathered in the studied populations (Gómez *et al.* 2007, 2009a). In total, we conducted 3–5 surveys per population. Most individual flower visitors were identified in the field, but some specimens were captured and sent to specialists.

We grouped the insects visiting *E. mediohispanicum* flowers in the following functional groups, according to their similarity in size, proboscis length, foraging behaviour and feeding habits: (i) large bees: mostly pollen- and nectar-collecting females  $\geq 10$  mm in body length, (ii) small bees: mostly pollen- and nectar-collecting females  $< 10$  mm, (iii) wasps: aculeate wasps, large parasitic wasps and kleptoparasitic bees collecting only nectar; (iv) ants: nectar-collecting worker ants, (v) beesflies: long-tongued nectar-collecting Bombyliidae, (vi) hoverflies: nectar- and pollen-collecting Syrphidae and short-tongued Bombyliidae; (vii) beetles: including species collecting nectar and/or pollen, (viii) butterflies: mostly Rhopalocera, all nectar collectors and (ix) others: nectar-collecting small flies, small parasitic wasps, bugs and grasshoppers.

Abundance of flower visitors was estimated by standardizing the number of visits per time unit (expressed as visits per population per hour). Flower visitor diversity was assessed by calculating species richness and evenness. Richness ( $S_{obs}$ ) was calculated as the number of flower-visiting species found visiting flowers in each population. In addition, we used EstimateS 7.5 software (Colwell 2005) to calculate two asymptotic richness estimates, the ACE Coverage Estimator and the Chao1 index. These are two robust estimates used to evaluate sample-size adequacy in calculation of diversity indices (Magurran 2004). Evenness was calculated as Hurlbert's PIE, the probability that two randomly sampled flower visitors from the community pertain to two different species. It is an evenness index that combines the two mechanistic factors affecting diversity: dominance and species

abundance. This index was generated by a randomization process using EcoSim 7 (Gotelli & Entsminger 2009).

Flower visitor assemblage composition was compared among populations using Bray–Curtis index (Magurran 2004). This index ranges from 0 (indicating no similarity in community composition between sites) to 1 (indicating complete overlap) and is considered the most robust measures of community similarity (Magurran 2004). The indices were obtained using EstimateS 7.5 software (Colwell 2005).

#### DATA ANALYSIS

We performed separate analyses for comparing between-treatment differences in reproductive output. To compare the PA and C treatments, since flowers belonged to the same individuals, we performed repeated-measures ANOVAS, using treatment as the within-subject factor and population as the between-subject factor. The comparisons between PA and CC treatments and between C and CC treatments were done with Generalized Linear Models (GLMs). The variables expressed as proportion (fruit production, seedling emergence and seedling survival) were compared using a binomial distribution and logit link function, and the remaining variables were compared using a Poisson distribution with log link function and including ovule number as a covariate in order to control for potential variation in reproductive capacity. We considered population as a fixed rather than a random factor because we were interested in determining the effect of pollen supplementation in those specific populations, from which we have the information on pollinators. All analyses were performed using the package *stat* in R (R Development Core Team 2008). Data from individual flowers belonging to the same treatment and plant were averaged.

Among-population differences in species composition were analysed by using an algorithm devised by Roff & Bentzen (1989) and implemented in the RXC program (G. Carmody, Carleton University). This program calculates Chi-square values for the observed contingency table and for 10 000 simulated tables obtained by permutation, and it calculates *P*-values with Monte Carlo methods. Among-population difference in pollinator abundance was tested by means of a GLM with negative binomial distribution and log link function, using the package MASS in R (Venables & Ripley 2002).

