

## POLLINATION INTENSITY INFLUENCES SEX RATIOS IN DIOECIOUS *RUMEX NIVALIS*, A WIND-POLLINATED PLANT

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**Abstract.**—Determining the mechanisms governing sex-ratio variation in dioecious organisms represents a central problem in evolutionary biology. It has been proposed that in plants with sex chromosomes competition between pollen tubes of female- versus male-determining microgametophytes (certation) causes female-biased primary sex ratios. Experimental support for this hypothesis is limited and recent workers have cast doubt on whether pollen-tube competition can modify sex ratios in dioecious plants. Here we investigate the influence of variation in pollination intensity on sex ratios in *Rumex nivalis*, a wind-pollinated alpine herb with strongly female-biased sex ratios. In a garden experiment, we experimentally manipulated pollination intensity using three concentric rings of female recipient plants at different distances from a central group of male pollen donors. This design enabled us to test the hypothesis that increasing pollen load size, by intensifying gametophyte competition, promotes female-biased sex ratios in *R. nivalis*. We detected a significant decline in pollen load at successive distance classes with concomitant reductions in seed set. Sex ratios of progeny were always female biased, but plants at the closest distance to male donors exhibited significantly greater female bias than more distant plants. The amount of female bias was positively correlated with the seed set of inflorescences. Hand pollination of stigmas resulted in ~100-fold higher stigmatic pollen loads than wind-pollinated stigmas and produced exceptionally female-biased progenies (female frequency = 0.96). Our results are the first to demonstrate a functional relation between stigmatic pollen capture, seed set, and sex ratio and suggest that certation can contribute towards female-biased sex ratios in dioecious plants.

**Key words.**—Certation, dioecy, female-biased sex ratios, pollen intensity, seed set, stigmatic pollen loads, wind pollination.

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In dioecious populations negative frequency-dependent selection generally acts to maintain unbiased sex ratios (Fisher 1930, Hardy 2002). Despite this fundamental principle, populations of many plant species exhibit biased sex ratios and a variety of ecological and genetic mechanisms have been proposed to explain deviations from equality (Delph 1999; de Jong and Klinkhamer 2002). These include gender-specific patterns of mortality due to different costs of reproduction in females versus males (Lloyd and Webb 1977; Bierzychudek and Eckhart 1988), local mate competition (de Jong et al. 2002), differential herbivory of the sexes (Ågren et al. 1999), selfish genetic elements (Taylor 1999; Taylor and Ingvarsson 2003), and gametophytic competition (Correns 1917; Conn and Blum 1981). These diverse mechanisms operate at different life-history stages and therefore determining when and how deviations from equality are established is a critical issue for research on the evolution of plant sex ratios.

Fourteen percent of dioecious plant species that have been surveyed exhibit female-biased adult sex ratios, with most reports concentrated in a few genera, particularly *Silene* and *Rumex* (reviewed in Delph 1999). Correns (1917, 1922, 1928) first proposed that in these taxa, pollen-tube competition in the style (*certation*: Heribert-Nilsson 1920; Baker 1975) between female- and male-determining gametophytes, could cause deviations from equal sex ratios (see also Webb 1992; Alström-Rapaport et al. 1997; Richards 1997; de Jong and van der Meijden 2004; Glawe and de Jong 2005). Correns experimentally manipulated the amount of pollen on stigmas (pollination intensity), estimated progeny sex ratios, and concluded that sex-ratio biases were usually stronger the larger the stigmatic pollen load (Correns 1928). Correns (1928) hypothesized that the earlier germination or faster growth of

pollen tubes might allow female-determining gametophytes to outcompete male-determining gametophytes in the pistils of female plants.

Recent experimental work on *Silene* has cast doubt on the role of certation as a mechanism contributing towards female-biased sex ratios (Carroll and Mulcahy 1993). At least in *S. latifolia*, biased sex ratios appear to be governed instead by the dynamics of selfish genetic elements involving a system of sex-ratio distorters and restorers (Taylor 1994, 1999). However, the certation hypothesis still remains a viable explanation for female-biased sex ratios in *Rumex*. In several species, stigmatic pollen loads have been experimentally manipulated giving female-biased sex ratios consistent with Correns' original hypothesis (Rychlewski and Zarzycki 1975; Conn and Blum 1981). However, attempts to demonstrate that certation influences sex-ratio variation in the field have been inconclusive and attempts to establish functional associations between pollination intensity and sex-ratio bias have given mixed results (Rychlewski and Zarzycki 1975, 1986; Conn and Blum 1981). As a consequence, the role of certation in affecting sex-ratio variation in dioecious plants has remained controversial (Richards 1997; de Jong and Klinkhamer 2002).

