

THE INFLUENCE OF POLLINATION INTENSITY ON FERTILIZATION SUCCESS, PROGENY SEX RATIO, AND FITNESS IN A WIND-POLLINATED, DIOECIOUS PLANT

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Variation in pollination intensity can influence the degree of gametophytic competition with diverse consequences for plant fitness. Such effects should be particularly important in wind-pollinated plants because they commonly possess uniovulate flowers. In several dioecious species, competition between female- and male-determining microgametophytes has been proposed as a cause of female-biased sex ratios. Here, we examine functional relations among pollination intensity, seed set, sex ratios, and sporophyte vigor in *Rumex hastatulus*, a wind-pollinated, dioecious plant. Using controlled pollinations, we applied a range of pollen grain numbers (1–71) to female flowers. Pollination intensity significantly predicted percent seed set, which was >80% when more than 15 pollen grains were added to stigmas. We estimated that the proportion of pollen grains participating in fertilization was 0.28 and the proportion of fertilized ovules developing into seed was 0.89. Overall, progeny sex ratios were female biased (0.62), with an increased probability of female offspring over the range of 1–15 pollen grains. We detected no effect of pollination intensity on sporophytic vigor. Our study is the first to investigate the relation between pollination intensity and female fertility in a wind-pollinated species and provides support for the contribution of gametophytic competition to sex ratio variation in plants.

Keywords: fertilization success, female-biased sex ratios, sporophyte fitness, gametophytic competition, wind pollination.

Introduction

Outcrossing in flowering plants depends on pollen dispersal between individuals by animals, wind, or water. The quantity and quality of pollen transferred from anthers to stigmas vary substantially among plants, with diverse consequences for fertility, mating, and sporophytic vigor (Mulcahy and Mulcahy 1975; Mitchell 1997; Delph and Havens 1998; Aizen and Harder 2007). Surveys of numerous species commonly demonstrate that insufficient pollen receipt limits seed production in plant populations (Burd 1994; Larson and Barrett 2000; Ashman et al. 2004; Knight et al. 2005). The vast majority of these studies have investigated pollen limitation in animal-pollinated species, and currently little is known about the extent of pollen limitation in wind-pollinated plants (reviewed in Friedman and Barrett 2009). Moreover, the functional relation between pollen load size (pollination intensity) and seed set has not been investigated in any wind-pollinated species. Such dose-response studies in animal-pollinated plants have provided valuable insights on the regulation of female fertility and on the consequences of pollination intensity for gametophytic competition and sporophytic fitness (Shore and Barrett 1984; Winsor et al. 1987; Snow 1990; Mitchell 1997; Kalla and Ashman 2002; Aizen and Harder 2007). Wind-pollinated species commonly possess uniovulate (single-ovule) flowers and brief floral longev-

ities (Friedman and Barrett 2011), and these features may potentially influence the extent to which variation in pollination intensity affects fertility, mating, and plant fitness.

Pollen load size can also affect progeny sex ratios in dioecious species by influencing the intensity of competition between female- versus male-determining pollen grains (microgametophytes). More intense competition is expected when pollen loads exceed that required for full seed set, because pollen grains compete to fertilize a limited number of ovules (Mulcahy 1975). Under these conditions, pollen tubes that grow faster through the style are more likely to fertilize ovules. As suggested by Mitchell (1997), the pollen load–seed set response curve can be used to identify the region of increasing pollen competition, which generally corresponds to the “sorting” stage before maximum seed set occurs (fig. 1). The degree of pollen tube competition will also depend on the amount of pollen deposited per ovule and should be greatest for species with uniovulate flowers. The generality of microgametophytic competition (also referred to as certation) in determining sex ratios in dioecious species has been controversial (Carroll and Mulcahy 1993), although several studies in wind-pollinated *Rumex* species have provided evidence consistent with the certation hypothesis (Rychlewski and Zarzycki 1975; Conn and Blum 1981). For example, in both experimental and natural populations of *Rumex nivalis*, female-biased progeny sex ratios are associated with higher pollen loads on stigmas of females and for females in which male proximity is highest (Stehlik and Barrett 2006; Stehlik et al. 2008). However, dose-response studies that examine the quantitative relations between pollination intensity and