We tested whether among-population changes in flower visitors may affect the intensity of pollen limitation by means of spatially-explicit models, since all the flower visitor assemblage descriptors were spatially autocorrelated among populations. We performed autoregressive models considering spatial-autocorrelation in both dependent and independent variables (lagged-predictor models, Haining 2002):

$$Y = \rho WY + X\beta + WX\gamma + e$$

where  $\rho$  is the autoregression parameter, the matrix **W** indicates the relationships among spatial units (populations) and contains neighbour (nearby populations) weights ( $w_{ij}$ ),  $\beta$  is a vector representing the slopes associated with the predictors in the original predictor matrix **X**, and  $\gamma$  represents the autoregressive coefficients of each of the predictors (Haining 2002). In these analyses, the observation unit was population ( $n = 8$ ). Spatial analyses were performed with SAM software (Rangel, Diniz-Filho & Bini 2006).

We corrected the *P*-values by sequential Bonferroni correction for those models performed several times. Throughout the manuscript we present means  $\pm$  SE.

## Results

#### POLLEN LIMITATION

Experimental pollen supplementation significantly increased the net reproductive rate of the plants, based on comparisons of the performance of supplemented flowers and their seeds with those of both control (C) flowers and CC flowers (Table 1). In fact, the  $PL_{CC}$  index for the whole life cycle and pooling all populations was  $0.063 \pm 0.064$  whereas the  $PL_C$  index was  $0.139 \pm 0.024$  (that is, pollen addition increased the expected net reproductive rate of the plants by 6% and 14%, depending on the control treatment considered). However, we found among-population differences in pollen limitation (Table 1), since only three populations (Em01, Em24 and Em25) were pollen-limited according to  $PL_C$  index estimates and only five populations (Em01, Em22, Em23, Em24, Em25) according to  $PL_{CC}$  index estimates (Table 2). Furthermore, for only two populations (Em22 and Em23) the values of  $PL_C$  and  $PL_{CC}$  indices were discordant (binomial test of equal probability, *P*-value = 0.144).

When each reproductive component was analysed separately, pollen limitation appeared to be proportionally more intense during the pre-dispersal stages than during the post-dispersal ones (Table 1, Fig. 1). Fruit production did not differ between PA and CC flowers (Table 1), since  $82.0 \pm 3.6\%$  of CC flowers, and  $85.8 \pm 2.3$  of PA flowers set fruit. However, fruit production differed significantly between PA flowers and C flowers (Table 1), as only  $75.1 \pm 2.2\%$  of C flowers produced fruits. The number of fertilized ovules per flower differed among all treatments (Table 1), with  $15.0 \pm 1.2$  fertilized ovules per fruit in CC flowers,  $18.6 \pm 0.7$  in C flowers and  $20.0 \pm 0.8$  in PA flowers. By contrast, no among-treatment differences were found in seed abortion (Table 1), there being  $1.9 \pm 0.3$  aborted seeds per fruit in CC flowers,  $1.7 \pm 0.2$  in C flowers and  $1.9 \pm 0.2$  in PA flowers. Pollen supplementation significantly increased seed production per flower (Table 1;  $15.5 \pm 0.8$  seeds per flower in PA flowers) compared to CC flowers ( $10.8 \pm 1.1$  seeds) and C flowers ( $12.7 \pm 0.7$  seeds; see Fig. S1).

Pollen supplementation did not affect post-dispersal components of *E. mediohispanicum* reproductive output. Thus, seedling emergence was  $55.1 \pm 4.8\%$  for CC seeds,  $59.7 \pm 3.0$  for C seeds and  $60.6 \pm 3.0$  for PA seeds (Table 1). Similarly, seedling survival was also similar between treatments (Table 1), with  $96.4 \pm 1.25\%$  for CC seedlings,  $97.3 \pm 11.8\%$  for C seedlings and  $94.5 \pm 18.1\%$  for PA seedlings.

