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MECHANISMS GOVERNING SEX-RATIO VARIATION IN DIOECIOUS *RUMEX NIVALIS*

IVANA STEHLIK[1] AND SPENCER C. H. BARRETT[2]
Department of Botany, University of Toronto, 25 Willcocks Street, Toronto, Ontario M5S 3B2, Canada
[2]*E-mail: stehlik@botany.utoronto.ca*
[3]*E-mail: barrett@botany.utoronto.ca*

Abstract.—Sex ratios of flowering individuals in dioecious plant populations are often close to unity, or are male biased owing to gender-specific differences in flowering or mortality. Female-biased sex ratios, although infrequent, are often reported in species with heteromorphic sex chromosomes. Two main hypotheses have been proposed to account for female bias: (1) selective fertilization resulting from differential pollen-tube growth of female- versus male-determining microgametophytes (certation); (2) differences in the performance and viability of the sexes after parental investment. Here we investigate these hypotheses in *Rumex nivalis* (Polygonaceae), a European alpine herb with female-biased sex ratios in which females possess XX, and males XY_1Y_2, sex chromosomes. Using field surveys and a glasshouse experiment we investigated the relation between sex ratios and life-history stage in 18 populations from contrasting elevations and snowbed microsites and used a male-specific SCAR-marker to determine the sex of nonflowering individuals. Female bias among flowering individuals was one of the highest reported for populations of a dioecious species (mean female frequency = 0.87), but males increased in frequency at higher elevations and in the center of snowbeds. Female bias was also evident in nonflowering individuals (mean 0.78) and in seeds from open-pollinated flowers (mean 0.59). The female bias in seeds was weakly associated with the frequency of male flowering individuals in populations in the direction predicted when certation occurs. Under glasshouse conditions, females outperformed males at several life-history stages, although male seeds were heavier than female seeds. Poor performance of Y_1Y_2 gametophytes and male sporophytes in *R. nivalis* may be a consequence of the accumulation of deleterious mutations on Y-sex chromosomes.

Key words.—Certation, dioecy, female-biased sex ratios, life-history traits, male-biased mortality, male-specific SCAR primers, *Rumex nivalis*.

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In species with separate sexes (dioecy), offspring sex ratios should generally be close to unity after the period of parental investment, as a result of negative frequency-dependent selection (Fisher 1930). However, sex-ratio bias may occur if there are differences in the cost of rearing the two sexes, or if sex-biased mortality occurs after parental investment. Since Fisher's classic work, sex-ratio theory has broadened considerably so that today there are a variety of additional models to explain the biased sex ratios that have been observed in natural populations of diverse organisms (reviewed in Hardy 2002). For example, local mate competition (Hamilton 1967; Antolin 1993), group selection in structured populations (Wilson and Colwell 1981), and the spread of selfish genetic elements (Sandler and Novitski 1957; Werren et al. 1988; Stouthamer et al. 2002) have been proposed as explanations for biased sex ratios.

Sex ratios in populations of dioecious flowering plants often appear to deviate from unity. A recent review (Delph 1999) reported that only 29% of species surveyed exhibited 1:1 sex ratios, with more than half (57%) having male-biased populations (but see Rottenberg 1998). Differences in the costs of reproduction between female and male plants have usually been proposed to account for male-biased sex ratios, particularly in long-lived species (Lloyd and Webb 1977). Female plants usually allocate a greater proportion of resources to reproduction than males. This can render them more susceptible to environmental stress, resulting in sex-biased mortality (Lloyd 1973; Meagher 1981), or cause differences in the frequency of flowering, or the age of first reproduction (Barrett and Helenurm 1981; Allen and Antos 1993). Female-biased sex ratios are less common in dioecious species, with most reports concentrated in a few genera (e.g., reviewed in Lloyd 1974). Local mate competition (de Jong and Klinkhamer 2002; de Jong et al. 2002), differential herbivory of the sexes (Ågren et al. 1999), sex-ratio distorters (Taylor 1999), or gametophytic selection through differential pollen-tube growth (Correns 1928) could cause females to be more numerous than males in dioecious populations. However, without sex-specific markers, only the gender of reproductive individuals can be determined. This has limited investigations of primary sex ratios in plants, and studies of the development of sex-ratio bias during early life-history stages (but see Lyons et al. 1995; Taylor 1996; Eppley et al. 1998; Korpelainen 2002).

Female-biased sex ratios are often reported in species with heteromorphic sex chromosomes (Lloyd 1974), suggesting a relation between the sex-determination system and either the performance of the sexes, or the sex-chromosomal genotype of gametophytes. Sex chromosomes are probably rare in angiosperms, having been reported from 11 often distantly related families, though many dioecious taxa have not been well studied (Charlesworth 2002; Vyskot and Hobza 2004). Despite independent origins, plant sex chromosomes have a number of shared features. Both X and Y chromosomes are larger than autosomes (Parker 1990), and in common with most animals, males are generally the heterogametic sex (except in *Fragaria*; Matsunaga and Kawano 2001). Due to the absence of recombination between the sex-determining loci, Y chromosomes are likely to accumulate deleterious mutations, a process that is accelerated because genes on the Y chromosome are present in only one sex. Therefore the effective population size of Y chromosomes is considerably reduced compared to autosomes. Muller's ratchet, background selection, the Hill-Robertson effect with weak selec-

tion, and the hitchhiking of deleterious alleles by favorable mutations represent additional mechanisms acting on nonrecombining Y chromosomes (Charlesworth and Charlesworth 2000). All of these processes involve a reduction in effective population size and the consequent weakening of the efficacy of selection. As a result, Y chromosomes should degenerate, and gene function may be impaired (Rice 1987; Charlesworth 2002; Vyskot and Hobza 2004).

A potential consequence of Y-chromosome degeneration is the poorer performance of male- versus female-determining microgametophytes. This could be manifested by slower pollen-tube growth of Y versus X chromosome bearing pollen grains, resulting in preferential fertilization (Smith 1963; Lloyd 1974; Charlesworth 2002). The competition between pollen tubes while growing in the style is a process known as certation (Heribert-Nilsson 1920) and is usually more intense the larger the stigmatic pollen load (Mulcahy 1975). Evidence for certation was first obtained by Correns (1917, 1922, 1928), from experimental manipulation of the amount of pollen on stigmas and assays of progeny sex ratios in *Silene* and *Rumex*, two genera with sex chromosomes. More recently, Rychlewski and Zarzycki (1975) and Conn and Blum (1981a) also altered primary sex ratios in *R. acetosa* and *R. hastatulus*, respectively, by manipulating pollination intensity. With small amounts of pollen, sex ratios were close to unity, whereas heavier pollen loads resulted in female-biased sex ratios. However, attempts to demonstrate that certation plays a role in governing sex-ratio variation under field conditions have largely been inconclusive (Rychlewski and Zarzycki 1975; Conn and Blum 1981a). Moreover, more recent work has cast doubt on the generality of Correns's certation hypothesis for *Silene* (Carroll and Mulcahy 1993).