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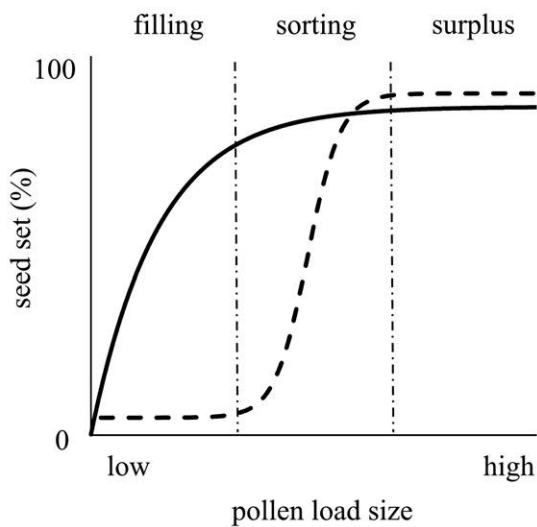


Fig. 1 A conceptual model for the effect of pollen load on seed set (solid line) and sporophyte fitness (dashed line) as related to the degree of pollen competition (following Mitchell 1997). The filling and sorting regions of the curve represent minimal and increasing levels of pollen competition, respectively. The surplus region represents maximum seed set and pollen competition.

sex ratio variation have not been conducted in any dioecious species. These experiments would be valuable for understanding the contribution of pollen competition to sex ratio variation in dioecious plants.

The intensity of microgametophytic competition may also influence sporophyte fitness through preferential fertilization by gametes of superior genetic quality when there is overlap in gene expression between the two life stages (Ottaviano and Mulcahy 1989). This hypothesis predicts that faster-growing pollen tubes will preferentially fertilize ovules and produce more vigorous offspring (Mulcahy and Mulcahy 1975). Most evidence for the effect of pollen load on sporophyte fitness has come from cultivated species (Winsor et al. 1987), whereas the results of experiments on wild species have been less consistent (Snow 1991; Mitchell 1997). The power to detect these effects depends on sampling across the pollen load–seed set response curve to ensure a sufficient spread of pollination intensities (fig. 1 in Mitchell 1997). There have been few investigations into these effects in wind-pollinated species (but see Davis 2004), despite the lower ovule number per flower in anemophilous species compared to many animal-pollinated species. Furthermore, in wind-pollinated plants pollen grains are dispersed independently (Whitehead 1983) and flowering can be highly synchronous (Dowding 1987), resulting in pollen loads from a diverse range of donors. These factors may establish the conditions for intense gametophytic competition in wind-pollinated species and the expectation of greater sporophyte vigor under high pollen loads.

Here, we investigate the effect of pollination intensity on seed set, sex ratios, and sporophyte vigor in *Rumex hastatulus*, a wind-pollinated, annual, dioecious plant. We examined these relations using a controlled pollination experiment conducted under glasshouse conditions in which we applied

specific numbers of pollen grains to female flowers and measured seed set, flowering sex ratios, and offspring fitness. If pollen loads influence fertilization success and the degree of microgametophytic competition, we expect a positive relation between the number of pollen grains applied to stigmas, seed set, sex ratio, and the vigor of offspring. Specifically, the certation hypothesis predicts a positive relation between pollen loads and the degree of sex ratio bias. We therefore predicted increasing female bias over the range of pollination intensities that characterize the sorting stage of fertilization success in *R. hastatulus*.

Material and Methods

Study System

Rumex hastatulus (Polygonaceae) is distributed across the southern regions of the United States from Texas to North Carolina and Florida. It has two distinct chromosome races, the North Carolina karyotype (females = XX, $2n=8$; males = XY₁Y₂, $2n=9$) and the Texas karyotype (females XX; males XY, $2n=10$; Smith 1963). Plants have a basal rosette and produce one to several inflorescences depending on resources. Female flowers are uniovulate and are produced progressively as the inflorescence expands. Plants are easily grown under glasshouse conditions and commence flowering ~6–8 wk after germination. Because germination is generally high (greater than ~95% of seeds sown) and virtually all plants of *R. hastatulus* survive to flowering, the adult sex ratios are likely to closely reflect primary sex ratios, at least under controlled growth conditions.