*Rumex nivalis* (Polygonaceae) is a wind-pollinated, dioecious herb endemic to the European Alps and mountains of Bosnia-Herzegovina (Wagenitz 1981). A survey of 18 populations from Switzerland revealed strongly female-biased adult (flowering) sex ratios (Stehlik and Barrett 2005). Using a male-specific sequence-characterized amplified-region (SCAR) marker, these authors demonstrated that open-pollinated seed sex ratios in these populations were also female biased (mean 0.59,  $N = 18$  populations), but less so than

non-flowering (0.78) or flowering individuals (0.87). A weak association was also evident between the degree of female bias in seed sex ratios and the flowering sex ratio of populations. Females in populations with strongly female-biased sex ratios produced less female-biased offspring, whereas populations with higher frequencies of flowering males produced more female-biased progeny (fig. 3, Stehlik and Barrett 2005). These patterns are predicted by the certation hypothesis if flowering sex ratios influence pollination intensity. For example, females in populations with few males might be expected to capture much less pollen than those in populations with higher frequencies of males. However, a direct functional link between flowering sex ratios and pollination intensity was not established in this study and because a range of demographic and environmental factors can influence pollination intensity, support for the certation hypothesis was weak at best.

Here, we investigate further the certation hypothesis by examining experimentally the effect of variation in pollination intensity on sex ratios in *R. nivalis*. In contrast to studies of pollination intensity in animal-pollinated plants in which the manual application of pollen grains to stigmas is relatively straightforward (e.g., Shore and Barrett 1984; Waser and Price 1991; Mitchell 1997), we used increasing spatial distances between males and females as a means of varying pollination intensity. In our experiments we sought to establish direct relations between the amount of pollen captured by stigmas, seed set, and sex ratio. Specifically, we predicted that if certation contributes towards the observed female-biased seed sex ratios in *R. nivalis*, we would expect significantly greater female bias in plants capturing the largest amounts of pollen on their stigmas. This prediction assumes that the size of the stigmatic pollen load is associated with the intensity of pollen-tube competition, which seems a reasonable assumption for a uniovulate-flowered species such as *R. nivalis*.

## MATERIALS AND METHODS

### *The Study Species*

*Rumex nivalis* (Polygonaceae) is an alpine perennial that grows above tree line (1900 to 2800 m a.s.l.) mostly in snowbeds composed of sparse, wind-swept vegetation in which it is sometimes the dominant species. Individuals are composed of a basal rosette of fleshy leaves and mostly unbranched inflorescences of approximately 30 cm height (Wagenitz 1981). Inflorescences flower for ~14 days, and the anthesis period of individual flowers lasts for two to three days (I. Stehlik, pers. obs.). Females produce taller inflorescences than males and mature up to 200 seeds per inflorescence (Stehlik and Barrett 2005). Females of *R. nivalis* possess one pair of X chromosomes (XX—homogametic sex;  $2n = 14$ ), whereas males have one X and two Y chromosomes (XY<sub>1</sub>Y<sub>2</sub>—heterogametic sex;  $2n = 15$ ; Wagenitz 1981).

### *Experimental Design*

We conducted six experimental trials to investigate the relation between pollination intensity and sex ratio using potted plants on an exposed rooftop garden atop the Botany

Department, University of Toronto, during August–September 2004. We used a donor-recipient design involving a central group of male plants surrounded by three concentric circles of eight female plants at three distance classes. Because male plants produced different numbers of inflorescences, we used either eight or nine plants to ensure similar numbers of inflorescences per trial. Conn and Blum's (1981) field study of *R. hastatulus* failed to detect any effect of increasing distance on sex ratios using relatively large distances (0.5–95 m minimum and maximum distance classes, respectively), we therefore used shorter distances between donor and recipient plants to ensure sufficient variation in pollen capture by stigmas to detect a signal from certation. For the minimum distance, we positioned females directly adjacent to males (5 cm distance) and used 80 cm and 150 cm as our intermediate and furthest distance class, respectively. These distances between male and female plants are representative of those commonly observed between neighbors in natural populations (I. Stehlik, unpubl. data;  $N = 6$  populations; total number of plants measured = 180; mean distance to closest neighbor = 109 cm; range: 1–500 cm). Individual trials were run for ~16 days and after each trial was complete, we moved females back into a glasshouse to mature seeds. Six weeks after the start of each trial we collected ripe seeds from inflorescences and stored them at room temperature.

We conducted supplemental ‘‘hand pollinations’’ on a group of seven females to compare the results with those obtained from the experimental trials. We repeatedly brushed individual female inflorescences against male inflorescences on six successive days. This procedure enabled us to assess the degree of pollen limitation in our experimental trials and to examine whether high pollen loads influence sex ratios.