#### FLOWER-VISITOR ASSEMBLAGE

We recorded more than 100 different species of insects visiting the flowers of *E. mediohispanicum* in the eight focal populations during 2008 (see Table S2 for the complete flower-visitor assemblage). In general, this assemblage was taxonomically diverse, composed of insects belonging to over 25 families and six orders. In addition, the assemblage was also functionally diverse, since we recorded insects belonging to all nine

**Table 1.** Effect of pollination treatments on *Erysimum mediolispanicum* reproductive output. Pollen addition treatment against Control treatment (PA vs. C) was tested with repeated-measures ANOVAS. Pollen addition treatment against Procedural control treatment (PA vs. CC) and Control treatment against Procedural control treatment (C vs. CC) were tested with Generalized Linear Models with Poisson distribution (ovule fertilization, seed abortion and seed production) or binomial distribution (fruit production, seedling emergence and seedling survival). Bold values are significant at  $\alpha < 0.05$  after sequential Bonferroni correction

		Fruit production			Ovule fertilization			Seed abortion			Seed production			Seedling emergence			Seedling survival			Overall life cycle ( $R_0$ )		
		d.f.	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P		
PA vs. C	Treatment	1	<b>22.66</b>	<b>0.0001</b>	<b>6.07</b>	<b>0.015</b>	0.95	0.33	<b>6.42</b>	<b>0.013</b>	0.04	0.844	0.82	0.368	<b>6.28</b>	<b>0.014</b>						
	Population	7	9.35	0.0001	2.00	0.061	7.65	0.0001	2.13	0.046	1.14	0.345	13.16	0.0001	5.10	0.0001						
	T × P	1	5.45	0.0001	0.24	0.97	0.80	0.59	0.47	0.856	1.28	0.267	0.33	0.936	2.09	0.050						
PA vs. CC	Treatment	1	0.04	0.83	<b>22.63</b>	<b>0.0001</b>	0.26	0.607	<b>22.29</b>	<b>0.0001</b>	0.63	0.428	1.76	0.180	<b>3.80</b>	<b>0.020</b>						
	Population	7	7.33	0.396	58.84	0.0001	43.47	0.001	94.05	0.0001	5.71	0.574	15.38	0.035	3.13	0.0001						
	T × P	7	3.86	0.794	9.96	0.191	37.94	0.0001	23.18	0.001	1.21	0.991	5.57	0.710	0.60	0.758						
C vs. CC	Ovule/flower*	1			290.44	0.0001	2.87	0.090	312.00	0.0001												
	Treatment	1	2.76	0.999	5.45	0.050	0.09	0.759	5.86	0.060	0.60	0.440	2.14	0.143	1.18	0.279						
	Population	7	18.10	0.001	2.91	0.014	47.51	0.0001	5.97	0.0001	4.94	0.667	17.96	0.012	4.55	0.0001						
	T × P	7	9.16	0.242	0.24	0.974	30.75	0.0001	1.66	0.366	1.79	0.970	4.53	0.717	1.27	0.268						
	Ovule/flower*	1			63.71	0.0001	6.79	0.009	56.43	0.0001												
	PL <sub>C</sub> index				0.136 ± 0.074	0.029 ± 0.012	0.079 ± 0.119	0.000 ± 0.345	0.214 ± 0.070	0.006 ± 0.050	0.006 ± 0.050	0.001 ± 0.040	0.001 ± 0.040	0.139 ± 0.024	0.139 ± 0.024							
PL <sub>CC</sub> index				0.043 ± 0.041	0.198 ± 0.038			0.297 ± 0.073	0.123 ± 0.056	0.123 ± 0.056	0.000 ± 0.032	0.000 ± 0.032	0.063 ± 0.064	0.063 ± 0.064								

PL index refers to pollen limitation index calculated comparing  $R_0$  of plants belonging to pollen addition treatment with plants belonging to procedural control treatment (PL<sub>CC</sub>) and with control plants (PL<sub>C</sub>).

\*Ovule number per flower was included as a covariate in the analyses of ovule fertilization, seed abortion and seed number to control for variation in reproductive capacity.