In April 2009, we collected open-pollinated families from 30 maternal plants from a population at Rosebud, Texas (31°07'02"N, 96°51'37"W), with a female-biased sex ratio of 0.65 ($n = 1622$). We germinated 60 seeds from each of 26 families in petri dishes on moist filter paper in a growth cabinet maintained at 20°C for 12 h and 10°C for 12 h with continuous light. After approximately 14 d, we transplanted seedlings to individual 3.8-cm pots containing Pro-Mix BX (peat moss, vermiculite, and perlite), and these were grown in a glasshouse maintained at 20°–24°C. We applied NPK fertilizer (10 : 52 : 10) to promote flowering. Female and male plants were kept in separate glasshouses for the duration of the pollination experiment to prevent pollen contamination of stigmas.

Pollination Experiment

After 6 wk, we selected 1–21 plants (average of 9.1) per maternal parent; these plants were used in controlled pollinations over a 14-d period. In total, 302 pollinations were undertaken on 8 d during this period, with an average of 40 pollinations conducted per day. We used fine forceps to remove all but one receptive flower on two separate flowering stems for each female. One flower was allocated to a particular pollination treatment, and the other was left as an un-pollinated control. On each day, we collected pollen on a microscope slide from at least five males at several time intervals during the day. A fine dissecting needle was used to collect pollen grains from the slide and the number of grains

adhering to the needle were immediately counted under a dissecting microscope ($\times 40$ magnification) before manual pollination. We deposited pollen grains on the stigmatic surface of female flowers under the dissecting microscope, and the needle was then recounted to ensure successful pollen transfer. For some pollinations involving stigmatic pollen loads >20 , multiple pollen additions were required to ensure accurate deposition of pollen numbers. We examined 58 pollination intensities on a continuous scale from 1 to 71 pollen grains (fig. 2A), with every pollen load between 1 and 30 pol-

len grains represented by 3–20 pollinations (mean = 8.5), whereas pollen loads between 31 and 71 involved 1–3 pollinations (mean = 1.6). We concentrated pollinations in the range from 3 to 20 because we were particularly interested in the sorting stage of fertilization success (fig. 1), identified by Mitchell (1997) as likely reflecting a steep gradient in microgametophytic competition. Also, this range of pollen load sizes is commonly found on open-pollinated stigmas of dioecious *Rumex* species in natural populations (see fig. 6 in Friedman and Barrett 2009).