Plants in our experiments originated from individuals used in Stehlik and Barrett (2005). In that study, open-pollinated seed from 18 populations in Switzerland were collected and grown in a randomized design in the glasshouse, and plants set seed through open wind pollination. From this glasshouse population we collected 42 seed families representing 16 populations and grew 32 seeds per maternal parent. Just prior to the onset of flowering of this large mixed filial generation, we selected females and males for our six trials. Because the genders of *R. nivalis* differ in their morphology it was possible to distinguish females and males before anthesis. Within individual trials we avoided half siblings and used females originating from as many populations as possible. By using a genetic admixture we avoided any potential population-specific influences on sex ratios.

### *Measurements*

#### *Stigmatic pollen loads*

We measured stigmatic pollen loads from flowers sampled from all female plants in the six pollination trials and of the seven hand-pollinated females. We excised three random stigmas per plant 7, 10, and 14 days after the onset of each trial, and on day 2, 4, and 6 of the hand-pollination experiment. Ovary swelling and female flower senescence was so rapid, especially in hand-pollinated females and females at the closest distances to males, that we were not able to collect the third stigma sample for some females (14% of the total

sample). We stored stigmas in 70% ethanol in microcentrifuge tubes before pollen loads were counted. We stained stigmas in 1% fuchsin and counted pollen grains on stigmas under a compound microscope with a 100× magnification. We also measured the maximum length of the three stigmatic lobes per stigma to investigate the influence of stigma size on the amount of pollen captured.

#### Seed set

We counted the number of seeds produced by all female inflorescences in the trials and calculated the total seed set of each female by dividing the number of fully developed seeds by the total number of flowers. Most females produced more than one inflorescence (mean inflorescence number per plant 3.36, range 1–8). Additionally, to explore the relation between inflorescence height and seed set we measured height from soil level to the top of the inflorescence.

#### Sex ratios

We calculated sex ratios from each of the three distance classes in each trial as the number of female offspring divided by the total number of offspring per plant. Progeny testing involved one inflorescence per female excluding those with too few seeds ( $N$  seeds < 20). We determined the sex ratio of progenies for each distance class per trial by growing 20 offspring per female to flowering. Our total sample used seed progenies for 132 of the possible 144 maternal parents with 12 females producing too little seed for sex ratio assessment. In these cases we compensated for missing families by doubling the number of seeds to 40 for a random female from the same distance class and trial. Germination rates were high (>90%) with on average 18.73 (SE = 0.12) seeds out of 20 producing seedlings. Our total sample size involved 2211 individuals and at the final census for sex determination only 1.3% of this total sample had not flowered. Our previous work on *R. nivalis* (Stehlik and Barrett 2005) established that the sex ratio of germinated versus ungerminated seeds was not significantly different. We are therefore confident that the flowering sex ratios in our experiment reflect seed sex ratios.

For the seven hand-pollinated females, we followed the same procedures with exception that all of the seeds produced were used for progeny testing in order to maximize sample sizes. This resulted in a sample of 172 flowering individuals.

#### Data Analysis

We investigated pollen deposition on stigmas, seed set, and sex ratios using generalized linear models in PROC GENMOD (SAS vers. 9.1; SAS Institute 2001) treating trial and distance to males as fixed factors. For the analysis of pollen capture, we applied the log-link function to account for the negative binomial data distribution (overdispersed Poisson distribution of pollen count data; SAS Institute 2001) and we treated stigma length as a covariate. We analyzed pollen capture data in two ways, including and excluding zero stigma pollen counts. We did not include height of inflorescences in these analyses because we removed stigmas from random inflorescences and flower positions.

We analyzed seed set for all inflorescences and also sep-

arately for the selected inflorescences used for progeny testing of sex ratios. We used logit transformations to accommodate the binomial distribution of data and used height of female inflorescences as a covariate, because we expected it to influence the ability of female plants to filter pollen from the airstream. Seed sex ratios of inflorescences chosen for sex determination were analyzed using logit transformations due to the binomial distribution of data with height of female inflorescences as a covariate. We also examined the overall relation between seed set and sex ratio irrespective of distance class using generalized linear models in PROC GENMOD (SAS vers. 9.1; SAS Institute 2001) and a binomial probability distribution on count data.

For all analyses, we excluded nonsignificant factors by stepwise backward elimination at  $\alpha = 0.05$ . We interpreted significant factors and their interactions with contrasts and corrected the probabilities for these contrasts using the Dunn-Šidák formula (Sokal and Rohlf 1995). For the three main analyses from the six trials (pollen capture, seed set, and sex ratios), we also compared the results with those obtained from hand-pollinated females.

