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# Density-dependent pollen limitation and reproductive assurance in a wind-pollinated herb with contrasting sexual systems

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## Summary

**1.** Wind pollination is thought to have evolved in response to selection for mechanisms to promote pollination success, when animal pollinators become scarce or unreliable. We might thus expect wind-pollinated plants to be less prone to pollen limitation than their insect-pollinated counterparts. Yet, if pollen loads on stigmas of wind-pollinated species decline with distance from pollen donors, seed set might nevertheless be pollen-limited in populations of plants that cannot self-fertilize their progeny, but not in self-compatible hermaphroditic populations.

**2.** Here, we test this hypothesis by comparing pollen limitation between dioecious and hermaphroditic (monoecious) populations of the wind-pollinated herb *Mercurialis annua*.

**3.** In natural populations, seed set was pollen-limited in low-density patches of dioecious, but not hermaphroditic, *M. annua*, a finding consistent with patterns of distance-dependent seed set by females in an experimental array. Nevertheless, seed set was incomplete in both dioecious and hermaphroditic populations, even at high local densities. Further, both factors limited the seed set of females and hermaphrodites, after we manipulated pollen and resource availability in a common garden experiment.

**4.** *Synthesis*. Our results are consistent with the idea that pollen limitation plays a role in the evolution of combined vs. separate sexes in *M. annua*. Taken together, they point to the potential importance of pollen transfer between flowers on the same plant (geitonogamy) by wind as a mechanism of reproductive assurance and to the dual roles played by pollen and resource availability in limiting seed set. Thus, seed set can be pollen-limited in sparse populations of a wind-pollinated species, where mates are rare or absent, having potentially important demographic and evolutionary implications.

**Key-words:** dioecy, local mating environment, *Mercurialis annua*, monoecy, reproductive ecology, resource availability, seed set

## Introduction

In many plant species, adequate pollen availability appears to be a key factor limiting seed production, with important demographic and evolutionary implications. In a review of the literature, Ashman *et al.* (2004) found that no fewer than 73% of the cases studied showed evidence for increased seed production by individuals experimentally given supplemental pollen. From a demographic point of view, reductions in seed set can reduce the growth or persistence of populations (Turnbull, Crawley & Rees 2000). This might be especially likely in small populations (e.g. Agren 1996; Groom 1998), or in populations of self-incompatible hermaphrodites or plants with separate sexes (Steven & Waller 2007; Wagenius, Lonsdorf & Neuhauser 2007; Shelton 2008). In the extreme, populations falling below a threshold size or density may experience an Allee effect (Allee *et al.* 1949), with ultimate extinction caused by reduced pollen availability at low densities (e.g. Groom 1998; Bessa-Gomes, Legendre & Clobert 2004; Davis *et al.* 2004; Morgan, Wilson & Knight 2005; Wagenius, Lonsdorf & Neuhauser 2007). Similarly, newly colonized populations may not establish if seed production is pollen-limited (Baker 1955; Pannell & Barrett 1998; Fausto, Eckhart & Geber 2001; Busch 2005; Pannell *et al.* 2008).

From an evolutionary point of view, chronic pollen limitation should select for mechanisms to promote pollen

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dispersal, e.g. by promoting enhanced allocation of resources to pollinator attraction (Haig & Westoby 1988) or bringing about evolutionary shifts from biotic to abiotic pollination (Culley, Weller & Sakai 2002; Friedman & Barrett 2009b). Alternatively, pollen-limited plants may evolve a capacity to self-fertilize their progeny, thereby acquiring reproductive assurance. Indeed, selection for reproductive assurance in the face of pollen or mate limitation is widely viewed as the prime reason for the frequent evolutionary transition from outcrossing to selfing in plants (e.g. Maurice & Fleming 1995; Barrett 2002; Kalisz, Vogler & Hanley 2004; Wolf & Takebayashi 2004). In support of this hypothesis, comparative analyses indicate that pollen limitation occurs more commonly in self-incompatible hermaphroditic populations or in populations with imperfect flowers, i.e. flowers that are either male or female (Larson & Barrett 2000; Knight *et al.* 2005), because the imposed separation of the sex roles precludes autonomous self-pollination. Here, wind pollination is thought to offer an alternative route to promote pollination success, when pollen dispersal and receipt are limited by a paucity of insect pollinators (e.g. Goodwillie 1999). For example, the rarity of females in self-compatible *Schiedea menziesii* has been attributed to a lack of available pollen, and there are some indications of morphological modifications in this species in keeping with a shift from biotic to wind pollination (Weller *et al.* 1998; Rankin, Weller & Sakai 2002). Importantly, although wind pollination might evolve as an outcrossing mechanism in the absence of insect pollinators, it can also allow self-fertilization between different flowers on the same plant (geitonogamy; Friedman & Barrett 2009a; Friedman & Barrett 2009b). Geitonogamy in wind-pollinated species may come at a selective advantage, because selfing may enhance a plant's total seed set under pollen-limited conditions, provided levels of inbreeding depression are sufficiently low (e.g. Lloyd 1980; Charlesworth & Charlesworth 1987).