**Table 2.** Abundance and diversity of the flower visitor assemblage visiting the flowers in each plant population and estimates of pollen limitation per population

Pop.	Number of insects	Abundance (Insects h <sup>-1</sup> )	S <sub>obs</sub>	S <sub>ACE</sub>	S <sub>Chao1</sub>	Hurlbert's PIE	PL <sub>CC</sub> index		PL <sub>C</sub> index	
							Value	95% Confidence interval	Value	95% Confidence interval
Em01	115	19.17 ± 21.48 <sup>2</sup>	25 <sup>2,4</sup>	42.25	35.00	0.9000 <sup>1,4</sup>	<b>0.207</b>	[0.112 to 0.303]	<b>0.148</b>	[0.037 to 0.259]
Em02	191	95.50 ± 26.31 <sup>1</sup>	44 <sup>3</sup>	58.24	52.58	0.9538 <sup>3</sup>	0.177	[-12.747 to 12.923]	0.018	[-0.020 to 0.056]
Em08	153	34.62 ± 18.60 <sup>2</sup>	23 <sup>6</sup>	27.47	28.00	0.8897 <sup>1,5</sup>	0.051	[-0.149 to 0.251]	0.134	[0.000 to 0.242]
Em21	166	52.42 ± 21.48 <sup>1,2</sup>	20 <sup>5</sup>	30.33	25.20	0.8695 <sup>4,5</sup>	0.095	[0.000 to 0.174]	0.023	[-1.166 to 1.189]
Em22	161	33.31 ± 18.60 <sup>2</sup>	30 <sup>4</sup>	32.74	31.11	0.9453 <sup>6</sup>	<b>0.175</b>	[0.133 to 0.217]	0.000	[-2.050 to 2.050]
Em23	173	62.91 ± 18.60 <sup>1,2</sup>	32 <sup>4</sup>	42.04	41.16	0.8755 <sup>4</sup>	<b>0.194</b>	[0.086 to 0.302]	0.013	[-3.303 to 3.316]
Em24	192	52.36 ± 26.31 <sup>2</sup>	27 <sup>2</sup>	37.19	38.25	0.8483 <sup>2</sup>	<b>0.181</b>	[0.165 to 0.197]	<b>0.062</b>	[0.039 to 0.084]
Em25	210	45.00 ± 26.31 <sup>1,2</sup>	42 <sup>1</sup>	63.66	62.12	0.9043 <sup>1</sup>	<b>0.036</b>	[0.006 to 0.065]	<b>0.064</b>	[0.050 to 0.078]

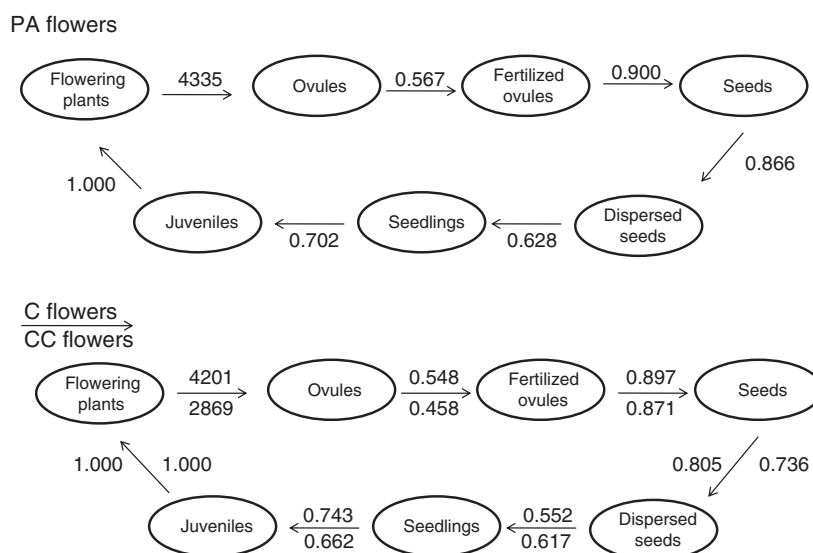
S<sub>obs</sub>, Observed flower visitor richness; S<sub>ACE</sub>, Expected flower visitor richness according to the ACE method; S<sub>Chao1</sub>, Expected flower visitor richness according to the Chao1 method.