## RESULTS

### Pollen Capture

The distance between male and female plants of *R. nivalis* had a significant effect on pollen capture (Fig. 1A, Table 1). Pollen loads on stigmas were largest at distance 1 (5 cm, mean 2.05, range 0–27), intermediate at distance 2 (80 cm, mean 0.31, range 0–4), and lowest at distance 3 (150 cm, mean 0.21, range 0–2). However, no pollen was detected on 68% of the sampled stigmas with the lowest fraction of zero counts at distance 1 (0.36, SE = 0.05) and equivalent values for the remaining two distance classes (distance 2: 0.82, SE = 0.04; distance 3: 0.82, SE = 0.04). Excluding stigmas that did not capture pollen, the mean number of pollen grains per stigma declined significantly among the three distance classes (Table 1: distance 1 = 3.2, SE = 0.5; distance 2 = 1.7, SE = 0.2; distance 3 = 1.1, SE = 0.1).

For the dataset including zero counts, stigma length had a significant effect on pollen capture (Table 1). Stigmas with very short or very long stigmatic lobes captured less pollen than stigmas of intermediate size. Additionally, there was a significant interaction of stigma length and distance to males (Table 1). Specifically, at distance 2, longer stigmas captured significantly more pollen ( $\chi^2_{1,119} = 11.64, P = 0.0006$ ), whereas at distance 1 and 3 there was no significant relation between pollen capture and stigma length.

As expected, hand pollination resulted in much higher pollen loads than occurred from wind pollination. Pollen was detected on all stigmas with an average of 344.1 pollen grains, SE = 67.8, and a range 2–1286.

### Seed Set

The distance between male and female plants of *R. nivalis* had a significant influence on percent seed set (Fig. 1B, Table 2). For the total inflorescence sample (all inflorescences produced, including multiple inflorescences per female), seed set was highest for females at the closest distance to males

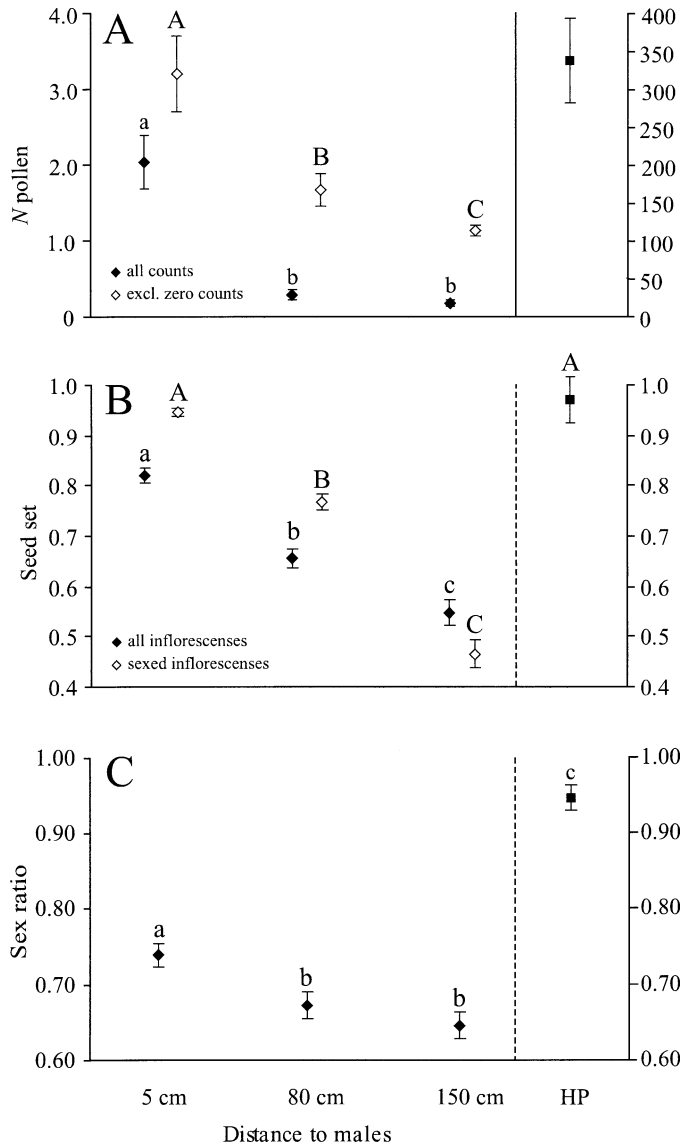


FIG. 1. (A) Mean (SE) number of pollen grains per stigma; (B) mean (SE) percent seed set, and (C) mean (SE) primary sex ratio for female plants of *Rumex nivalis* at three distances (5, 80, and 150 cm) to males in six trials involving wind pollination. The results from seven hand-pollinated (HP) females are also illustrated. For further details of the experimental trials see Methods. (A) Filled diamonds: all stigmas; open diamonds: subset of stigmas excluding zero pollen counts. Note the different scale of the y-axis for the six trials versus the hand-pollinated females. (B) Filled diamonds: all inflorescences; open diamonds: subset of inflorescences used for sex determination. (C) Note that all sex ratios were significantly female-biased (G-tests rejected unbiased sex ratios at  $P < 0.0001$ ; Sokal and Rohlf 1995). Letters above means indicate significant differences.