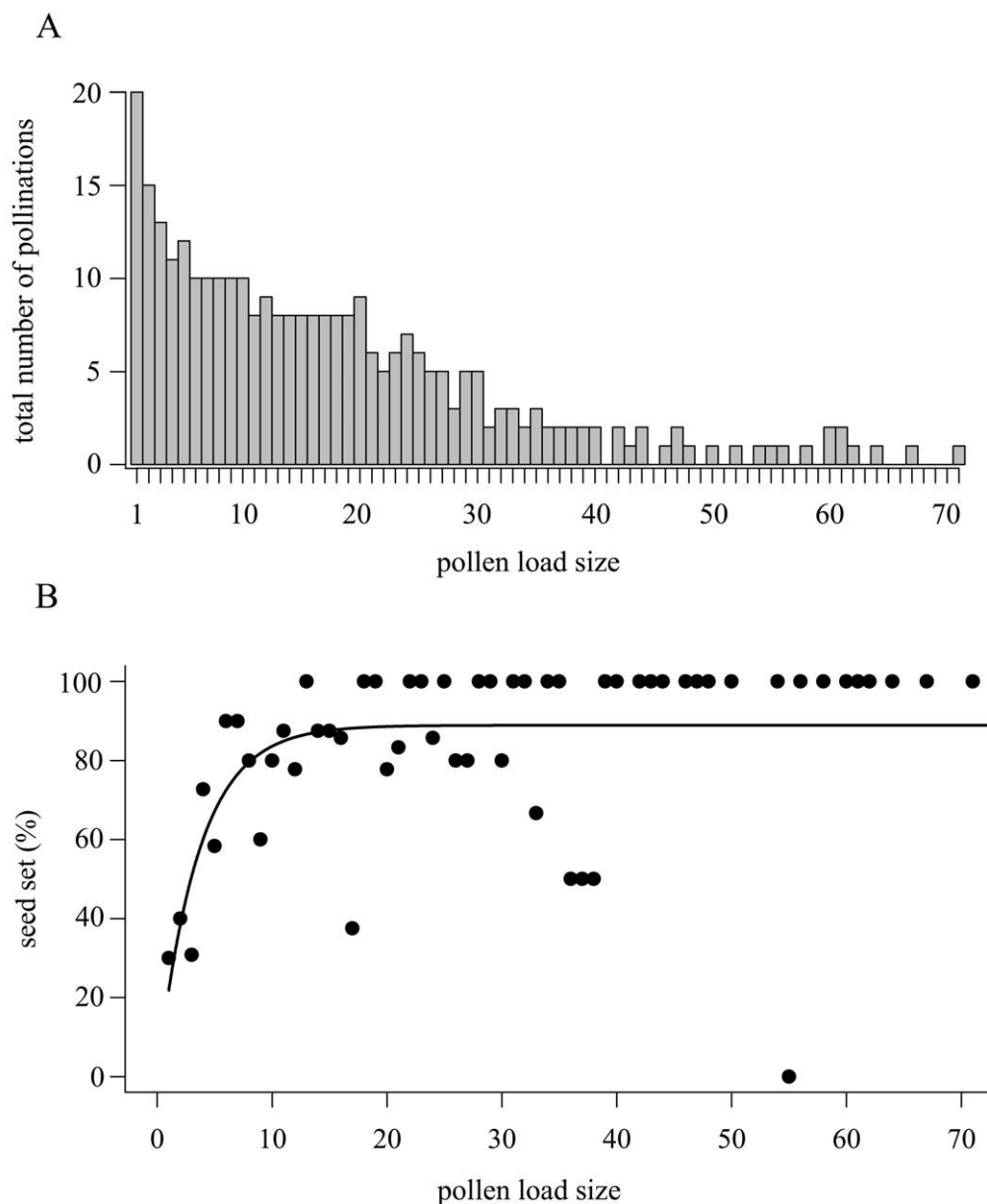


Fig. 2 A, Distribution of pollination intensities (pollen load size) and their sample sizes for controlled pollinations of *Rumex hastatulus* in this study ($n = 302$). B, Dose-response relation between pollen load size and seed set for controlled pollinations of *R. hastatulus*. The curve represents the nonlinear regression of $S = dO(1 - e^{-bp})$, where S is seed set (%), $d = 0.89$ (95% CI, 0.804–0.973), $O = 1$, $b = 0.28$ (95% CI, 0.279–0.286), and p is pollen load size.

Fertilization success was easily identified by the swelling of the ovaries within 24 h of pollination, and fruits were matured in the glasshouse for ~4 wk and seed set was scored (0 = no seed, 1 = filled seed) for pollinated and unpollinated flowers. We collected fruits when they were mature, and they were dried at room temperature for 8 wk. Only 1.5% of the unpollinated flowers set seed, compared with 74.1% of the experimental pollinations, indicating that there was no significant contamination of pollinated flowers by airborne pollen in our experiment.

Sex Ratios and Sporophyte Vigor of Progenies from Controlled Pollinations

We germinated seed from controlled pollinations and transplanted seedlings using the methods described above for the parental generation. The number of progeny for each pollen load ranged from 1 to 9 (mean = 3.9). Pots were positioned in a complete randomized design on a glasshouse bench. Unlike the parental generation, no fertilizer was applied to plants for the duration of the experiment. At flowering we recorded the gender of each individual, and at 8 wk the following fitness components were measured: (i) number of leaves, (ii) plant height, (iii) number of inflorescences, (iv) total length of the inflorescences, and (v) total aboveground biomass after oven-drying (55°C for 72 h). There is a significant correlation between inflorescence length and flower number for females and males of *R. hastatulus* (females: $r = 0.84$, $P < 0.001$; males: $r = 0.78$, $P < 0.001$; M. Pickup and S. C. H. Barrett, unpublished data, 2010). We therefore used inflorescence length as an estimate of total flower production. Seed set was high in the glasshouse because of the intermingling of females and males and low-level air currents produced by fans.