Other mechanisms could also play a role in causing female-biased sex ratios in plant species with sex chromosomes. In experimental populations of *R. thyrsiflorus* and *R. acetosa*, adult sex ratios were more female biased than sex ratios of seeds and seedlings, determined cytologically (Zarzycki and Rychlewski 1972; Rychlewski and Zarzycki 1986) or by using Y chromosome-specific DNA markers (Korpelainen 2002). These results indicate that male-biased mortality can influence postparental care sex ratios. Patterns of sex-ratio variation in natural populations of *Rumex* spp. suggest that competition during succession may play a role in causing increased male mortality (Harris 1968; Putwain and Harper 1972; Zarzycki and Rychlewski 1972). In contrast, in *Silene latifolia* there is no evidence for differences in mortality between females and males (Purrington and Schmitt 1998; but see Delph 1999, p.166). Rather, sex-ratio variation in natural populations reflects seed sex ratios and these appear to be governed by the dynamics of selfish genetic elements involving a system of sex-ratio distorters and restorers (Taylor 1994; 1999).

Here we investigate the genetic and ecological mechanisms governing patterns of sex-ratio variation in the alpine herb *R. nivalis* (Polygonaceae), a European endemic that grows predominantly in snowbeds above timberline from 1900 to 2800 m (Wagenitz 1981). Preliminary field observations conducted during a phylogeographic study of *R. nivalis* (Stehlik 2002) revealed that the female-biased sex ratios commonly reported in several other *Rumex* species also occur in this species. Because of the steep environmental gradients that characterize alpine environments, and the possibility that ecological factors influence sex ratios, we chose 18 populations distributed between low and high elevation sites to examine patterns of sex-ratio variation. At each site, we sampled plants from early- and late-melting snowbed microsites to investigate how both local conditions, as well as broader elevational gradients might influence sex-ratio variation. Having established that female-biased sex ratios are a general feature of flowering portions of *R. nivalis* populations, we then investigated the sex of vegetative individuals using male-specific sequence-characterized amplified-regions (SCAR) primers (Stehlik and Blattner 2004). This marker allowed us to determine whether sex ratios change during the species' life history.

We specifically examined two hypotheses to explain female-biased sex ratios in *R. nivalis*.

Certation.—If certation occurs, as reported in other *Rumex* species (Rychlewski and Zarzycki 1975; Conn and Blum 1981a), the sex ratio of open-pollinated seeds sampled from natural populations should be female biased. We further predicted that the degree of female bias in a particular population should be associated with its flowering sex ratio. This is because flowering sex ratios seem likely to influence pollination intensity (pollen loads on stigmas) and hence the degree of competition between male gametophytes. Populations with low frequencies of flowering males should have less female-biased seeds, whereas populations with higher flowering male frequencies should have more strongly female-biased seed sex ratios.

Gender-specific mortality.—If a difference between the sexes in postparental care mortality contributes towards female-biased sex ratios, the sex ratio should change during the life-history of plants in natural populations. Moreover, if ecological stress plays a role in causing differential mortality, we should observe different degrees of sex bias across the larger-scale (elevation) and smaller-scale (snowbed microsites) environmental gradients that we sampled. We used field sampling of seed families and vegetative plants to examine the sex ratios of nonreproductive individuals, and a glasshouse experiment that compared the performance of female and male plants under contrasting nutrient regimes.

Materials and Methods

Study Species and Population Sampling

Rumex nivalis section *Acetosa* (Polygonaceae) is a wind-pollinated, perennial dioecious herb restricted in distribution to the European Alps and mountains of Bosnia-Herzegowina. The principal habitat of *R. nivalis* is snowbeds. The growing season is from July through September but is shortened in the center of snowbeds, where snowmelt is delayed by up to three weeks. Plants are composed of a basal rosette of fleshy leaves and mostly unbranched inflorescences on stems with no, or rarely up to two, leaves (Wagenitz 1981). Clonal expansion via limited basal branching is restricted in *R. nivalis* and genets are easy to delimit. Females have uni-ovulate flowers and mature adults produce up to 200 seeds each season. As in most other species of *Rumex*, females of *R. nivalis* possess one pair of X chromosomes (homogametic sex; $2n = 14$), whereas males are characterized by one X and two Y

chromosomes (heterogametic sex; $2n = 15$; Wagenitz 1981). Kihara and Ono (1925) observed that in *R. acetosa* pollen mother cells comprise either Y_1 and Y_2 chromosomes or a single X chromosome, of which female-determining pollen has the X chromosome and male-determining pollen the two Y chromosomes. *Rumex* species in section *Acetosa* possess the XX and XY_1Y_2 sex chromosome system (Zuk 1963; Vyskot and Hobza 2004), therefore we assume that Kihara and Ono's (1925) observations also pertain to *R. nivalis*.

We chose nine regions in Switzerland where populations of *R. nivalis* are distributed across a steep elevational gradient. In summer 2002, we selected one high elevation and one low elevation population in each region. The specific localities and elevations of each population are listed in the Appendix. The average difference in altitude between high and low elevation populations was 330 m (range 170–540). In each of the 18 populations, we determined the sex ratio of the flowering portion of the population and collected leaf material to determine the sex ratio of the nonflowering portion. Average sample sizes for flowering and nonflowering portions were 66.6 and 32.4, respectively. To facilitate sampling we used grids, sampled the nearest individual to each intersection of the grid and recorded its sex if flowering, or collected leaf material in silica gel if it was vegetative. We mapped the position of all plants according to the grid in relation to the centre of the snowbed and observed the local pattern of snowbed melt and its influence on phenology (see Galen and Stanton 1995) through repeated visits to each population in 2001–2002. In September 2002, we collected seed families from open-pollinated females in each population and stored them at room temperature. The average number of seed families per population was 46, range 20–50.

DNA Isolation and PCR

To determine whether seeds and vegetative individuals sampled from the field and in the glasshouse experiment (described below) were female or male, we used male-specific sequence-characterized amplified-regions (SCAR) primers following the methods detailed in Stehlik and Blattner (2004). To avoid false negative scoring of sex, we co-amplified the nuclear rDNA internal transcribed spacer 2 (ITS2) together with the male-specific fragment as an internal control for successful PCR reactions. We subjected genomic DNA amounts of 3 ng to PCR in 12.5 μl reaction volumes with 1.25 μl 10× PCR reaction buffer, 3 μl of 10 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 0.1 μl of 25 pM of RnivYf and RnivYr (SCAR primers; Stehlik and Blattner 2004), 0.1 μl of 5 pM ITS-B and ITS-D (Blattner 1999), 0.05 μl 100× BSA and 0.1 μl 5 U/μl Taq, adjusted with ddH_2O. Cycling conditions were: 4 min at 94°C followed by 35 cycles of 45 sec at 92°C, 45 sec at 48°C, and 30 sec at 72°C and a final extension time of 10 min at 72°C. We visualized PCR products on 1.6% agarose gels stained with ethidium bromide.

Glasshouse Experiment Comparing Females and Male Performance

To investigate whether female and male plants of *R. nivalis* differ in growth, reproduction, and viability, we conducted a glasshouse experiment at the University of Toronto from January 2003 to June 2004 using open-pollinated families sampled from the 18 populations described above. Populations were grown under two contrasting nutrient regimes with the expectation that this would lead to differences in the degree of environmental stress experienced by plants and potentially result in sex-dependent mortality. Prior to germination, we vernalized all seeds at −20°C for two weeks. Germination rates differed among populations preventing a balanced number of families and progenies in the experiment. The average number of families and progeny per population represented in each treatment was 46.0 (8–75) and 66.2 (56–80), respectively.