(distance 1: 0.82, SE = 0.09) and decreased with increasing distance from males (distance 2: 0.65, SE = 0.11; distance 3: 0.54, SE = 0.03). The same general pattern was evident among the inflorescences that were selected for progeny sex ratios (144 females, excluding the 12 females with too little seed set).

There was significant variation in percent seed set among the six trials for both the entire dataset and also for the se-

lected inflorescences (Table 2). Height of inflorescence was only significant for the total inflorescence sample (Table 2), with taller inflorescences setting proportionally more seed than smaller inflorescences. However, for both datasets there was a marginally significant interaction between height and distance ( $P = 0.053$  for the full dataset;  $P = 0.051$  for selected inflorescences; Table 2). In the total inflorescence sample, the relation between height and seed set increased with increasing distance to males (distance 1:  $\chi^2_{1,139} = 5.67$ ,  $P = 0.0172$ ; distance 2:  $\chi^2_{1,146} = 12.56$ ,  $P = 0.0004$ ; distance 3:  $\chi^2_{1,130} = 13.52$ ,  $P = 0.0002$ ), whereas for selected inflorescences there was only a significant relation between seed set and height at the furthest distance class, with taller inflorescences exhibiting the highest seed set.

Mean seed set in hand-pollinated females (0.97, SE = 0.07) was significantly higher than for all distance classes except for selected inflorescences at the closest distance to males (pair-wise comparisons, adjusted for multiple comparisons using sequential Bonferroni tests at  $\alpha = 0.05$ ; distance 1 versus hand-pollinated:  $\chi^2_{1,50} = 1.28$ ,  $P = 0.258$ ; distance 2 versus hand-pollinated:  $\chi^2_{1,52} = 135.78$ ,  $P < 0.0001$ ; distance 3 versus hand-pollinated:  $\chi^2_{1,62} = 460.44$ ,  $P < 0.0001$ ; Fig. 1B).

#### Sex Ratios

All sex ratios obtained for the three distance classes were significantly female biased (G-tests rejected an unbiased sex ratio at  $P < 0.0001$ ; Sokal and Rohlf 1995). The distance between female and male plants had a significant influence on the degree of female bias (Fig. 1C, Table 3). The strongest female bias was evident for female plants at the closest distance to males (0.74, SE = 0.01). This sex ratio was significantly higher than the sex ratio obtained for progenies at distance class 2 (0.67, SE = 0.02;  $\chi^2_{1,114} = 7.58$ ,  $P < 0.0059$ ). Sex ratios of distance classes 2 and 3 (0.64; SE = 0.02) were not significantly different.

The seed set of inflorescences was significantly associated with variation in sex ratios (Fig. 2, Table 3). Inflorescences with high proportional seed set produced more female-biased sex ratios than inflorescences with lower seed set, although there was considerable scatter in this relation because of the relatively small family sizes used per inflorescence. In this and the preceding analysis of sex ratio, a significant component of the variation was explained by the factor trial, whereas the height of inflorescences was not significant (Table 3).

The mean sex ratio of hand-pollinated inflorescences was highly female biased (0.96, SE = 0.02). This value was significantly different from female-biased sex ratios of all other distance classes in experimental trials (pair-wise comparisons, adjusted for multiple comparisons by sequential Bonferroni tests at  $\alpha = 0.05$ ; distance 1 vs. hand-pollinated:  $\chi^2_{1,46} = 44.78$ ,  $P < 0.0001$ ; distance 2 vs. hand-pollinated:  $\chi^2_{1,51} = 67.00$ ,  $P < 0.0001$ ; distance 3 vs. hand-pollinated:  $\chi^2_{1,49} = 79.33$ ,  $P < 0.0001$ ; Fig. 1C). Among the seven hand-pollinated plants, one female produced only daughters ( $N = 25$ ).

TABLE 1. Analysis of pollen loads on stigmas of *Rumex nivalis* following six experimental trials involving wind pollination (see Methods for details). The data were tested against a negative binomial distribution by fitting generalized linear models with the log-link function in PROC GENMOD (SAS vers. 9.1; SAS Institute 2001). Distance to males and trial were treated as fixed factors and stigma length as a covariate. Nonsignificant factors were excluded by stepwise backward elimination at  $\alpha = 0.05$ .