Statistical Analyses

We investigated the relation between stigmatic pollen load, seed set, and sex ratio using a generalized linear model (GLM) with a binomial distribution and logit link function (logistic regression). Logistic regression is the most appropriate model for analyzing proportion data (seed set or sex ratio) because it uses weighted regression to include individual sample sizes as weights in the analysis (Wilson and Hardy 2002). For our data, where we had multiple observations for most pollen load numbers (mean = 8.5 observations per pollen load), logistic regression enabled us to examine whether the probability of setting seed (or being female or male) varied with pollination intensity while accounting for variation in sample size among the different pollen load numbers. Log transformation of the number of pollen grains significantly improved the fit of the model for both seed set and sex ratio. Given the potential contribution of maternal family to variation in seed set and sex ratio, we included maternal parent in the analysis of both variables. Accordingly, for each analysis, the log number of pollen grains, maternal plant, and their interaction were added sequentially to the model. For the sex ratio data, we conducted two separate analyses: (i) one for pollen loads up to 15 pollen grains, because this was identified from the seed set analysis as the pollen load at which

maximum seed set was achieved, and (ii) one for the entire range of pollen load sizes (1–71). Including all pollinations, we tested for significant deviations in sex ratio from 0.5 using a binomial test.

We used the dose-response relation between pollination intensity and seed set to estimate pollen grain fertilization efficiency (b) and the fraction of fertilized ovules that develop into seeds (d) using the nonlinear regression model of Aizen and Harder (2007). Using this approach, total expected seed set (S) is

$$S = dO(1 - e^{-bp}),$$

where d is the proportion of fertilized ovules that develop into seed, O is the number of ovules per flower, b is the proportion of the pollen grains producing pollen tubes that reach the ovary, and p is the stigmatic pollen load (per ovule). This model was fitted and parameters b and d were estimated (with 95% confidence intervals [CIs]) using the package “DoseFinding” (Bornkamp et al. 2011) in R (ver. 2.12.1).

We tested the effect of gender (male, female) and stigmatic pollen load on each fitness-related trait using analysis of covariance (ANCOVA), which allows the analysis of data with both categorical (gender) and continuous (pollen grain number) explanatory variables. We assessed assumptions of normality and homogeneity of variances by plotting residuals. We tested the significance of statistical tests at $\alpha = 0.05$ and conducted the GLM, binomial test, and ANCOVA using Genstat for Windows (12th ed., VSN International, Oxford).

Results

Relation between Pollination Intensity and Seed Set

There was a significant positive relation between stigmatic pollen load size (log) and seed set in *Rumex hastatulus* ($\chi^2_{1,297} = 35.57$, $P < 0.001$; fig. 2B). Seed set was >80% when pollen loads were >15 and 30%–40% when <3 pollen grains were applied to stigmas. Maternal parent had no effect on seed set ($\chi^2_{1,297} = 0.23$, $P = 0.632$), and there was no significant interaction between maternal parent and stigmatic pollen load size ($\chi^2_{1,297} = 2.98$, $P = 0.085$). Following Aizen and Harder (2007), the proportion of pollen grains that reached the ovary (b) was 0.28 (95% CI, 0.279–0.286), and the proportion of fertilized ovules that developed into seed (d) was 0.89 (95% CI, 0.804–0.973).

Relation between Pollination Intensity and Progeny Sex Ratios

Overall there was significant female bias in progeny of *R. hastatulus* (sex ratio = 0.62, $n = 205$; $P < 0.001$, 95% CI, 0.56–0.69) when all pollinations in the experiment were combined. In addition, there was a significant positive relation between stigmatic pollen load (log) and sex ratio for pollen loads between 1 and 15 pollen grains ($\chi^2_{1,96} = 7.46$, $P = 0.008$; fig. 3). Over these pollination intensities, increasing the number of pollen grains applied to stigmas resulted in greater female bias. Progeny sex ratios were male biased