If wind pollination evolves when pollinators are unreliable, wind-pollinated plants should be less prone to pollen limitation than their insect-pollinated counterparts (Whitehead 1969; Regal 1982; Weller *et al.* 1998; Goodwillie 1999; Culley, Weller & Sakai 2002). In a recent study of several wind-pollinated herbs, including both monoecious and dioecious species, Friedman & Barrett (2009b) found that supplemental hand pollination of stigmas did not increase seed set, and that pollen loads on open-pollinated stigmas were surprisingly similar to those of animal-pollinated species, pointing to the efficacy of wind pollination as an outcrossing mechanism. However, some evidence indicates pollen limitation in wind-pollinated trees (e.g. Fox 1992; Knapp, Goedde & Rice 2001; Totland & Sottocornola 2001; Sork *et al.* 2002), as well as in marginal populations of invasive species (Davis *et al.* 2004). For instance, Fox (1992) found evidence for pollen limitation in several willow species and suggested that dual pollination by insects and wind ensures reproductive success in these species, e.g. when transfer by wind alone is not effective.

Although wind pollination may often promote outcrossing, its efficacy depends on the relative proximity of pollen donors and thus on population density (e.g. Rognli, Nilsson & Nurminiemi 2000; Stehlik, Caspersen & Barrett 2006; Steven & Waller 2007). Thus, even wind-pollinated species that typically receive adequate pollen for maximal seed set may be pollen-limited in sparse or newly established populations when mates are rare or absent, and where the degree of pollen-limitation varies over time. Such a situation may occur in marginal populations (e.g. Knapp, Goedde & Rice 2001), in populations on the edge of a range expansion (e.g. Davis *et al.* 2004) or in species that occupy frequently disturbed habitats (e.g. Somanathan & Borges 2000), e.g. species subject to metapopulation dynamics, in which frequent local extinction is balanced by colonization (Pannell & Barrett 1998). There is little doubt that variation in plant density affects mating opportunities, but the extent to which this has substantive demographic and evolutionary consequences is unclear (Ashman *et al.* 2004; Friedman & Barrett 2009b).

Here, we consider the demographic and evolutionary implications of pollen-limited seed production in the wind-pollinated herb *Mercurialis annua*. *Mercurialis* comprises about eight exclusively wind-pollinated species, all but two of which are dioecious (Tutin *et al.* 1968; Obbard *et al.* 2006). In the clade of annual species to which the species complex *M. annua* belongs, self-compatible monoecy is hypothesized to have evolved from dioecy in response to selection for reproductive assurance, notably through bouts of colonization during range expansion and/or ongoing metapopulation dynamics, when population densities are low (Pannell 1997a; Pannell 1997b; Pannell *et al.* 2008). Accordingly, we predicted that dioecious populations of *M. annua* should be more prone to pollen limitation at low densities than monoecious populations, in which individuals may self-fertilize via geitonogamy.

We assessed pollen limitation in *M. annua* under both conditions in the field as well as under controlled conditions in experimental mating arrays. We first assessed seed set in natural monoecious and dioecious populations of *M. annua* in north-eastern Spain, near the transition between sexual systems, where we considered the dependence of seed set on both neighbourhood density and composition (in terms of the local sex ratio). We found clear evidence for pollen limitation in isolated females in dioecious populations, but not in monoecious populations. To characterize the spatial scale over which females become pollen-limited, we further assessed seed set in an artificial array, in which distance to the nearest pollen source was experimentally controlled.

Despite pollen limitation in dioecious *M. annua* populations at low density, seed set was less than complete in populations of both sexual systems, even at very high densities where pollen was unlikely limiting. We therefore hypothesized that seed set in these populations could also have been limited by resources available for seed provisioning (see Haig & Westoby 1988; Campbell & Halama 1993). We tested this hypothesis by experimentally manipulating both available resources and pollen for seed production in females and hermaphroditic individuals. Rather than controlling the amount of pollen deposited on stigmas via hand pollinations (Wesselingh 2007), we altered the pollination environment indirectly through manipulation of the sexual identity of neighbouring plants; focal plants neighbouring a male were thus viewed as corresponding to a

'pollen-supplementation' treatment. Although this method did not allow precise manipulation of pollen loads and thus leaves certain questions unaddressed (see Discussion), it mimicked the context dependence of mating opportunities in a relatively natural way. Our results suggest that seed set of *M. annua* is limited by both pollen and resources.