Pollen load	Distance	Trial	Stigma length	Stigma length $\times$ distance
All stigmas	$\chi^2_{2,339} = 21.20^{***}$	$\chi^2_{3,339} = 6.99$ ns	$\chi^2_{1,339} = 12.20^{***}$	$\chi^2_{2,339} = 9.29^{**}$
Stigmas excluding zero counts	$\chi^2_{2,103} = 16.70^{***}$	$\chi^2_{5,103} = 17.61^{**}$	$\chi^2_{1,103} = 0.66$ ns	ns

\*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; ns, nonsignificant.

## DISCUSSION

Our study demonstrates that primary sex ratios in the alpine herb *R. nivalis* deviate substantially from the expected 1:1 sex ratios for dioecious organisms and involve consistent female bias. By manipulating the distance between male pollen donors and female recipients we established for the first time in dioecious plants a functional relation between pollination intensity, seed set, and sex ratios. Females positioned in close proximity to males captured the most pollen (Fig. 1A), produced the greatest amount of seed (Fig. 1B), and exhibited the most female-biased sex ratios (Fig. 1C). More distant females had lower capture rates of pollen resulting in a correlated decline in seed set, and progeny with less female bias. Significantly, when females of *R. nivalis* were hand-pollinated, receiving pollen loads that were  $\sim 100$ -fold greater than in the wind pollination experiment, sex ratios were exceptionally female biased and were composed of only 4% males on average. The extent of this female bias is striking and raises important questions concerning the influence of postpollination mechanisms on sex-ratio variation in dioecious plants.

### *Pollen Capture, Seed Set, and Sex Ratio*

Although the patterns of pollen capture, seed set, and sex ratio bias that we obtained were in general accord with the certation hypothesis, there were several discrepancies between measured variables that require explanation. For example, the overall seed set of female inflorescences at distance 1 was 0.82 (Fig. 1B), substantially higher than the fraction of stigmas (0.64, Fig. 1A) on which pollen was detected. This discrepancy was even more pronounced for distances 2 and 3, in which seed set was 0.65 and 0.54, respectively (Fig. 1B), but only a small subset of stigmas had pollen on them (0.18 in both cases). These results suggest that the procedures used in our study may have underestimated the total amount of pollen captured by stigmas.

Two explanations may account for underestimates of stigmatic pollen loads in our experiment. First, the low values may have been a consequence of the way we chose to sample stigmas during each trial. Of the three dates of stigma collection, pollen counts were highest for the second collection in four out of six trials, whereas the third collection was always lowest (data not shown). The last collection of stigmas was after peak flowering in males, whereas the first collection was somewhat earlier than peak flowering. Using only pollen loads for the second sample reduces considerably the disparity between the fraction of stigmas with pollen and percent seed set. It is also possible that some pollen may have been inadvertently washed off stigmas during the process of pre-

serving stigmas in ethanol and during slide preparation. Regardless of the accuracy of our estimates it is worth noting that the values that we obtained in our experiment are not substantially different from pollen loads that we have estimated from natural populations of *R. nivalis*. In a study of seven populations from the Swiss Alps, average pollen loads per stigma ranged from 0.18–3.10 with 47% of all stigmas sampled without pollen (I. Stehlik, J. Friedman, S. C. H. Barrett, unpubl. data). Low pollen loads are not unexpected in wind-pollinated plants, which are commonly uniovulate (Pohl 1929; Dowding 1987; but see Honig et al. 1992; Linder and Midgley 1996). However, in comparison to animal-pollinated plants (Burd 1994; Larson and Barrett 2000; Ashman et al. 2004), the extent to which wind-pollinated populations are pollen limited is poorly understood.

We found that sex ratios exhibited the highest female bias in seed progenies of females closest to males (Fig. 1C). Almost three-quarters of all seeds produced at distance 1 were female (0.74), whereas at distance 2 (0.65) and 3 (0.63) the degree of female bias was similar and closer to values obtained in natural populations (Stehlik and Barrett 2005). We also compared sex ratios and seed set of individual inflorescences without using distance as a surrogate for pollination intensity. Seed set was a significant predictor of the primary sex ratio (Table 3); inflorescences with high seed set produced significantly more female-biased sex ratios than inflorescences with lower seed set (Fig. 2). This pattern probably reflects the overall pollen environment of plants at different distances from male donors. Inflorescences that received sufficient pollen to produce near maximal seed set also had higher individual pollen loads on stigmas. In contrast, flowers on inflorescences with lower percent seed set captured significantly less pollen. The relation between percent seed set and female bias therefore represents the correlated outcome of variation in pollination intensity and its probable effect on pollen-tube competition. To our knowledge these functional relations among components of reproduction and sex ratios have not been previously considered for dioecious plants.