In January 2003, seeds were germinated in plastic petri dishes on moist filter paper in the glasshouse between 17° and 22°C. To keep track of the origin (population and family) of each seed, we placed a circular paper sheet with a numbered grid under the filter paper of the petri dishes. Following germination, when seedlings exhibited expanded cotyledons and roots, we planted each seedling into a 7.6 cm diameter peat pot. We considered seeds that had not germinated after one month to be inviable and stored them for later sex determination using SCAR-markers.

To produce the two nutrient regimes, we varied the proportion of sand in the soil in which plants were grown and applied contrasting fertilizer regimes. In the ''high-nutrient'' treatment the soil consisted of a mixture of 50% equal parts peat, sand, loam, and 50% sand, whereas in the ''low-nutrient'' treatment the ratios were 25% soil mix and 75% sand. In addition, plants grown in the high-nutrient treatment received three applications of 1% 20:20:20 = NPK fertilizer during spring to autumn 2003, whereas no fertilizer was provided to plants in the low-nutrient treatment. Early in the summer of 2003, we transplanted all plants into 15.2 cm diameter plastic pots with the same soil type per treatment. We randomly assigned plants to trays containing either low- or high-nutrient pots to prevent fertilizer effects on low nutrient plants. Each tray was assigned to a random position within a single greenhouse and rerandomized every two to three weeks throughout the duration of the experiment to minimize position effects within the glasshouse and reduce environmental heterogeneity in the experiment. By the time plants had expanded their third leaf, we removed a small tissue sample from all plants in the experiment for sex determination. We also collected tissue from dead seedlings (during germination) and from seedlings that failed to establish in soil.

We measured the following traits for each individual in the experiment: seed weight, days to germination, days to develop first leaf, number of basal shoots, length of the longest leaf, days to flowering, number and height of flowering shoots, aphid damage, and mortality. We measured the weight of each individual seed to the nearest μg on a Mettler-Toledo UMT2 balance (Mettler-Toledo Gmbh, Greifensee, Switzerland). To record the day of germination and development of the first leaf, we monitored the plants on a daily basis. Later, we assessed plants every two weeks throughout the duration of the experiment. We counted the number of shoots in March 2003, approximately two months after germination. We measured the length of the longest leaf from the soil level to the tip of the leaf on four occasions in March, May, July, and

October 2003. Flowering individuals often produced sequential flowering stalks. We recorded the date flowering commenced and counted the total number of flowering stalks and the length of the longest stalk. During September–October 2003, plants were subjected to a significant unplanned aphid outbreak that damaged leaves and caused senescence. We quantified the extent of leaf damage by classifying plants into five categories based on the percentage of green leaves: (1) 5%, (2) 25%, (3) 50%, (4) 75%, and (5) undamaged. We recorded mortality throughout the duration of the experiment until June 2004.

Statistical Analysis

We investigated the influence of ecological gradients on sex ratios in natural populations of *R. nivalis*, and also whether the sex ratios of flowering and vegetative portions of populations differed, using generalized multiple linear models (GLMs; Wilson and Hardy 2002). We used logit transformations of sex ratio data to account for the binomial error distribution (PROC GENMOD, SAS Institute 2001a) and considered elevation (high versus low), microsite (center versus periphery of snowbed), and life-history stage (flowering versus nonflowering) as fixed factors in the analyses.

In the glasshouse experiment, we initially tested whether seed sex ratios were unbiased, and then whether there was any change in the sex ratio during the duration of the experiment. Additionally, we assessed whether there was any evidence for sex-biased mortality resulting from increased stress in the low-nutrient treatment. We considered region, elevation, life-history stage, and treatment as fixed factors. We determined sex ratios at nine time intervals corresponding to different developmental stages (weeks after the start of germination in brackets): seeds (0), seedlings (4), age when plants developed their first true leaf (6), when basal shoots were evident (9), and at five subsequent intervals during flowering and postflowering (16, 24, 30, 38, 60). This sampling procedure involved repeated measures, so we used generalized estimating equations (Liang and Zeger 1986; PROC GENMOD, REPEATED option, SAS Institute 2001a) to model the associated variances and covariances.

For both the sex ratios of natural populations and plants in the glasshouse experiment, we excluded nonsignificant factors by stepwise backward elimination. We interpreted significant factors and their interactions with contrasts. The probabilities of these contrasts were corrected using the Dunn-Šidák formula (Sokal and Rohlf 1981), with $P = 1 - (1 - 0.05)^{1/N}$, where N is the total number of comparisons. To examine sex ratios R and their standard errors (upper *uSE*, lower *lSE*) for different factor levels, we back-transformed the parameter estimates L and SE provided by the GEE analysis (SAS Institute 2001a) as: $R = e^{L}/(1 + e^{L})$, $uSE = e^{L+SE}/(1 + e^{L+SE})$, and $lSE = e^{L-SE}/(1 + e^{L-SE})$. To investigate the relation between sex ratios of flowering portions of natural populations and the sex ratios of seed progenies we regressed the proportion of females in seed against the proportion of males in the population.

We evaluated differences in the performance of females and males for the measured traits by analysis of variance using JMP 4.0.4 (SAS Institute 2001b). We treated sex (female vs. male), nutrient regime (low vs. high), region (nine regions), and elevation (low vs. high) as fixed effects in a full-factorial design and removed nonsignificant interactions with stepwise backward elimination retaining two-way interactions. We transformed data as necessary to meet assumptions of ANOVA.

RESULTS

Sex Ratios of Natural Populations

Flowering individuals in populations of *R. nivalis* had strongly female-biased sex ratios (Fig. 1A). The average frequency of females was 0.87 (SE = 0.01; range 0.72–0.99; N = 18 populations, 1199 individuals). There was a significant difference in the frequency of flowering females between low and high elevation sites, with greater bias among low than high elevation populations (average female frequency: low elevation = 0.93, SE = 0.01; high elevation = 0.78, SE = 0.02; $\chi^2 = 35.04$, $P < 0.0001$; Table 1, Fig. 1A). Flowering sex ratios were also significantly more female biased at the periphery of snowbeds than in the center (average female frequency: periphery = 0.87, SE = 0.09; center = 0.72, SE = 0.06; $\chi^2 = 50.04$, $P < 0.0001$).

Sex-specific markers revealed that sex ratios among nonflowering plants were significantly less female biased than for flowering plants (mean female frequency = 0.78, SE = 0.02; range = 0.36–0.94; N = 18 populations, 583 individuals; $\chi^2 = 16.83$, $P < 0.0001$; Table 1, Fig. 1B). The degree of female bias was significantly greater among low elevation populations than among high elevation populations (average female frequency: low elevation = 0.79, SE = 0.02; high elevation = 0.68, SE = 0.03; $\chi^2 = 13.99$, $P = 0.0002$; Fig. 1B), and at the periphery of snowbeds than in the center (average female frequency: periphery = 0.73, SE = 0.21; center = 0.69, SE = 0.28; $\chi^2 = 4.24$, $P = 0.0395$).