## Materials and methods

### STUDY SPECIES

*Mercurialis annua* is a wind-pollinated, facultative annual plant that occurs in disturbed habitats across Europe and the Mediterranean Basin. The species is a polyploid complex, with a loose correspondence between ploidy and the sexual system. Diploid populations, which occur throughout western, central and eastern Europe and around the eastern Mediterranean Basin, are exclusively dioecious, with sex ratios typically close to 1:1. In contrast, tetraploid and hexaploid populations, which occur in the Iberian Peninsula and North Africa, are either monoecious (hermaphroditic) or androdioecious (males coexisting with monoecious individuals).

Flowering in *M. annua* is indeterminate: new inflorescences continue to be produced in leaf axils throughout the growing season. Thus, at any time, females or hermaphrodites may have the: (i) newly formed pistillate flowers in the leaf axils bunched at the tip of the growing stem; (ii) developing fruits, with swelling carpels; (iii) fully developed fruits; and (iv) swollen fruit pedicels whose fruits have already been dispersed (these remain visible for the life of the plant and differ in size from flower pedicels that failed to develop fruits). Although fruits of hermaphrodites begin to swell several days before the first male flower in the same inflorescence sheds its pollen, geitonogamous pollination can occur between inflorescences from different leaf axils.

Because hermaphrodites of *M. annua* strongly resemble females in their sex allocation (Hesse & Pannell 2011), we do not expect there to be a strong trade-off between resources available for pollen production vs. seed provisioning. Carpels of *M. annua* typically contain three locules, each with one ovule. Usually, only two ovules develop as seeds (i.e. one is aborted), although three-seeded fruits occasionally occur. Under pollen- and/or resource-limited conditions, fruits may remain completely unfilled or develop with only one seed; such one-seeded fruits are conspicuously asymmetrical in their morphology.

### POLLEN LIMITATION IN NATURAL POPULATIONS

We surveyed naturally occurring variation in seed set in 11 dioecious and 11 hermaphroditic populations of *M. annua* in Catalonia in north-eastern Spain, close to a natural transition zone between sexual systems. In each population, we recorded the seed set of five plants growing in each of a low- and high-density patch, as well as the seed set of two to five additional plants that were more isolated from one another (specifically, at least 2 m from the nearest non-self pollen donor). Low- and high-density patches were selected subjectively, but they always differed at least eightfold in density (see Results). For each patch, we recorded the density, and, in dioecious populations, the local sex ratio. Plants within patches were randomly selected from areas of relatively even density; the total area sampled per patch differed across sites because of variation in density. We counted the flowers, fruits and seeds produced on the two main secondary branches of each plant, until at least 100 flowers, fruits or seeds had been sampled. We discarded the top two nodes of each branch to ensure that we counted only older flowers that had been exposed to potential pollen (i.e. we did not record the numbers of fresh flowers, for which pollination could not be assessed). Seed set was calculated as the proportion of ovules producing seeds. In addition, we computed incomplete seed set at the fruit level as the proportion of all fruits that had produced only one seed. Because fruits are bi- or tri-ovulate, the proportion of one-seeded fruits can be viewed as a measure of incomplete seed set (see previous section).

To evaluate whether density differentially affected the seed and fruit set of females and hermaphrodites, we used a two-way ANOVA, with two sexual systems (dioecy and monoecy) and three densities (high density, low density and isolated plants). Similarly, a two-way ANCOVA was used to test whether the proximity to potential mates (i.e. distance) affected female and hermaphroditic seed set differentially. All measures of female reproductive output were calculated using individuals pooled for their density level, so that replication was provided by sampling multiple populations. We adopted this approach to maintain a balanced data set, largely because $n$ varied between two and five individuals for the class of isolated plants. For dioecious populations, we also tested the effects of local male frequency on seed set, using a linear model. To achieve normality of standardized residuals and equality of variance, response variables were transformed, where appropriate (see Results). Tests of significance were carried out using $F$-tests of likelihood ratios. In case of significant differences, we used treatment contrasts to compare differences between means, with $\alpha < 0.05$. In particular, we first compared means between density classes (I = isolated, L = low density and H = high density) for each sexual system independently and subsequently compared means between sexual systems for each of the three density classes. For all analyses, here and later, we used the statistical package R Version 2.2.2 (R Development Core Team; http://www.r-project.org).