Our assumption that pollination intensity influences competition between female- and male-determining pollen tubes was corroborated by the differences we obtained between the sex ratios of open- and hand-pollinated flowers of *R. nivalis*. Average stigmatic pollen loads were  $\sim 100$  times larger in hand-pollinated females than for open-pollinated females at distance 1 (excluding zeros; Fig. 1A). Both treatments received sufficient pollen for near full seed set (distance 1: 0.94; hand pollination: 0.97) and the values we obtained were not significantly different. However, the average primary sex ratio of the two treatments was strikingly different (distance

TABLE 2. Analysis of seed set in *Rumex nivialis* following six experimental trials involving wind pollination (see Methods for details). Data were tested against the binomial distribution by fitting generalized linear models with the logit-link function in PROC GENMOD (SAS vers. 9.1; SAS Institute 2001). Distance to males and trial were treated as fixed factors and height of inflorescences as a covariate. Nonsignificant factors were excluded by stepwise backward elimination at  $\alpha = 0.05$ .

Seed set	Distance	Trial	Height	Distance × experiment	Distance × height
All inflorescences	$\chi^2_{2,400} = 13.21^{**}$	$\chi^2_{2,400} = 103.68^{***}$	$\chi^2_{1,400} = 17.73^{***}$	$\chi^2_{10,400} = 44.59^{***}$	$\chi^2_{2,400} = 5.88^a$
Sexed inflorescences	$\chi^2_{2,138} = 28.24^{***}$	$\chi^2_{5,138} = 49.22^{***}$	$\chi^2_{1,138} = 0.40$ ns	—	$\chi^2_{2,138} = 5.93^b$

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; ns, nonsignificant.

<sup>a</sup>  $P = 0.053$ .

<sup>b</sup>  $P = 0.051$ .

1: 0.74; hand pollination: 0.96). The exceptionally strong female bias in hand-pollinated females, including one individual that produced all female offspring, implies, under the certation hypothesis, that almost every male-determining pollen tube was outcompeted by female-determining pollen. However, the magnitude of pollen loads may not have been the only factor influencing competitive effects among pollen tubes in the two treatments. Pollen deposition in hand-pollinated females was simultaneous, accentuating any competition between female- and male-determining pollen tubes. By contrast, pollen deposition in the wind pollination trials was likely to have been more gradual with earlier arriving pollen grains having a fertilization advantage regardless of gender determination.

*General Patterns and Mechanisms*

Our results demonstrating that pollination intensity causes biased sex ratios provide more convincing results than earlier findings on *R. acetosa* (Rychlewski and Zarzycki 1975) and *R. hastatulus* (Conn and Blum 1981), two species that also display female-biased sex ratios. These authors conducted pollination experiments by applying either ‘‘sparse’’ and ‘‘abundant’’ pollen (Rychlewski and Zarzycki 1975), or by diluting *Rumex* pollen with dead pine pollen (Conn and Blum 1981). Additionally, they also carried out field studies with female plants positioned at different distances from males. The results of their field studies were inconclusive. Sex ratio biases were generally small and unrelated to the distance between males and females, probably because the distances were too great to cause the consistent biases we detected in our experiments. However, evidence for a relation between female bias and pollination intensity was obtained in the controlled pollination studies conducted by these authors. For example, hand pollination of eight female plants of *R. acetosa* with ‘‘excess pollen’’ produced strongly female-biased sex ratios (mean 0.82), including an individual that produced only female offspring (Rychlewski and Zarzycki 1975), a result strikingly similar to what we obtained. Collectively these studies of *Rumex* therefore establish a relation between pollination intensity and sex-ratio bias that so far has no parallel in other dioecious plants.

Variation in the primary sex ratios of dioecious organisms may also result from sex-chromosome meiotic drive or cytoplasmic elements (de Jong and Klinkhamer 2002; Stouthamer et al. 2002). Meiotic drive or segregation distortion can result in the selective abortion of male-determining gametes resulting in a bias in the frequency of female-determining gametes (Taylor and Ingvarsson 2003). Segregation distortion has been detected in *Silene latifolia* (Taylor 1994, 1999; Taylor et al. 1999; Ingvarsson and Taylor 2002), the other well-studied dioecious species with heteromorphic sex chromosomes (Charlesworth 2002). Although primary sex ratios in *S. latifolia* are often female biased, the degree of bias is far less pronounced in comparison with *Rumex* (Carroll and Mulcahy 1993; Taylor 1994). In controlled crosses males produce heritable differences in sex ratios ranging from slightly male biased to strongly female biased (Taylor 1994, 1999; Taylor et al. 1999; Ingvarsson and Taylor 2002). This heritable variation is unlikely to be subject to environmental

TABLE 3. Analysis of sex ratio in *Rumex nivalis* following six experimental trials involving wind pollination (see Methods for details). Data were tested against the binomial distribution by fitting generalized linear models with the logit-link function in PROC GENMOD (SAS vers. 9.1; SAS Institute 2001). Distance to males and trial were treated as fixed factors and height of inflorescences and seed set as covariates. Nonsignificant factors were excluded by stepwise backward elimination at  $\alpha = 0.05$ .