Flower visitor abundance was compared among populations by means of a Generalized Linear Model with negative binomial distribution.

Flower visitor diversity and richness was compared among populations by a rarefaction process using EcoSim software (see Materials and methods)

Different superscript numbers indicate significant differences at  $\alpha < 0.05$ .

PL index refers to pollen limitation index calculated comparing R<sub>0</sub> of plants belonging to pollen addition treatment with plants belonging to procedural control treatment (PL<sub>CC</sub>) and with control plants (PL<sub>C</sub>). The 95% confidence interval was generated by bootstrapping. When the interval does not cross the zero, the PL estimate is significant and it is shown in bold.



**Fig. 1.** The life cycle graphs of pollen-supplemented and control individuals of *Erysimum mediohispanicum*. Arrows denote observed transitions during 2 years and numbers denote transition probabilities or effective fecundities. All populations were pooled. PA flowers = flowers belonging to pollen addition treatment. C flowers = flowers belonging to control treatment. CC flowers = flowers belonging to procedural control treatment.

functional groups considered. Plant populations differed in the composition of their flower-visitor assemblages both taxonomically and functionally (Monte Carlo contingency test = 461.4,  $P \pm SE = 0.0001 \pm 0.0001$ ). In fact, similarity between populations in terms of insect specific composition was very low, since the average Bray–Curtis index was only  $0.29 \pm 0.08$  ( $n = 28$  pairwise comparisons; see Table S3). Similarly, populations differed in the relative importance of flower visitor functional groups. Plants belonging to Em02,

Em23 and Em25 populations were visited mostly by large bees, whereas those belonging to the Em21 population were visited by small bees and beeflies, those belonging to Em01 were visited by large bees and beetles, and those belonging to Em08, Em22 and Em24 populations were visited mostly by beetles (see Fig. S2).

There were also significant among-population differences in the diversity of the flower-visitor assemblages (Table 2). Thus, flower-visitor richness S<sub>obs</sub> ranged between 20 (Em21) and 44

species per population (Em02), whereas Hurlbert's PIE was between 0.85 (Em24) and 0.95 (Em02) (Table 2). The values calculated for the two richness estimators, ACE and Chao1, suggest that flower-visitor assemblages were richer than inferred from our sampling.

Finally, flower-visitor abundance also varied among populations (log-likelihood = 234.23,  $P < 0.0001$ , GLM with negative binomial distribution), ranging from  $19.17 \pm 21.48$  insects  $\text{h}^{-1}$  in the population Em01 to  $95.50 \pm 26.31$  insects  $\text{h}^{-1}$  in the population Em02 (Table 2).

We found no spatial correlation across plant populations in flower-visitor abundance, diversity and composition ( $P > 0.1$ ,  $n = 8$  populations, Mantel  $r$ ).

#### EFFECTS OF FLOWER VISITORS ON POLLEN LIMITATION

The intensity of pollen limitation was negatively related to flower-visitor abundance, this effect being significant only for  $\text{PL}_C$  index (Table 3). We also found a significant, negative effect of flower-visitor richness and diversity on pollen limitation, since populations with less diverse flower-visitor fauna showed stronger pollen limitation (Table 3). Finally, the type of insect visiting the flowers also affected the strength of pollen limitation at the population level. Populations where large bees were proportionally more abundant had lower levels of pollen limitation measured as  $\text{PL}_{CC}$  index, whereas those where the most abundant flower visitors were beetles and others had greater pollen limitation measured as  $\text{PL}_C$  index (Table 3). We found a marginally negative relationship between bee fly, wasp and ant abundance and the degree of pollen limitation and a marginally positive relationship between hoverfly and small bee abundance and pollen limitation intensity (Table 3).