### EXPERIMENTAL ASSESSMENT OF DISTANCE-DEPENDENT POLLEN LIMITATION

Seeds for this study were bulk-collected from > 50 females from a dioecious population of *M. annua* near Montestigliano Rosia, Italy. Seeds were sown in seed trays in a glasshouse and, at the onset of flowering, males and females were transplanted separately into 10-cm pots containing peat-based soil. Plants in their pots were then placed in an experimental mating array in a farmland field site near Oxford (UK), where *M. annua* is naturally absent. We placed 10 males in the centre of the mating array and groups of females at a range of distances (20, 50 cm, 1, 2, 5, 10, 15 and 25 m) radiating out in eight directions from the centre. Following Bullock & Clarke (2000), we increased the number of females with increasing distance from the centre to increase the total number of flowers that might set seed. Within each station, females were approximately spaced 25 cm apart to limit the opportunity for interference in pollen receipt.

Before being transplanted into the mating array, several female plants had produced seeds. Accordingly, we removed the above-ground tissue of all females at the start of the experiment and allowed them to re-grow and reproduce during 6 weeks, i.e. all seed set assays were of flowers and fruits developed after females were in place (see Discussion). During the experiment, plants received slow-release fertilizer. At 6 weeks, we harvested all plants and tallied the flowers, fruits and seeds on a subset of females at each distance along each transect ('station'), ensuring that at least five females were sampled per station. Measures of seed set were calculated as described earlier for the pooled individuals per station.

To assess distance-dependent pollen dispersal, female fertility was modelled as a function of inter-plant distance ('station') using a range of different functions (Tufto, Engen & Hindar 1997). The best model

was identified using *F*-tests of likelihood ratios. The minimal adequate models for seed set and the percentage of one-seeded fruits were: $y = ae^{-cx}$ and $y = ax^{-c}$, respectively, where $y$ is seed set or percentage of one-seeded fruits, $a$ the intercept, $c$ a constant, and $x$ the distance from the pollen source.

### EXPERIMENTAL ASSESSMENT OF POLLEN VS. RESOURCE LIMITATION

We conducted two experiments at the Oxford University Field Station at Wytham near Oxford (UK) to examine the factors limiting seed set of (i) females and (ii) hermaphrodites. In both the experiments, we manipulated pollen availability by growing the females or hermaphrodites alone, with another female or hermaphrodite, as appropriate, or with a male (from either a dioecious or androdioecious population, respectively).

Seeds for the experiments were bulk-collected from 50 seed-producing individuals in a dioecious and an androdioecious population of *M. annua*: the dioecious population was located near Sitges in north-eastern Spain; the androdioecious population was located at Nave, in southern Portugal. Seeds were sown in seed trays in a glasshouse, and at the onset of flowering, males, females and hermaphrodites were identified and transplanted into 10-cm pots containing nutrient-poor sandy soil. The experiments were each set up outdoors in a blocked, fully crossed two-factorial design. For each sexual system, plants were randomly assigned to the following treatments in each of the 30 blocks that were spaced 2 m apart, yielding a total of 180 pots: pollen availability (no neighbour, hermaphroditic or female neighbour, and male neighbour) and resource availability (high-nutrient vs. low-nutrient soils). High-nutrient soils received 100 mL of 1 mL $L^{-1}$ Phostrogen (Cambridge, UK) fertilizer every 2 days (Phostrogen contains equivalent molarities of nitrogen, potassium and phosphorus, plus micronutrients); low-nutrient soils received 100 mL of water every 2 days and 100 mL of 0.125 mL $L^{-1}$ Phostrogen fertilizer once a week. Because of space limitations, we had to place plants within their block fairly closely together (*c.* 30 cm apart). This meant that plants were grown within reasonable (and perhaps typical) background pollen availability, and that, consequently, our experiment likely underestimates pollen limitation that might occur under low-density conditions in natural populations.

After 6 weeks, we harvested the above-ground portions of the focal plants, and their female reproductive output was recorded as described earlier. For each sexual system, we used linear mixed-effect models, with block treated as a random effect, to evaluate the effects of the availability of pollen (N = no, M = male and F/H = female or hermaphroditic neighbour, where appropriate) and resources (P = poor, R = rich soils) on seed set.