Sex ratio	Distance	Seed set	Trial	Height
By distance	$\chi^2_{1,114} = 17.56^{***}$	na	$\chi^2_{3,114} = 14.71^*$	$\chi^2_{1,114} = 0.37^{ns}$
By seed set	na	$\chi^2_{1,114} = 12.99^{***}$	$\chi^2_{3,114} = 19.66^{**}$	$\chi^2_{1,114} = 0.89^{ns}$

\*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ ; ns, nonsignificant; na, not applicable.

modification in a manner similar to the way that pollination conditions alter sex ratios in *Rumex*. For example, the finding that there is a relation between the degree of female bias in seeds and the flowering sex ratio of populations (fig. 3 in Stehlik and Barrett 2005) would not be predicted by the sex-ratio distorter hypothesis. Although the contribution of sex-ratio distorters cannot be entirely ruled out in *Rumex* species, it seems unlikely that they play the primary role in governing the female bias that characterizes seed sex ratios.

We are currently investigating through flow cytometry whether there is evidence of sex ratio bias in female- versus male-determining pollen (I. Stehlik, B. C. Husband, and S. C. H. Barrett, unpubl. data). Pollen inviability could be the result of trivalent formation and nondisjunction of the sex chromosomes during meiosis as a result of the  $XY_1Y_2$ -system of sex determination in *R. nivalis* (B. P. Pickersgill, pers. comm.). Alternatively, variation in pollen quality may result from Y-chromosome degeneration resulting from the accumulation of deleterious mutations (Smith 1963; Lloyd 1974; Charlesworth 2002; Stehlik and Barrett 2005). Differences among pollen donors in the quality of female- versus male-determining gametes could then provide the necessary variation for the competitive interactions that are the basis for certation.

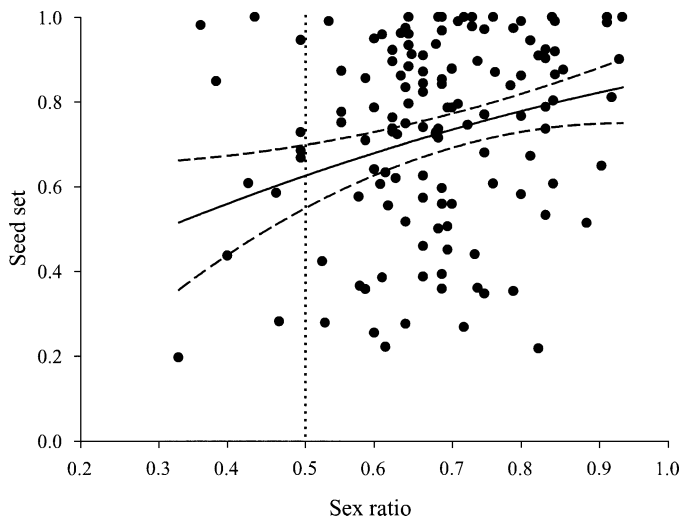


FIG. 2. The relation between the sex ratio and seed set of inflorescences in experimental trials involving wind pollination in *Rumex nivalis*. Each point involves an inflorescence from a different maternal plant. The solid line indicates the predicted relations based on logistic regression, the dashed lines are 95% confidence intervals, and the dotted line represents an unbiased sex ratio. Intercept =  $-0.78$ , SE =  $0.57$ ,  $\chi^2 = 1.82$ , df = 1; sex ratio =  $2.54$ , SE =  $0.84$ ,  $\chi^2 = 9.17^{**}$ , df = 1.

Our ability to manipulate the degree of female bias in seed sex ratios of *R. nivalis* by varying pollination intensity is consistent with Correns' certation hypothesis. However, we cannot provide definitive evidence for this hypothesis because pollen-tube competition was only inferred and not measured directly in our experiments. Because of the technical difficulties of distinguishing female- and male-determining pollen tubes in styles of female plants obtaining direct evidence for certation will remain a challenge. However, viable alternate hypotheses have little support and given the available evidence from pollination studies of three *Rumex* spp. we consider that the certation hypothesis currently provides the best explanation for female-biased primary sex ratios in this genus. Future studies could profitably explore the influence of postpollination mechanisms on sex-ratio variation in other dioecious taxa with biased sex ratios, particularly species with sex chromosomes.

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