By combining the data on sex ratios of flowering and vegetative individuals of *R. nivalis*, it is evident that female-biased sex ratios are a general feature of *R. nivalis* populations (mean female frequency = 0.82, SE = 0.01, range = 0.69–0.95; N = 18 populations, 1782 individuals). Both elevation (average female frequency: low elevation = 0.88, SE = 0.01; high elevation = 0.75, SE = 0.01; $\chi^2 = 47.18$, $P < 0.0001$; Table 1) and snowbed microsite (average female frequency: snowbed periphery = 0.88, SE = 0.02; snowbed center = 0.74, SE = 0.02; $\chi^2 = 36.13$, $P < 0.0001$; Table 2, Fig. 2) influenced sex ratios.

Seed Sex Ratios and Relation with Flowering Sex Ratios

The average sex ratio of open-pollinated seed sampled from natural populations of *R. nivalis* was female biased (mean female frequency = 0.59, SE = 0.01, range = 0.55–0.64; N = 18 populations, 2253 seeds). The percentages of female seeds at low (0.57, SE = 0.01) and high (0.62, SE = 0.01) elevation sites were significantly different ($\chi^2 = 17.69$, $P < 0.0001$; Fig. 1C). The relation between the proportion of flowering males in populations and the proportion of female seeds in open-pollinated families (ln-transformed) was marginally significant ($r^2 = 0.21$, $P = 0.058$; Fig. 3). Removal of an outlier population strengthened this relation con-

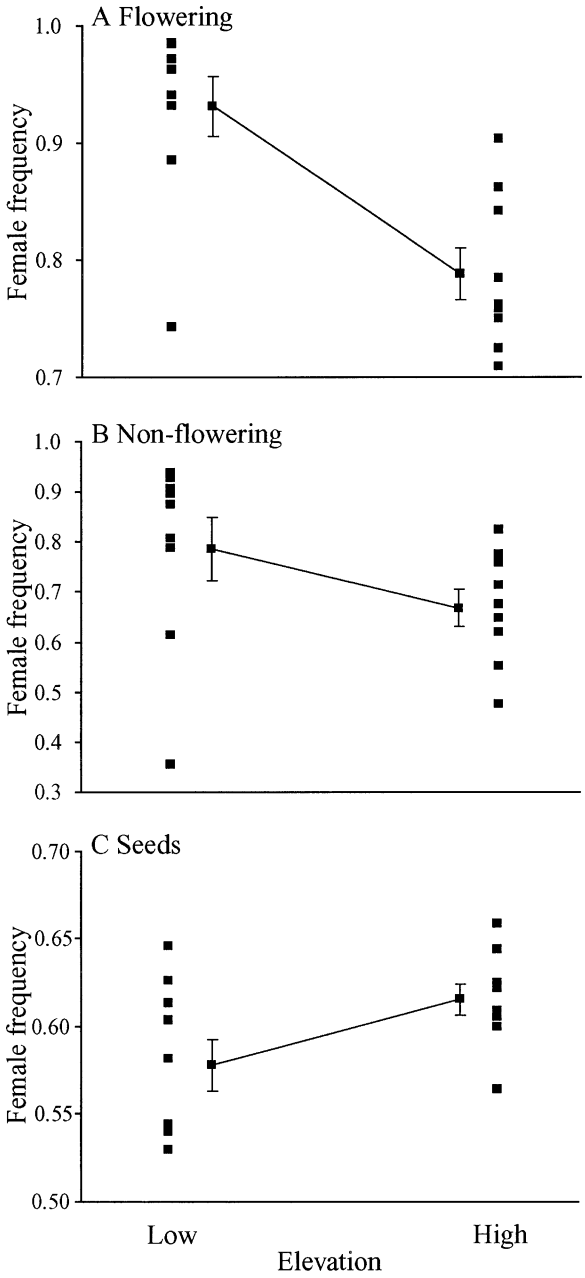

FIG. 1. Relations between sex ratio and life-history stage in 18 populations of *Rumex nivalis* occurring in low and high elevation sites in the Swiss Alps. The female frequencies are plotted for (A) flowering plants; (B) nonflowering plants; (C) open-pollinated seed. Mean female frequencies and their standard errors are joined by lines. Note the different scales on the vertical axes. Statistical comparisons are presented in Table 1.

siderably (see Fig. 3). Seed sex ratios in populations with higher frequencies of flowering males tended to be more female biased than in populations with lower male frequencies. However, there was considerable variation in seed sex ratios, particularly in populations with few males (Fig. 3).

Changes in Sex Ratio in the Glasshouse Experiment

A total of 2382 seeds were used to initiate the glasshouse experiment, and the sex was determined for 93.2% of these.

TABLE 1. Analysis of sex ratios in natural populations of *Rumex nivalis* tested against the binomial distribution by fitting generalized multiple linear models (GLMs; Wilson and Hardy 2002) with binomial error and logit function in GENMOD (SAS Institute 2001a). Elevation (high vs. low), microsite (center vs. periphery of snowbeds), and reproductive state (flowering vs. vegetative) were treated as fixed factors and tested against the three-way interaction. Nonsignificant factors were excluded by stepwise backward elimination.

Source	df	χ^2	*P*
Elevation	1	36.13	<0.0001
Microsite	1	16.83	<0.0001
Reproductive state	1	47.18	<0.0001
Microsite × reproductive state	1	8.00	0.0047

Most individuals whose sex was not determined were ungerminated seeds or weak seedlings that yielded insufficient or degraded DNA, and uninterpretable PCR results. Of the individuals whose sex was determined, 84.5% germinated, 37.3% flowered by the end of the experiment, and 47.2% died.

Male plants had significantly higher mortality than females in the experiment. The increase in female bias was gradual, rather then being associated with any particular life cycle stage. The frequency of females among all surviving individuals by June 2004 was 0.64 (SE = 0.01), significantly more female-biased than the seed sex ratio of 0.59 (SE = 0.01, $\chi^2 = 7.78$, $P = 0.0053$; Table 2, Fig. 4D). Significantly more males died in the low-nutrient than in the high-nutrient treatment, whereas females were not affected by nutrient conditions ($\chi^2 = 17.69$, $P < 0.0001$, Table 2, Fig. 4D).

Glasshouse Comparisons of Female and Male Performance

On average, male seeds of *R. nivalis* were significantly heavier than female seeds (males = 0.969 mg, SE = 0.008; females = 0.937, SE = 0.006; Table 3, Fig. 5). This was not

TABLE 2. Analysis of sex ratios during the growth of *Rumex nivalis* in the glasshouse experiment. Sex ratios were tested against the binomial distribution by fitting generalized multiple linear models (GLMs; Wilson and Hardy 2002) with binomial error and logit function in GENMOD (SAS Institute 2001a). The following ten sequential stages were examined over an 18-month period: seeds, seedlings, age when plants developed their first true leaf, and on seven subsequent intervals corresponding to various developmental stages including the timing of basal shoot production, peak flowering, postflowering and at the termination of the experiment in June 2004. Region, elevation, life stage, and treatment (nutrient regime) were treated as fixed factors and tested against the four-way interaction. Because the comparison of sex ratios at different times involved repeated measures, we used the REPEATED option (SAS Institute 2001a). Nonsignificant factors were excluded by stepwise backward elimination.