## Results

The survey of natural populations demonstrated that low- and high-density patches differed more than eightfold in density (mean ± SE plants $m^{-2}$: 7.79 ± 2.21 vs. 72.79 ± 27.62 and 8.50 ± 2.86 vs. 68.09 ± 9.57 in low- and high-density patches in dioecious and hermaphroditic populations, respectively). Seed set of isolated females was significantly lower than that of females at higher densities (I–L and I–H contrasts: $P < 0.001$; L–H contrast: $P = 0.19$) or that of isolated hermaphrodites (I–I contrast: $P = 0.01$; Table 1; Fig. 1a). Hermaphroditic seed set was density-independent (all contrasts: $P > 0.05$) and was similar to that of females growing at higher densities (L–L contrast: $P = 0.37$; H–H contrast $P = 0.71$). When growing in isolation, hermaphrodites set significantly more seeds than females ($F_{1,49} = 11.51$; $P < 0.01$; Fig. 1a), independent of pollen donor proximity ($F_{1,49} = 0.72$; $P = 0.40$). Finally, male frequency did not significantly affect ($F_{1,20} = 0.08$, $P = 0.78$) the seed set of females in dioecious populations (mean male frequency ± SE: 0.43 ± 0.01 vs. 0.45 ± 0.01 in low- and high-density patches, respectively).

**Table 1.** Summary of ANOVA models for seed set ($x^2$-transformed) and incomplete seed set at the fruit level as a function of patch density in dioecious and monoecious populations (system) in Spain

| | Seed set | Incomplete seed set |
|---|---|---|
| Density | $F_{2,60} = 2.35$ | $F_{2,60} = 1.25$ |
| System | $F_{1,60} = 4.98$* | $F_{1,60} = 0.52$ |
| Density × System | $F_{2,60} = 3.53$* | $F_{2,60} = 3.2$* |

*$P < 0.05$.

The proportion of incomplete seed set was significantly higher for isolated females compared with that of isolated hermaphrodites (I–I contrast: $P = 0.01$; Table 1; Fig. 1c), or that of females growing in high-density patches (I–L and L–H contrasts: $P > 0.05$; I–H contrast: $P = 0.01$). Incomplete seed set did not vary across density classes in hermaphroditic populations (all contrasts: $P > 0.05$), but it was significantly higher for hermaphrodites in high-density patches compared with females growing at an equivalent density (L–L contrast: $P = 0.19$; H–H contrast: $P = 0.03$).

The dispersal array demonstrated that pollen dispersal was mainly local, and that it declined quickly with increasing distance from the pollen source, as indicated by the reduced seed set by females at greater distances from the males. Distance-dependent pollen dispersal could be described by an exponential function, which explained 68% of the total variation in seed set among stations (Fig. 1b). Surprisingly, at >20 m from the point source, seed set was still *c.* 5%. In contrast, incomplete seed set increased less than linearly with inter-plant distance and could be described by a power function, which explained 54% of variation in the data (Fig. 1d).

The common garden experiment showed that seed set in dioecious populations was significantly higher for females grown in rich soils (P–R contrast: $P = 0.04$) and near a male (N–M contrast: $P = 0.03$; other contrasts: $P > 0.05$; Table 2; Fig. 2a). In androdioecious populations, resource and pollen availability jointly affected hermaphroditic seed set (Table 2; Fig. 2b). That is, pollen availability limited the seed set of hermaphrodites, but only when grown in rich soils (N–M contrast in rich soils: $P = 0.01$; P–R contrast with male neighbour: $P = 0.02$; other contrasts: $P > 0.05$).

## Discussion

Natural levels of seed set in hermaphrodites of *M. annua* never exceeded *c.* 75%, independent of patch density. In dioecious populations, seed set by females was similar across a wide