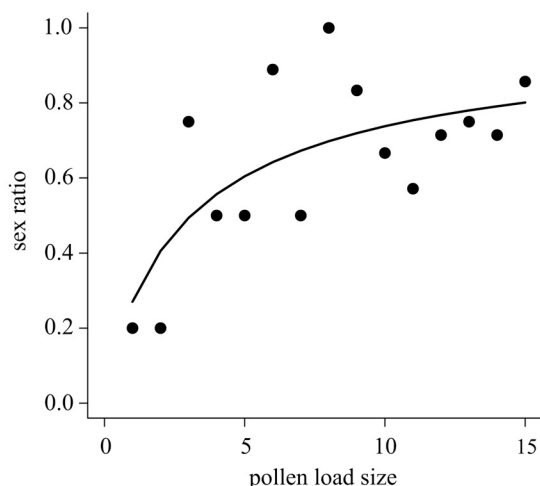


Fig. 3 Dose-response relation between stigmatic pollen load and sex ratio for controlled pollinations of *Rumex hastatulus* over the range of 1–15 pollen grains. The equation for this relation is $y = e^{-0.993 + 2.028x} / (1 + e^{-0.993 + 2.028x})$, where x is the log number of pollen grains.

when 1 and 2 pollen grains were applied to stigmas and either displayed equality or female bias from pollen load sizes of 3–7 pollen grains. In comparison, progeny sex ratios from 8 to 15 pollen grains were all female biased (fig. 3). Maternal plant had no effect on progeny sex ratio ($\chi^2_{1,96} = 0.82$, $P = 0.367$), and there was no significant interaction between pollen grain number and maternal plant ($\chi^2_{1,96} = 0.08$, $P = 0.784$). In contrast, when the entire range of pollen load sizes was examined, there was no significant relation between the probability of producing female offspring and stigmatic pollen load size ($\chi^2_{1,197} = 0.57$, $P = 0.451$). Similar to the analysis using pollen loads of up to 15 pollen grains, maternal plant had no effect on sex ratio ($\chi^2_{1,197} = 2.00$, $P = 0.159$), and there was no interaction between maternal plant and pollen grain number ($\chi^2_{1,197} = 0.31$, $P = 0.578$).

Comparisons of Fitness Components in Progeny from Controlled Pollinations

Stigmatic pollen load size had no significant effect on any of the fitness-related traits measured for progenies grown from controlled pollinations (table 1; $P > 0.05$). Progenies

produced from a range of pollen loads (1–71 pollen grains) had a similar number of leaves, plant height, inflorescence traits and total biomass, and this was consistent for both females and males (number of pollen grains \times sex; $P > 0.05$). However, for several traits there were significant differences between females and males ($P < 0.001$ – 0.015). Female plants produced significantly more leaves and inflorescences than males and had greater biomass. In contrast, males were significantly taller at first flowering, but there was no difference in plant height at harvest. Total inflorescence length, a measure of total flower production, was also similar for females and males (table 1).

Discussion

Our study is the first to examine the dose-response relation between pollination intensity and female fertility in a wind-pollinated plant. By manipulating the number of pollen grains deposited on stigmas of female flowers in *Rumex hastatulus*, we determined the functional relation between pollen load size and seed set (fig. 2B). This species is characterized by relatively high fertilization efficiency and asymptotic seed set, with maximum seed fertility achieved with pollen loads >15 . We found that seed progenies from all controlled crosses exhibited an overall female bias (0.62) but that the probability of producing female offspring increased only over the range of pollination intensities from 1 to 15 pollen grains (fig. 3), presumably reflecting the region over which gametophytic competition increases most steeply up to maximum seed set. We found no evidence that pollen load size influenced the sporophytic vigor of offspring resulting from controlled pollinations. We focus our discussion on the implications of our results for the pollination biology of anemophilous plants and evaluate the potential contribution of gametophytic competition to sex ratio variation in dioecious species.

Pollen Limitation in Wind-Pollinated Plants

Understanding the functional relation between pollination intensity and seed set is important for determining the conditions under which pollen limitation occurs and its ecological and evolutionary consequences (Aizen and Harder 2007). By using dioecious, wind-pollinated *R. hastatulus*, we were able to isolate the effects of pollen quantity on seed set and mini-

Table 1

Results of the Statistical Analyses (ANCOVA) of the Influence of Stigmatic Pollen Load Size (log), Sex (Male or Female), and Their Interaction on a Range of Fitness-Related Traits in *Rumex hastatulus*

	df	No. pollen grains (log)		Sex		No. pollen grains (log) \times sex	
		F	P	F	P	F	P
No. leaves	3, 199	3.00	.085	<u>46.57</u>	<.001	1.00	.319
Plant height (mm)	3, 199	.11	.738	3.15	.078	1.20	.274
No. inflorescences	3, 199	.09	.766	<u>15.08</u>	<.001	.03	.872
Total inflorescence length (mm)	3, 195	1.97	.162	3.67	.057	1.51	.220
Total biomass (mg)	3, 183	.17	.681	<u>6.07</u>	.015	.20	.651