Source	df	χ^2	*P*
Region	8	16.79	0.0324
Elevation	1	17.50	<0.0001
Life-history stage	9	12.62	0.1804
Treatment	1	15.51	<0.0001
Treatment × region	8	15.52	0.0497
Treatment × elevation	1	17.29	<0.0001
Region × elevation	8	14.15	0.0780
Treatment × region × elevation	8	17.57	0.0247

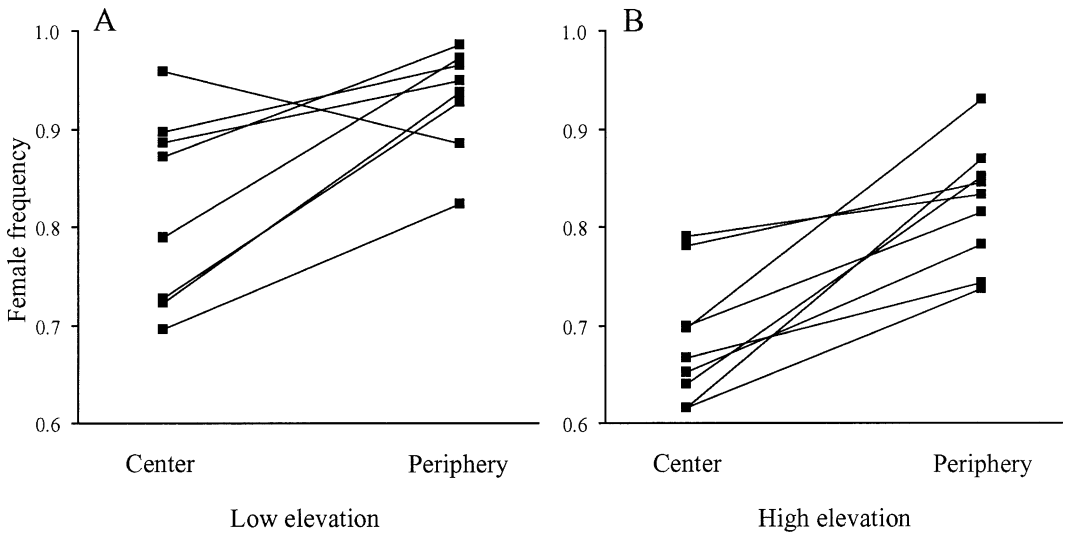

FIG. 2. Relations between sex ratio and snowbed microsite in 18 populations of *Rumex nivalis* occurring in (A) low and (B) high elevation sites in the Swiss Alps. Center and periphery refer to the location of plants in snowbeds. Lines join plants growing in the same population. Statistical comparisons are presented in Table 1.

associated with any difference in percent germination (Table 1), although, on average, male seeds germinated significantly earlier than female seeds (females = 14.35 days, SE = 0.20; males = 13.24 days, SE = 0.22; Table 3). Seeds of low elevation populations (1.012 mg, SE = 0.007) were significantly heavier than from high elevation populations (0.889 mg, SE = 0.006; Table 3), and they also germinated earlier than seeds from high elevation populations (Table 3).

Female plants of *R. nivalis* developed their first leaf faster than male plants (females = 12.87 days, SE = 0.07; males = 13.25 days, SE = 0.09; Table 3). This was also true for seedlings of high elevation populations compared with those from low elevation populations (high = 12.82 days, SE = 0.08; low = 13.87 days, SE = 0.08; Table 3). After two months of growth in the glasshouse, female plants had on average 1.29 basal shoots (SE = 0.02), whereas males had 1.15 (SE = 0.02; Table 3, Fig. 4A). Plants in the low-nutrient treatment had fewer basal shoots than those in the high-nutrient treatment (low nutrient = 1.17 branches, SE = 0.02; high nutrient = 1.26, SE = 0.02; Fig. 4A). For two of the four measurements of the length of the longest leaf, there was a significant effect of sex, with females possessing longer leaves than males (week 16: females = 2.86 cm, SE = 0.04; males = 2.65 cm, SE = 0.04; week 24: females = 6.29 cm, SE = 0.05; males = 5.87 cm, SE = 0.07; Table 3, Fig. 4B). On average, leaves were longer in length in the high-nutrient treatment for three of the four measurements (Table 3, Fig. 4B).

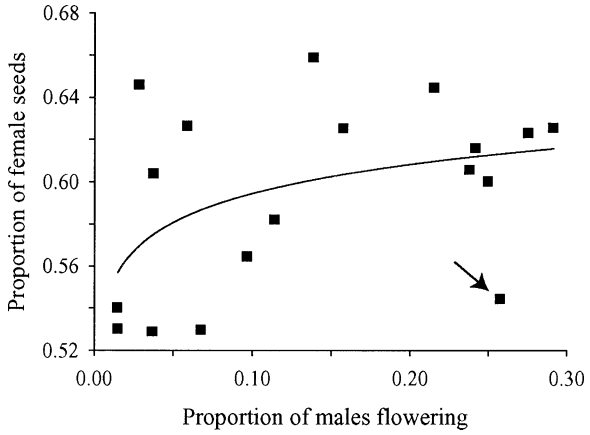

FIG. 3. Relation between the proportion of flowering males and the proportion of females in open-pollinated seed in 18 populations of *Rumex nivalis* occurring in the Swiss Alps. The regression equation is: $S = 0.640 + 0.0198 \ln(M)$, where S is the proportion of female seeds and M is the proportion of males in the population. For this model, $R^2 = 0.21$, SE = 0.0097, $F_{1,16} = 4.17$, $P = 0.0579$. Exclusion of the outlier indicated by the arrow resulted in a significantly better fit to the model ($R^2 = 0.32$, $F_{1,15} = 6.92$, $P = 0.0189$).

There was no effect of gender on either the onset of flowering, or the proportion of plants that flowered in the experiment (Table 3, Fig. 4C). Plants growing in the high nutrient regime flowered earlier and produced more flowering shoots than plants in the low-nutrient regime. The overall flowering sex ratio of the glasshouse population of *R. nivalis* did not differ from the sex ratio of germinated seeds, confirming that there was no difference in the proportion of females and males that flowered during the experiment ($\chi^2 = 0.12$, $P = 0.944$; Fig. 4C). Although there was no significant difference in the number of flowering shoots produced by females and males (Table 3), female plants had significantly longer flowering shoots than males (females = 21.05 cm, SE = 0.44; males 15.79, SE = 0.37).

There was a marginally significant difference in the response of female and male plants of *R. nivalis* to damage caused by an aphid infestation in the glasshouse ($F_{1,1304} = 2.788$, $P = 0.108$; Table 3). Females suffered less damage (percent of undamaged leaves in females = 49.5%, males = 47.5%) and had a lower percent of damaged leaf area in seven out of the nine regions from which seed progenies originated.