## Discussion

Our experiment demonstrates that *E. mediohispanicum* is pollen-limited at the regional level – that is, when considering all studied populations together, as suggested by the significant differences found between the PA treatment and any of the two control treatments across populations (Table 1). This was unexpected, since this plant species, a mega-generalist, is pollinated by many different species of insects that abundantly visit its flowers (Gómez *et al.* 2007), and it can also produce some seeds by selfing without any pollinator (Gómez 2005; Muñoz-Pajares *et al.*, unpubl. data). According to the reproductive-assurance hypothesis (Burd 1994) self-compatible plants would suffer from less pollen limitation than self-incompatible ones because they can mitigate the effects of pollinator scarcity by autogamy (Galen & Newport 1988; Hill, Brody & Tedesco 2008). Similarly, most reviews on pollen limitation have found that specialist and self-incompatible plants are more prone to being pollen-limited than are generalist and/or self-compatible plants (Larson & Barrett 2000; Ashman *et al.* 2004; Knight *et al.* 2005). The present study nevertheless demonstrates that generalist self-compatible plants can also be pollen-limited.

The outcomes of pollen-supplementation experiments may give misleading results because plants can reallocate resources among flowers in response to experimental pollinations. Unfortunately, we could not submit whole individual plants to control or experimental treatments, since an individual can produce several hundred flowers. However, following Wesselingh (2007), we used two complementary controls, one from treated plants and the other from untreated ones. Notably, we found that control flowers from non-manipulated plants had lower reproductive output than did control flowers from manipulated plants, suggesting that pollen added to some flowers did not divert resources from accompanying flowers.

**Table 3.** Summary of the univariate spatially explicit lagged models examining the across-population relationship between the characteristics of the flower visitor assemblages and the intensity of pollen limitation (calculated as in Table 2) in *Erysimum mediohispanicum*.  $n = 8$  populations. Bold values are significant at  $\alpha < 0.05$  after sequential Bonferroni correction

	$\text{PL}_C$			$\text{PL}_{CC}$		
	Coefficient $\pm$ SE	$t$	$P$	Coefficient $\pm$ SE	$t$	$P$
Flower visitor abundance	$-0.076 \pm 0.028$	<b>2.66</b>	<b>0.038</b>	$-0.090 \pm 0.230$	0.39	0.710
Flower visitor richness ( $S_{\text{obs}}$ )	$-0.544 \pm 0.232$	2.22	0.070	$-0.045 \pm 0.293$	0.16	0.883
Flower visitor diversity (Hurlbert's PIE)	$-0.789 \pm 0.375$	<b>3.15</b>	<b>0.020</b>	$-0.205 \pm 0.355$	<b>3.40</b>	<b>0.027</b>
Flower visitor identity						
Large bees	$0.043 \pm 0.928$	0.05	0.941	$-0.896 \pm 0.280$	<b>3.20</b>	<b>0.012</b>
Small bees	$2.828 \pm 0.1.28$	2.20	0.032	$-1.805 \pm 1.589$	1.14	0.183
Wasps	$-0.195 \pm 0.232$	0.84	0.301	$-0.380 \pm 0.182$	2.09	0.036
Ants	$-0.443 \pm 0.191$	2.33	0.030	$0.209 \pm 0.154$	1.35	0.101
Bee flies	$-1.716 \pm 1.119$	1.53	0.077	$1.381 \pm 1.156$	1.19	0.166
Hoverflies	$2.551 \pm 1.430$	1.78	0.054	$1.765 \pm 1.474$	1.20	0.166
Beetles	$1.706 \pm 0.541$	<b>3.15</b>	<b>0.012</b>	$-0.886 \pm 0.812$	1.09	0.183
Butterflies	$-0.291 \pm 0.216$	1.35	0.130	$-0.150 \pm 0.189$	0.79	0.264
Others	$0.515 \pm 0.186$	<b>2.77</b>	<b>0.017</b>	$0.939 \pm 0.240$	<b>3.91</b>	<b>0.007</b>

$S_{\text{obs}}$ , Observed flower visitor richness; Hurlbert's PIE, Probability of Interspecific Encounter.