Note. Significant P values ($\alpha = 0.05$) are underlined.

mize the potential influences of pollen quality limitation. In dioecious species, unisexuality precludes opportunities for self-pollen to occur on stigmas, a major source of low-quality pollen in hermaphroditic species. As a result, pollen quality is less likely to influence the shape of the dose-response relations between pollen receipt and seed set (see fig. 1a in Aizen and Harder 2007). Moreover, pollen quality limitation may be lower in wind-pollinated plants because, unlike animal-pollinated species, in which pollen commonly arrives in clumps, anemophilous pollen grains arrive independently and so are more likely to be drawn from a larger sample of potential paternal parents in a population (Hamrick et al. 1979; Friedman and Barrett 2009). These features, combined with the strong association between anemophily and uniovulate flowers (Friedman and Barrett 2008), suggest that the functional relations between pollination intensity and seed set and their influence on pollen limitation may be fundamentally different between wind- and animal-pollinated plants.

A nonlinear relation between pollination intensity and seed (or fruit) set has been observed for several animal-pollinated species (Shore and Barrett 1984; Kalla and Ashman 2002; Cane and Schiffhauer 2003; Aizen and Harder 2007). For *R. hastatulus*, we also found a nonlinear pollen load–seed set response curve with maximum seed set when more than 15 pollen grains were applied to stigmas. In our study, we found higher fertilization efficiency ($b = 0.28$) and asymptotic seed set ($d = 0.89$) compared to previous reports for animal-pollinated species (e.g., $b = 0.19$, $d = 0.60$; Aizen and Harder 2007). This raises the possibility that wind-pollinated plants may generally exhibit steeper dose-response relations and hence a lower potential for pollen limitation than occurs in animal-pollinated species. Unfortunately, few data are available for wind-pollinated plants to evaluate this idea. Recent surveys of pollen loads ($n = 19$ species) and the degree of pollen limitation ($n = 10$ species) in herbaceous wind-pollinated plants have reported pollen loads in excess of the number of available ovules (mean pollen load, 34.1; Friedman and Barrett 2009) and little evidence for pollen limitation among the species examined (Friedman and Barrett 2008). Future investigations of dose-response relations between pollination intensity and seed set over a broader range of life histories (e.g., shrubs and trees) would be valuable for determining the incidence of pollen limitation in wind-pollinated plants.

Mechanisms Causing Biased Sex Ratios

Biased sex ratios are commonly found in dioecious plant populations, and a variety of mechanisms contribute to deviations from equality (reviewed in Barrett et al. 2010). Female-biased sex ratios are ~50% less frequent than male-biased ratios and are often associated with the occurrence of sex chromosomes (Lloyd 1974; D. L. Field, M. Pickup, and S. C. H. Barrett, unpublished manuscript, 2011). It has been proposed that gametophytic competition (certation) between female- and male-determining pollen tubes contributes to female-biased ratios in dioecious *Rumex* species (Correns 1922, 1928; Smith 1963; Conn and Blum 1981; Stehlik and Barrett 2005), with female-determining pollen tubes prefer-

entially fertilizing ovules when stigmas receive multiple pollen grains. Pooling progeny from all controlled pollinations, we found an overall female bias (0.62), which was close to that observed in the population from which families were sampled (0.65) and identical to the average bias that occurs in populations of *R. hastatulus* (0.62; $n = 46$, range = 0.5–0.71; M. Pickup and S. C. H. Barrett, unpublished data, 2009). In contrast to perennial *Rumex* species, where the gender-based mortality of males contributes substantially toward female-biased sex ratios (see, e.g., fig. 1 in Stehlik and Barrett 2005), *R. hastatulus* is an annual, and the vast majority of plants that germinate go on to flower. In our experiment, germination and survival were high (97% and 98%, respectively), and virtually all plants flowered. Thus, in this experiment, the scope for gender-based mortality in *R. hastatulus* is highly restricted, and female bias appears to result largely from processes operating during the progamic phase of the life cycle. However, we cannot discount that differences in gender-based mortality may become apparent under the more stressful conditions that commonly occur in the field.