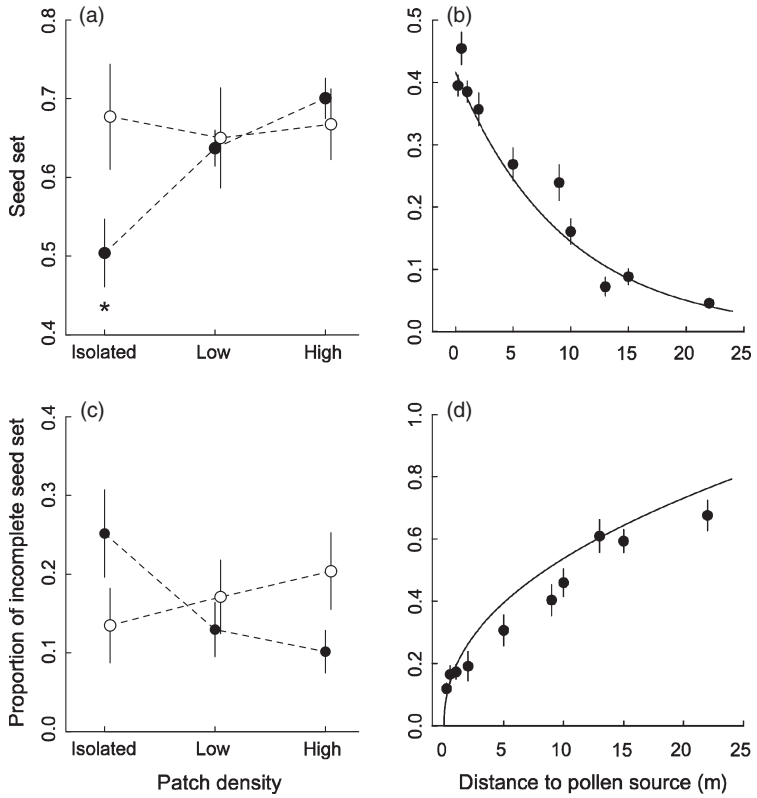



**Fig. 1.** (a) Mean seed set ± SE as a function of density in natural dioecious (filled symbols) and monoecious populations (open symbols). Asterisks denote significant differences ($P < 0.05$), calculated using treatment contrasts; (b) Mean seed set ± SE as a function distance to the nearest male pollen source in an artificial dioecious population. The fitted curve is: $y = ae^{-cx}$, where $a = 0.43 \pm 0.02$ and $c = 0.11 \pm 0.01$ (estimate ± SE); (c) Mean percentage of incomplete seed set at the fruit level ± SE as a function of density in natural dioecious (filled symbols) and monoecious populations (open symbols); (d) Proportion of incomplete seed set (± SE), calculated as the number of fruits with one seed over the total number of fruits produced, as a function of distance to the nearest pollen donor in an artificial dioecious population. The fitted curve is $y = ax^{-c}$, where a $= 0.19 \pm 0.02$ and $c = 0.44 \pm 0.04$ (estimate ± SE).

**Table 2.** Summary of linear mixed-effect models for seed set as a function of pollen and resource availability in dioecious (*a*sin $\sqrt{x}$-transformed) and androdioecious ($x^2$-transformed) populations of *Mercurialis annua* in a common garden experiment

| | Dioecious | Androdioecious |
|---|---|---|
| Pollen | $F_{2,112} = 4.85$** | $F_{2,147} = 1.17$ |
| Resource | $F_{1,112} = 8.10$** | $F_{1,147} = 0.04$ |
| Pollen × Resource | $F_{2,112} = 0.46$ | $F_{2,147} = 3.18$* |

*$P < 0.05$, **$P < 0.01$.

range of densities, equivalent to that of hermaphrodites (*c*. 75%), when growing in denser patches. However, there was a clear reduction in seed set when females were growing more than *c*. 2 m from the nearest male. Our mating array experiment detected an even more rapid decline in total seed set by females and an increase in the level of incomplete seed set, with distance from the nearest pollen source. Although we did not measure stigmatic pollen loads directly, these results strongly suggest that the fertility of females, but not of hermaphrodites, of *M. annua* can be pollen-limited at low density. Manipulation of both resource and pollen availability revealed pollen limitation at even smaller spatial scales than in the field, as well as some resource limitation.

## EVOLUTION AND EFFICACY OF WIND POLLINATION IN HERBACEOUS PLANTS

The context-dependent nature of the success of wind pollination for *M. annua* parallels results for other wind- (Rognli, Nilsson & Nurminiemi 2000; Davis *et al*. 2004; Steven & Waller 2007; Stehlik, Friedman & Barrett 2008; Friedman & Barrett 2009b) and animal-pollinated plants (Kunin 1997; Larson & Barrett 2000; Stehlik, Caspersen & Barrett 2006; Aizen & Harder 2007) that have demonstrated increasing mate limitation with decreasing density. Thus, while wind pollination must often be an effective pollen vector, we should expect it to evolve only under conditions in which plant densities are typically high (Levin & Kerster 1974). This is no doubt one reason for the relative low frequency of wind-pollinated species in tropical forests, where population densities tend to be low (Regal 1982).