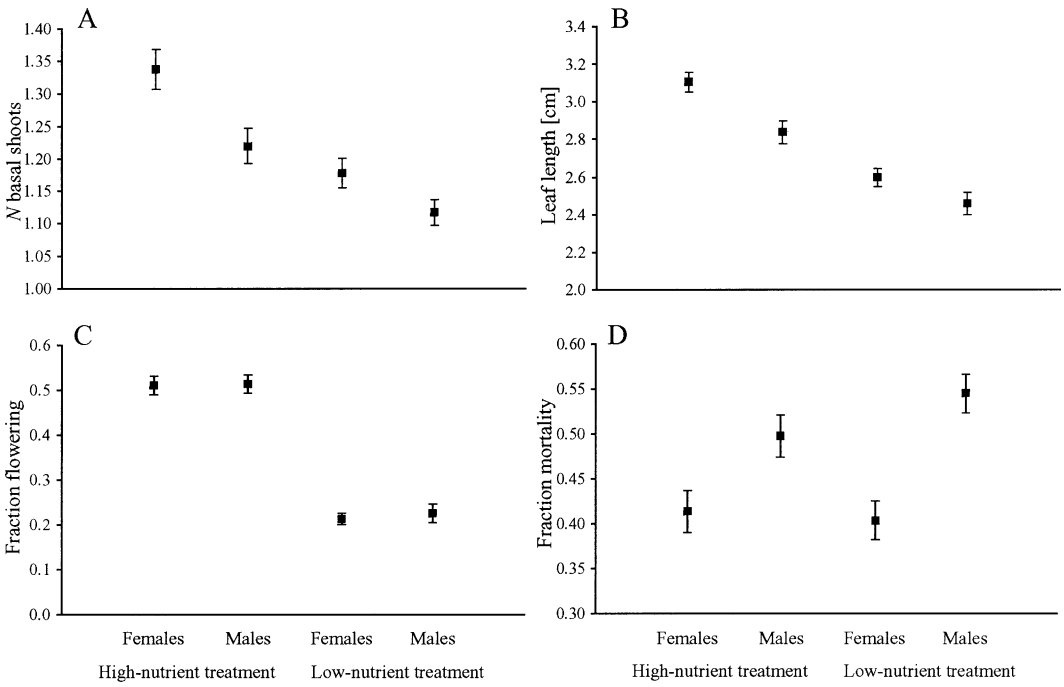

FIG. 4. Performance of female and male plants of *Rumex nivalis* originating from 18 populations from the Swiss Alps when grown in a glasshouse experiment under low and high nutrient regimes (see methods for details). The values presented are grand means and their standard errors. (A) Number of basal shoots; (B) leaf length (cm); (C) fraction of plants that flowered; (D) fraction of plants that died. Statistical comparisons are presented in Table 3.

DISCUSSION

Our investigation of the alpine plant *Rumex nivalis* has revealed four important findings concerning the mechanisms governing sex-ratio variation. First, field surveys and the use of sex-specific genetic markers revealed that female-biased sex ratios characterize both the flowering and vegetative portions of populations (Fig. 1A, B). Second, the degree of sex-ratio bias varies with ecological conditions, with females attaining their highest frequencies at lower elevation sites (Fig. 1) and at the periphery of snowbeds (Fig. 2). Third, the sex ratios of open-pollinated seeds were female biased, and associated with the flowering sex ratios of populations (Fig. 3), suggesting that certation plays a role in determining primary sex ratios. Finally, comparisons of sex ratios at different life-history stages in the field and glasshouse indicated that male plants suffer greater mortality than female plants (Figs. 1, 4D). We now discuss the implications of these results and propose that several of our findings may be a consequence of the sex-chromosome system in *R. nivalis*.

Female-Biased Primary Sex Ratios

The mean frequency of females in open-pollinated seeds of *R. nivalis* was 0.59, with all populations exhibiting some degree of female bias (Fig. 1C). There was a weak statistical association between the degree of bias in seed sex ratios and the flowering sex ratio of populations in the direction predicted by the certation hypothesis (Fig. 3). The highest variation in seed sex ratios was evident in populations with very low frequencies of flowering males. This pattern is probably caused by the local nature of pollen dispersal in these populations resulting in considerable stochasticity in both mating and sampling. Natural pollen loads are influenced by diverse environmental and demographic factors and additional information on the local density of flowering males and the temporal dynamics of pollen-load size would be necessary to directly confirm the certation hypothesis under field conditions.

Gametophytic competition is likely to be severe in *R. nivalis*, because like other *Rumex* species and many other wind-pollinated plants, flowers possess a single ovule. However, other potential reproductive mechanisms could also influence seed sex ratios in *Rumex* species. These include differences in the viability of female- versus male-determining microgametophytes and megagametophytes, or the very early abortion of embryos. There are no reports of these phenomena in *Rumex*, and it is difficult to imagine how these potential mechanisms would result in the association we observed between female bias in seeds and the flowering sex ratios of populations. Moreover, experimental evidence from other *Rumex* species implicates certation as the mechanism causing biased seed sex ratios. For example, experimental manipulation of pollination intensities in *R. thyrsiflorus* (Rychlewski and Zarzycki 1986) and *R. hastatulus* (Conn and Blum 1981a) resulted in female frequencies in seedlings of 0.83 and 0.62, respectively, following high pollen-load treatments. Open-pollinated seeds collected from populations of *Rumex* species are also often female biased (Zarzycki and Rychlewski 1972; Rychlewski and Zarzycki 1986), although this is not always the case (Putwain and Harper 1972; Korpelainen 2002), suggesting that environmental conditions influence the extent to which certation occurs in natural populations. However, as

TABLE 3. Analysis of variance of 12 measures of performance during different life stages in *Rumex nivalis* in the glasshouse experiment. Sex, treatment (nutrient regime), region, and elevation were treated as fixed effects in a full factorial design. We removed nonsignificant interactions with stepwise backward elimination keeping two-way interactions. For details on the measurements refer to the text.

	Sex	Treatment	Region	Elevation
Seed weight[1]	$F_{1,2139} = 7.92^{**}$	$F_{1,2139} = 2.62^{ns}$	$F_{8,2139} = 29.16^{***}$	$F_{1,2139} = 213.42^{***}$
Days to germinate[2]	$F_{1,1852} = 12.53^{***}$	$F_{1,1852} = 13.85^{***}$[8]	$F_{8,1852} = 11.83^{***}$	$F_{1,1852} = 5.08^{*}$
Days to develop first leaf[3]	$F_{1,1752} = 9.50^{**}$	$F_{1,1752} = 0.50^{ns}$	$F_{8,1752} = 5.10^{***}$	$F_{1,1752} = 5.77^{*}$
Number of basal shoots[4]	$F_{1,1791} = 11.59^{***}$	$F_{1,1791} = 24.56^{***}$	$F_{8,1791} = 0.89^{ns}$	$F_{1,1791} = 0.02^{ns}$
Length of the longest leaf (March)[5]	$F_{1,1776} = 14.36^{***}$	$F_{1,1776} = 67.43^{***}$	$F_{8,1776} = 11.59^{***}$	$F_{1,1776} = 1.34^{ns}$
Length of the longest leaf (May)	$F_{1,1675} = 46.37^{***}$	$F_{1,1675} = 958.70^{***}$	$F_{8,1675} = 10.63^{***}$	$F_{1,1675} = 0.14^{ns}$
Length of the longest leaf (July)	$F_{1,1638} = 1.65^{ns}$	$F_{1,1638} = 1.44^{ns}$	$F_{8,1638} = 1.70^{ns}$	$F_{1,1638} = 1.85^{ns}$
Length of the longest leaf (October)[6]	$F_{1,1314} = 1.14^{ns}$	$F_{1,1314} = 6.82^{**}$	$F_{8,1314} = 3.22^{**}$	$F_{1,1314} = 4.12^{*}$
Days to flower	$F_{1,668} = 0.202^{ns}$	$F_{1,668} = 76.95^{***}$	$F_{8,668} = 3.16^{**}$	$F_{1,1668} = 0.38^{ns}$
Number of flowering shoots	$F_{1,668} = 0.03^{ns}$	$F_{1,668} = 33.67^{***}$	$F_{8,668} = 1.45^{ns}$	$F_{1,668} = 0.03^{ns}$
Length of flowering shoot	$F_{1,668} = 56.65^{***}$	$F_{1,668} = 3.55^{ns}$	$F_{8,668} = 3.17^{*}$	$F_{1,668} = 0.08^{ns}$
Aphid damage[7]	$F_{1,1304} = 2.59^{ns}$	$F_{1,1304} = 3.13^{ns}$	$F_{8,1304} = 4.16^{***}$	$F_{1,1304} = 0.57^{ns}$