Consistent with the certation hypothesis, involving preferential fertilization of ovules by female-determining pollen tubes, we found that female bias increased with pollen load size when we applied between 1 and 15 pollen grains to the stigmas of female flowers (fig. 3). Even though this relation is driven primarily by the progeny sex ratios for the treatments involving the addition of 1 and 2 pollen grains, the probability of producing female progeny increased with pollen load, providing some support for the certation hypothesis. Intermediate pollen load treatments (3–7 pollen grains) were either at equality or female biased, whereas female bias was consistent for treatments between 8 and 15 pollen grains. Although we have no data on pollen loads in *R. hastatulus* populations, this range is likely to be biologically realistic and similar to that found in natural populations of other dioecious *Rumex* species (Stehlik et al. 2008; Friedman and Barrett 2009). When we included in our analysis all pollen grain number treatments (1–71), we found no significant relation between pollen load size and sex ratio bias. This result occurred primarily because female bias was established for treatments between 10 and 15 pollen grains and pollen loads >30 produced considerable scatter in the degree of bias but with no consistent trend of increasing female bias. This suggests that there may be an upper limit to the bias that can be achieved in this species compared to what has been documented in other *Rumex* species (see below).

The presence of sex ratio distorters (modifiers and restorers) has been documented in several dioecious plant species (Taylor 1999; Glawe and De Jong 2007), whereby maternal and/or paternal genetic effects contribute to variation in primary sex ratios. Although we found no effect of maternal parent on sex ratio variation, larger progeny sample sizes at each pollen load would provide greater power to detect the presence of sex ratio distorters and their potential contribution to sex ratio variation in *R. hastatulus* populations. In our experiment, the most likely explanation for this variation is the smaller sample sizes at higher pollination intensities (fig. 2A). Alternatively, pollen loads may have a predictable effect on sex ratio only when there is variation in

the strength of gametophytic competition, as might occur in the sorting stage of pollen competition up to maximum seed set. However, this explanation is not altogether satisfactory, as previous pollination studies of *Rumex* species have given results inconsistent with these findings. For example, in *Rumex acetosa* (Rychlewski and Zarzycki 1975) and *Rumex nivalis* (Stehlik and Barrett 2006) the application of very large amounts of pollen to stigmas resulted in strongly female-biased ratios. In contrast, a study on *R. hastatulus* involving four levels of pollen dilution found that the most biased sex ratio was 0.62 (Conn and Blum 1981), the overall value we obtained from all pollinations in this study. Strong sex ratio bias of the magnitude reported in perennial *Rumex* species may not be obtainable in *R. hastatulus*, although why this should be so is at the moment unclear. Taken together, these results and those of previous studies leave little doubt that variation in pollination intensity plays an important role in causing female-biased sex ratios in *Rumex*.

Pollination Intensity and Sporophyte Fitness

The ecological and evolutionary significance of gametophytic competition on sporophyte fitness has been a controversial issue (Mulcahy 1979; Charlesworth 1988). A criticism of experimental studies of the effects of pollination intensity on sporophyte fitness has been the lack of spread of pollen loads across the filling (low-competition), sorting (moderate-competition), and surplus (high-competition) regions of the pollen load–seed set dose–response curve (Mitchell

1997). In our study, we used a continuous range of pollen grain numbers (fig. 2A) and identified the number of pollen grains required for maximum seed set to ensure that progeny represented all three stages. Despite sampling across all three regions, variation in pollen loads had no effect on sporophytic fitness, a result also reported from several animal-pollinated (Snow 1990, 1991; Mitchell 1997) and one wind-pollinated (Davis 2004) species. The resource levels in which progeny are grown can affect the outcomes of gametophytic competition, with high resources obscuring the influence of pollination intensity on fitness components (Kalla and Ashman 2002). In our experiment, progeny were grown under low-resource conditions to limit such an effect, but we were still unable to demonstrate any influence of pollen load size on sporophytic fitness. The occurrence of uniovulate flowers and a potentially high diversity of pollen donors should lead to intense gametophytic selection in wind-pollinated plants. However, whether such effects mediated by variation in pollination intensity commonly influence plant fitness remains to be demonstrated.

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