Whereas wind is an effective mediator of outcrossing in dioecious populations of *M. annua*, in hermaphroditic (or androdioecious) populations, wind mediates both outcrossing and/or self-pollination. In a previous study, Eppley & Pannell

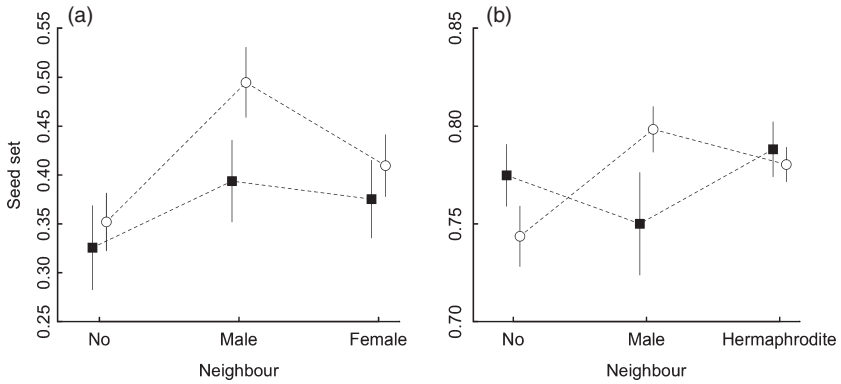



**Fig. 2.** Mean seed set ± SE in (a) dioecious and (b) androdioecious populations as a function of the availability of resources (squares = poor soils and circles = rich soils) and pollen (no, male or female/hermaphroditic neighbour, respectively).

(2007a) demonstrated that hermaphrodites do sire their own ovules when local densities are low, i.e. that the selfing rate is strongly density-dependent. With this in mind, it would seem likely that isolated hermaphrodites set as many seeds as those growing in dense patches because of their capacity to self-fertilize their ovules. The alternative hypothesis, that elevated seed set by hermaphrodites was the result of greater outcross pollen availability in the androdioecious array (where both sexual morphs produce pollen), seems less likely, because hermaphrodites produce so much less pollen than do males (Hesse & Pannell 2011; relative pollen production of males: 0.20; and hermaphrodites: 0.03). This would imply that elevated seed set by hermaphrodites is largely the result of geitonogamy, rather than a consequence of differences in the availability of outcross pollen between sexual systems. The fact that wind-mediated geitonogamy in *M. annua* confers reproductive assurance accentuates the importance of distinguishing between biotic vs. abiotic vectors, when modelling the evolution of selfing. Lloyd (1992) drew attention to the fact that geitonogamy cannot provide reproductive assurance in animal-pollinated plants, largely because it implies pollen and seed discounting. However, it would seem that geitonogamy might provide reproductive assurance in wind-pollinated populations, such as those of *M. annua* studied here. Pollen discounting seems unlikely for a wind-pollinated species, and the strong within-inflorescence protogyny displayed by *M. annua* suggests that ovules are unlikely to be selfed, if outcross pollen is available (i.e. seed discounting is likely to be low; Friedman & Barrett 2009a). Protogyny in general may act as a delayed selfing mechanism, because delayed pollen dispersal allows outcrossing, but ensures seed set in the absence of compatible mates (Lloyd & Schoen 1992; Herlihy & Echert 2002).

### POLLEN VS. RESOURCE LIMITATION

That seed set in natural populations of *M. annua* was incomplete, even at high densities, where pollen limitation seemed unlikely, suggested the possible importance of resource limitation (e.g. Campbell & Halama 1993). We tested this hypothesis by manipulating both pollen availability and soil nutrients and found that both females and hermaphrodites growing in rich soils and with a male neighbour had increased seed set. This result points to the possibility that both pollen and resources limited seed set in these individuals, as predicted by Haig & Westoby (1988). The result also points to the possible importance of the sex ratio in dioecious populations, where females in patches with a high male frequency should be less prone to pollen limitation.

Haig & Westoby's (1988) graphical model has stimulated considerable empirical work on factors limiting seed set in plant populations (reviewed in Ashman *et al*. 2004). Much of this work has recently been criticized, because it relied on pollen supplementation experiments, which have drawbacks that can overestimate pollen limitation (Knight, Steets & Ashman 2006; Wesselingh 2007). Key concerns with these experiments are that supplemental pollen is typically applied to stigmas all at once, often only treating a subset of flowers on the plants, and from a pollen source that is likely to be of better quality than is likely to be experienced naturally (Ashman *et al*. 2004). We attempted to avoid some of these problems by altering the pollination environment indirectly through manipulation of the identity of a plant's direct neighbours. The power to detect pollen limitation with this design was no doubt compromised, because females growing on their own in the common garden experiment would still have received pollen from males in other blocks. However, our method probably reflects the context dependence of mating opportunities and pollen receipt relatively well, and it is difficult to interpret the patterns of pollination without invoking pollen abundance as causative.