PL index refers to pollen limitation index calculated comparing  $R_0$  of plants belonging to pollen addition treatment with plants belonging to procedural control treatment ( $\text{PL}_{CC}$ ) and with control plants ( $\text{PL}_C$ ).

In fact, under resource allocation, controls in non-manipulated plants would be expected to have higher rather than lower reproduction than controls in manipulated plants. Two additional reasons suggest that resource reallocation did not significantly alter our results. First, by locating control flowers under the pollen-added flowers along the flowering stalk, we sought to ensure that they would start developing fruits at the same time or before we supplemented pollen to pollen-added flowers (since *E. mediohispanicum* flowers acropetally). This presumably decreased the ability of resource redistribution. Secondly, we did not find any effect of pollen supplementation in any component of progeny quality (germination, survival, etc.), as would be expected with resource reallocation (Knight, Steet & Ashman 2006).

#### POLLEN LIMITATION DURING DIFFERENT REPRODUCTIVE COMPONENTS

The intensity of pollen limitation in *E. mediohispanicum* varied between reproductive components, being more intense during seed ripening (pre-dispersal stage) than during seedling recruitment (post-dispersal stage). It is remarkable that pollen limitation was weak during fruit production, a widely used component for studying pollen limitation (Burd 1994; Knight *et al.* 2005; Knight, Steet & Ashman 2006). Knight *et al.* (2005) found a good correlation between magnitude of pollen limitation during fruit production and seed production across different plant species, concluding that fruit set may be an appropriate and easy proxy for exploring pollen limitation. However, our study advises caution with this conclusion, since we found strong pollen limitation during seed set but a weak one during fruit set. Several non-exclusive reasons may explain our finding. First, *E. mediohispanicum* flowers bear 30–40 ovules, but fruit can probably be produced in this species even when fewer pollen grains land on flower stigmas. In this case, since several to many ovules remain unfertilized, fruit set becomes an inaccurate estimate of reproductive success. A good correlation between fruit and seed set may occur in plant species displaying a small and fixed number of ovules per flower. Secondly, pollen limitation estimated at fruit and seed set may also be inconsistent because *E. mediohispanicum* is self-compatible and can probably initiate fruits after autogamous pollination, irrespective of the future fate of the seeds developing inside. This feature is presumably common to other self-compatible species (Fenster & Martín-Rodríguez 2007). We think that an accurate picture of pollen limitation requires study beyond fruit production.

The strongest pollen limitation in *E. mediohispanicum* occurred during ovule fertilization and seed ripening. In fact, our experiment shows that pollen limitation starts to be significant during these two phases. It was clear that artificial supplementation of pollen resulted in more ovules being fertilized per flower and, consequently, more seeds per fruit. In contrast, the addition of extra pollen from different individuals did not decrease the abortion rate. We found no effect of experimental supplementation of cross pollen either on seed germination or on seedling vigour. Taken together, these findings suggest that

pollen limitation in *E. mediohispanicum* occurs mostly through the quantity rather than the quality of pollen grains reaching the stigma (Aizen & Harder 2007).