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, nonsignificant.
[1] Region × elevation: $F_{8,2139} = 28.937^{***}$.
[2] Region × elevation: $F_{8,1852} = 9.692^{***}$.
[3] Region × elevation: $F_{8,1752} = 4.214^{***}$.
[4] Treatment × elevation: $F_{8,1791} = 4.092^{*}$; region × sex: $F_{8,1791} = 4.329^{*}$.
[5] Treatment × region: $F_{8,1776} = 2.052^{*}$; region × elevation: $F_{8,1776} = 6.421^{***}$.
[6] Region × elevation: $F_{8,1314} = 2.325^{*}$.
[7] Sex × region: $F_{8,1304} = 2.171^{*}$.
[8] This result was due to experimental error as a consequence of differences in water supply to petri dishes during germination.

discussed below, the relative contribution of certation to female bias in *R. nivalis* appears to be relatively small in comparison with the different responses of female and male plants to ecological conditions.

Sixteen percent of the open-pollinated seeds of *R. nivalis* we used for the glasshouse experiment did not germinate. Nevertheless, we were able to determine the sex of the vast majority using the SCAR marker. This is significant because the female bias detected in seedlings in previous studies of *Rumex* species through karyological analyses (Zarzycki and Rychlewski 1972; Rychlewski and Zarzycki 1986) could have resulted from male seeds failing to germinate; not an improbable scenario given the generally poorer performance of males that we detected in our study. However, in *R. nivalis* this can be ruled out since the sex ratios of seeds and young seedlings were not significantly different. This indicates that there is no difference in viability between female and male plants at this particular life stage and that a component of female bias is established earlier in the life cycle, prior to fertilization.

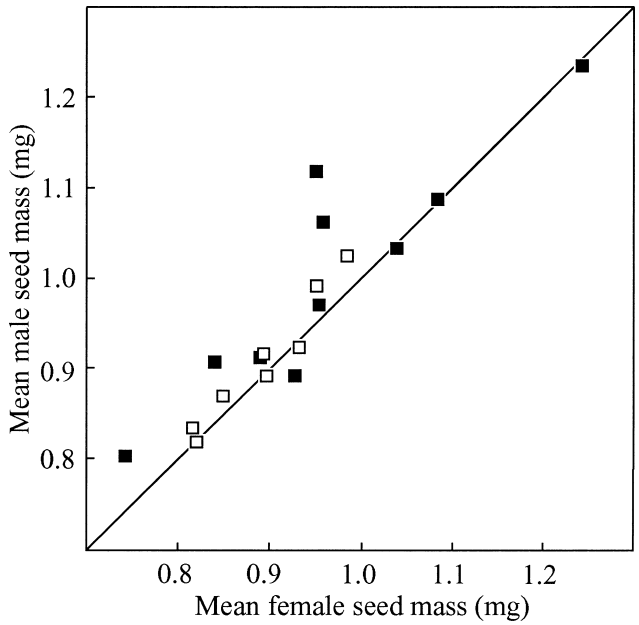

FIG. 5. Relation between the mean seed weight (mg) of female and male seeds in 18 populations of *Rumex nivalis* occurring in the Swiss Alps. The line represents equal seed weight of female and male seeds. Closed squares are low elevation populations; open squares are high elevation populations.

An unexpected finding of our study was that male seeds were significantly heavier than female seeds (Table 3, Fig. 5). Male seeds also germinated significantly faster than female seeds (Table 3). To our knowledge, this is only the second report of sexually dimorphic seeds in flowering plants. Freeman et al. (1994) also reported that male seeds were heavier than female seeds in spinach (*Spinacia oleracea*). However, spinach has environmental sex determination and seed size can be altered by interactions between external conditions and hormones (Freeman and Vitale 1985; Chailakhyan and Khrianin 1987). A study of the mass of female (XX) and male (XY) seeds within families of *Silene latifolia* detected no difference (Taylor 1996). It is possible that the difference in seed weight we detected between females and males of *R. nivalis* is associated with contrasting amounts of nuclear DNA in the sex chromosomes through its effect on cell size and number (see Bennett 1972), or this pattern results from sexually dimorphic seed provisioning by maternal parents.

Our finding of gender dimorphism of seed mass in *R. nivalis* is of interest to theories of parental expenditure and offspring sex ratios. In cases of equal overall expenditure to sons and daughters but different costs to producing female versus male offspring, more offspring of the cheaper sex should be produced (Fisher 1930; Hamilton 1967; de Jong and Klinkhamer 2002). As female seeds of *R. nivalis* weigh

less than male seeds, these theories predict that females should be produced in larger numbers. Indeed, this is the pattern we observed in *R. nivalis*, assuming that seed mass is directly associated with the cost of producing seeds. Can the proportional difference in seed mass account for the proportions of females and males in seed progenies? Following de Jong and Klinkhamer (2002),

$$r = C_f/(C_m + C_f) \quad (1)$$

where r is the seed sex ratio, and C_f and C_m are the costs of producing female and male offspring, respectively, this relation predicts an expected frequency of 50.84% females in seed progenies, considerably lower than the 59.62% females we observed in *R. nivalis*. It therefore seems unlikely that female bias is the outcome of selection for equivalent parental expenditure to sons and daughters that differ in cost.