Although females growing with a male neighbour increased their seed set when growing in rich soils more than those growing in poorer soils, seed set, nonetheless, remained incomplete, and indeed lower than in natural populations (50% vs. 75% seed set in experimental vs. natural populations of dioecious *M. annua*, respectively). We do not know the reason for the continued low seed set under resource-rich conditions. One possibility is that low seed set is simply an artefact of the pruning treatment or a plastic response to the growth conditions in our experiment (see Materials and methods). Another possibility is that pollen flow may have been inadequate to ensure maximal potential seed set, even when focal plants were neighbouring a male. Yet a third possibility is that seed set in

*M. annua* is constrained physiology, e.g. because of limits acting on transport of resources to individual fruits (e.g. Watson & Casper 1984). It is difficult to rule out these explanations on the basis of our results here, and more detailed work including measures of stigmatic pollen loads would be worthwhile. Nevertheless, it remains possible that the lower than complete seed set we observed may actually have resulted from resource limitation, consistent with Haig & Westoby's (1988) model that predicts simultaneous pollen and resource limitation. Under such a scenario, it might increase a plant's fitness to allocate a large proportion of its resources towards vegetative growth, when growing under competitive conditions rather than towards reproduction. This might occur in plants such as *M. annua*, which display indeterminate flowering and continue to grow and reproduce throughout their adult life.

In principle, a plant's seed set might also be limited by pollen quality (e.g. Herlihy & Eckert 2004; Aizen & Harder 2007). For instance, those ovules that failed to develop into seeds might, in fact, have been fertilized, only to abort prematurely because of the expression of deleterious alleles, e.g. as an outcome of inbreeding depression (Charlesworth & Charlesworth 1987; Husband & Schemske 1996). We cannot rule out this possibility. However, recent studies have found no evidence for substantial inbreeding depression in dioecious (Eppley & Pannell 2009) and hermaphroditic (Pujol *et al.* 2009) populations of *M. annua* from north-eastern Spain. Pollen quality limitation would thus seem to be unlikely for these populations.

### IMPLICATIONS OF POLLEN LIMITATION FOR THE EVOLUTION OF SEXUAL SYSTEMS IN *M. ANNUA*

Pollen limitation is widely cited as the leading hypothesis for evolutionary transitions from outcrossing to self-fertilization (Barrett 2002; Harder & Aizen 2010). This hypothesis pertains both to the breakdown of outcrossing mechanisms, such as self-incompatibility in hermaphroditic populations (Wagenius, Lonsdorf & Neuhauser 2007; Busch & Schoen 2008), as well as to the evolution of self-fertile hermaphroditism from dioecy (Maurice & Fleming 1995; Wolf & Takebayashi 2004). *Mercurialis annua* represents one of a few cases in which both dioecy and self-fertile hermaphroditism occur in different parts of a species' range. In a handful of other such cases, the transition between combined and separate sexes has been attributed mainly to plants responding to environmental stress and/or intensity of competition, with increased gender separation occurring in harsher (e.g. *Wurmbea dioica*: Barrett 1992; *Ecballium*: Costich 1995) or more competitive environments (e.g. *Sagittaria latifolia*: Dorken & Barrett 2004). In contrast, there is little evidence for such divergence between dioecious and monoecious *M. annua* (Buggs & Pannell 2007). Rather, the evolution of hermaphroditism from dioecy in *M. annua* may have been a response to selection for reproductive assurance under pollen limitation, specifically during colonization (Pannell *et al.* 2008).

Our findings are largely consistent with the reproductive assurance hypothesis: in sparse populations, the fertility of females, but not that of hermaphrodites, seemed to be pollen-limited. Similarly, patterns of habitat occupancy and abundance (Eppley & Pannell 2007b) and sex allocation in *M. annua* (J. R. Pannell *et al.* unpubl. data) are consistent with the metapopulation model. Nevertheless, it is important to note that dioecious and hermaphroditic *M. annua* populations occupy very similar habitats at similar densities (M. E. Dorken & J. R. Pannell, unpubl. data). So why has dioecy been maintained?

Dioecious and hermaphroditic populations of *M. annua* have different ploidy levels (diploid and hexaploid, respectively; Durand & Durand 1992) and different phylogeographic histories (Obbard, Harris & Pannell 2006). Notwithstanding the evidence supporting reproductive assurance, the shift from dioecy to hermaphroditism could thus have occurred as a direct consequence of polyploidization. That males have re-invaded hermaphroditic populations in large parts of the hexaploid range (Pannell *et al.* 2008), and that hermaphrodites have become almost entirely female in parts of North Africa (Durand 1963; Pannell 1997b), might argue for dioecy being the ultimate stable strategy for *M. annua* (Charnov, Bull & Maynard Smith 1976). In this case, hermaphroditism might simply represent a transient stage resulting from a perturbation caused by polyploid hybridization. It remains a challenge to disentangle these two possibilities.

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