#### RELATIONSHIP BETWEEN FLOWER VISITORS AND POLLEN LIMITATION IN *E. MEDIOHISPANICUM*

Our study has shown significant spatial variation in pollen limitation across *E. mediohispanicum* populations. Spatial variation in pollen limitation has seldom been explored (Burd 1994; Knight *et al.* 2005). Several genetic and environmental factors have been proposed as driving spatial, between-population differences in pollen limitation (Baker, Barrett & Thompson 2000; Jakobsson, Lázaro & Totland 2009). However, a reason causing spatial variation in pollen limitation is related to the quality and quantity of pollinators (Wilcock & Neiland 2002; Knight *et al.* 2005; Knight, Steet & Ashman 2006). Pollinator abundance is a primary factor driving pollen limitation in many plant species, both specialists (Duan, Zhang & Liu 2007; Cosacov, Naretto & Cocucci 2008) and generalists (González-Varo, Arroyo & Aparicio 2009). Supporting this idea, we found a negative relationship across populations between flower visitor abundance and *E. mediohispanicum*  $PL_C$  index. This relationship may suggest that increased flower-visitor abundance may decrease the intensity of pollen limitation among the plants, although we did not find any relationship between flower visitor abundance and  $PL_{CC}$  index. Nevertheless, our study suggests that flower visitor diversity, rather than abundance, is a main factor determining pollen limitation, since this variable related to both pollen limitation estimates. Populations with lower flower visitor diversity had stronger pollen limitation. Higher pollinator diversity is associated with higher reproductive success in several plant species (Klein, Steffan-Dewenter & Tscharntke 2003; Ashman *et al.* 2004; Knight *et al.* 2005; but see Hegland & Totland 2008). Our results support this idea, suggesting that flower visitor diversity benefits *E. mediohispanicum* reproduction. In fact, in a previous study we have shown that the diversity of pollinators visiting *E. mediohispanicum* flowers in a given population is positively related with reproductive output in that population (Gómez *et al.* 2007). As *E. mediohispanicum* is a pollination-generalist species, many of the insects visiting its flowers can act as pollinators. For this reason, increased flower visitor diversity usually entails a concomitant boost in the probability of being visited by effective pollen vectors (Perfectti, Gómez & Bosch 2009). In addition, we have found that pollinators also differ in foraging traits determining the pattern of pollen transfer (Gómez *et al.*, unpubl. data). Therefore, high pollinator diversity probably augments diversity in the stigmatic pollen load, with pollen grains coming from a wide range of donors. This probably favours pollen competition and enhances the likelihood of successful pollinations, ultimately benefiting flower reproductive success.

Finally, we also found that the strength of pollen limitation was related to the presence of some types of flower visitors. Specifically, populations where large bees were abundant showed low levels of pollen limitation, whereas populations



where beetles or others were abundant showed higher levels of pollen limitation. Although many of those insects can act as pollinators in *E. mediohispanicum*, our analysis of their foraging behaviour suggests inequalities in pollination efficiency. For example, most beetles and insects belonging to the functional group 'others' (bugs, small flies, etc.) entered the flower from the bottom, robbing the nectar without touching the anthers or stigma. In addition, the duration of a single flower visit, positively related in many plants with the proportion of selfed pollen deposited, was much shorter for large bees than for these kinds of insects. We did not find a similar positive relationship between the degree of pollen limitation and the abundance of ants, wasps or other low-efficiency insects, probably because of their scarcity in our populations (see Table S2). Nevertheless, as a whole, our results imply that populations in which the pollinator assemblage was dominated by efficient pollen vectors were less limited than when the assemblage was predominantly inefficient. This finding is noteworthy because this effect of pollinator composition on pollen limitation, although frequently considered in theoretical approaches, has been seldom shown in the wild (Ward & Johnson 2005; McMullen 2009).

## Conclusions

In this study, we have shown that even in a plant with a mega-generalist self-compatible pollination system, reproduction can be pollen-limited. We also demonstrate that in generalist plants the strength of pollen limitation may depend on the local diversity and composition of the pollinator assemblages. Due to the usual spatial variability occurring in the pollinator fauna interacting with generalist plants, our results reflect that, for an accurate estimate of pollen limitation, pollen limitation should be examined throughout the diverse habitats occupied by the plant under study. Most importantly, our findings indicate that any impoverishment in pollinator diversity or any change in the identity of flower visitors may have negative consequences for the reproduction of some generalist plants.

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## Supporting Information

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

**Table S1.** Description of the studied *Erysimum mediohispanicum* populations.

**Table S2.** Flower visitor assemblage of the eight studied plant populations during 2008.

**Table S3.** Pairwise comparisons in flower visitor assemblage composition by means of Bray–Curtis similarity indices.

**Figure S1.** Per-population among-treatment differences in pollen limitation.

**Figure S2.** Among-population differences in the composition of the flower visitor assemblage.

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