The Increase in Female Bias Postparental Care

During the 16-month glasshouse experiment, female bias in *R. nivalis* increased significantly from 0.59 to 0.64 as a result of the higher mortality of male than female plants, particularly under low nutrient conditions (Fig. 4D). The duration of this experiment was relatively short, in comparison to the total life span of *R. nivalis*, and it is probable that increased levels of male mortality would have occurred if our experiment had been conducted for a longer period. Evidence to support this comes from our analysis of sex ratios in open-pollinated seeds, vegetative and flowering plants in natural populations. These comparisons demonstrate a striking increase in the degree of female bias with the life stage of plants as a result of differential mortality between the sexes. The higher mortality of males in the field versus glasshouse is likely to be a result of the more stringent growing conditions of alpine environments, and the difficulty of replicating these conditions in glasshouse experiments. Rychlewski and Zarzycki (1986) demonstrated a decrease in the proportion of male plants over a ten-year period in an experimental population of *R. thyrsiflorus*. These results from *Rumex* contrast with data for *S. latifolia* in which sex-differential mortality has not been detected (but see Delph 1999), and adult sex ratios do not differ from primary sex ratios (Taylor 1994, 1999; Purrington and Schmitt 1998).

We detected several differences in the performance of female and male plants of *R. nivalis* in our glasshouse experiment (Table 3, Fig. 4). Although males germinated earlier than females, probably because of their larger seeds, females produced their first leaf more rapidly and developed more shoots and longer leaves than males. There were no differences between the sexes in the onset of flowering, or the proportion of plants that flowered, and both developed similar numbers of flowering shoots. However, females had longer flowering shoots, and because of seed production, almost certainly invested more resources into reproduction. Similar gender-specific differences in growth and allocation patterns have also been documented in other *Rumex* species (Conn and Blum 1981b; Korpelainen 1992a, b), but it is unclear whether these differences are functionally associated with the differential mortality of the sexes that occurs under field conditions.

The patterns of sex-ratio variation in natural populations of *R. nivalis* implicate ecological factors as playing an important role in governing sex ratios. However, it is difficult to identify the specific features of the local environment responsible for differences in the performance of female and male plants. Because males suffered more mortality than females in the glasshouse, sites with higher male frequencies might be interpreted as less stressful. However, male frequencies were highest in the centers of snowbeds and in high elevation sites, that is, the environments that we might expect to experience more severe environmental conditions. This ''paradox'' can be resolved by considering the arguments of Körner (2003) on the direction of stress gradients in alpine plants. According to this perspective, for an alpine snowbed specialist, such as *R. nivalis*, conditions at lower elevations and at the periphery of snowbeds close to alpine meadows probably represent more stressful conditions. Additional support for this interpretation comes from our analysis of the sex ratios among life-history stages in low versus high elevation populations. At low elevations, seed sex ratios were the least biased but the sex ratios of adults were strongly female biased indicating that ecological conditions in these populations must promote significant male mortality. In contrast, at higher elevations the difference in sex ratios between seeds and adults was not as pronounced indicating that male mortality was less severe under more alpine conditions.

Why Do Males of Rumex Perform Poorly?

Our studies of sex ratios among different life-history stages in the field, and comparisons of the growth and viability of females and males in the glasshouse generally indicate that males of *R. nivalis* performed less well relative to females. This pattern is the reverse to that found in most dioecious species where females often suffer greater mortality (Lloyd and Webb 1977; Meagher and Antonovics 1982; Delph 1999). What mechanism(s) might account for the generally weaker male performance, which may also occur in other *Rumex* species (Smith 1963; Zarzycki and Rychlewski 1972; Rychlewski and Zarzycki 1975, 1986; Conn and Blum 1981a; Korpelainen 2002)? Poor male performance in *R. nivalis* was not associated with any specific life-history stage. Instead it was manifested throughout the life history, and probably also during competition between microgametophytes in the pistils of female plants. This suggests that the differences between females and males may be an inherent feature of *R. nivalis* populations, perhaps associated with the particular sex-chromosome system that this species possesses.

We favor the hypothesis that Y-chromosome degeneration may be responsible for the poor male performance at different life-cycle stages in *R. nivalis*. Y chromosomes in *Rumex*, as well as in other species with heterogametic sex chromosomes, do not recombine, and are therefore sheltered from purifying selection. This can result in the accumulation of deleterious mutations and Y-chromosome degeneration (Charlesworth and Charlesworth 2000; Charlesworth 2002; Vyskot and Hobza 2004). Such degeneration may occur in *Rumex* and could be associated with the high frequency of repetitive elements on Y chromosomes (Wilby and Parker 1988; Matsunaga and Kawano 2001). Y-chromosome degeneration may

explain the slower pollen-tube growth of male-determining microgametophytes under competitive situations, because pollen is haploid and deleterious genes will therefore be fully expressed (Smith 1963; Lloyd 1974; Charlesworth 2002). However, since a significant proportion of genes are expressed in both the gametophytic and sporophytic portions of the life cycle in plants (Ottaviano and Mulcahy 1989; Honys and Twell 2003), the poor performance of male plants in the field and glasshouse may also be linked to the deleterious consequences of Y-chromosome degeneration, including reduced gene function. This of course assumes that at least some of the genes involved are not fully recessive and that purging during the haploid phase is not complete. Alternatively, female bias could involve genetic elements associated with the X chromosome that have a transmission advantage but are associated with deleterious pleiotropic effects on Y chromosomes. Clearly, determining the underlying genetic mechanisms responsible for poor male performance requires further work. Specifically, molecular evidence for Y-chromosome degeneration in *Rumex* species and the causes and consequences of this phenomenon would be of particular value.

Matsunaga and Kawano (2001) proposed that Y chromosomes in *Rumex* subgenus *Acetosa* (including *R. nivalis*) are more differentiated than in *Rumex* subgenus *Acetosella*, and also in comparison with *S. latifolia* (Löve 1969). In *Rumex* subgenus *Acetosa*, sex is determined by a dosage system dependent on the ratio of X chromosomes and autosomes (Żuk 1963), whereas in the other two taxa, Y chromosomes determine sex. Interestingly, populations of *R. acetosella* and *S. latifolia* do not show consistent female-biased primary sex ratios and there is no evidence for postparental care male-biased mortality (*Rumex*: Putwain and Harper 1972; Korpelainen 1991; *Silene*: Taylor 1994, 1999; Purrington and Schmitt 1998; but see Carroll and Mulcahy 1990; Delph 1999). In contrast, the occurrence of female-biased seed or seedling sex ratios in subgenus *Acetosa*, including *R. acetosa*, *R. thyrsiflorus*, and *R. nivalis*, and postparental care male-biased mortality in *R. thyrsiflorus* (Zarzycki and Rychlewski 1972; Rychlewski and Zarzycki 1986) and *R. nivalis* (present study) raises the intriguing possibility that there may be a functional association between the degree of sex-chromosome differentiation, Y-chromosome degeneration, and sex-ratio variation in dioecious plants.

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APPENDIX 1.

Nine regions in Switzerland where populations of *Rumex nivalis* were investigated in this study. In each region high and low elevation sites were sampled.

Region	Altitude m a.s.l.	Coordinates (E/N)
Arosa	2500	9°37′7.2″, 46°46′6.1″
	2250	9°37′7.3″, 46°46′9.3″
Avers	2540	9°32′31.2″, 46°28′54.6″
	2340	9°35′15.8″, 46°29′7.7″
Brigels	2400	9°2′5.2″, 46°49′6.6″
	1920	9°1′2.0″, 46°48′2.7″
Davos	2500	9°49′20.0″, 46°41′32.3″
	2330	9°48′33.7″, 46°41′49.4″
Engelberg	2400	8°25′10.3″, 46°47′5.8″
	2100	8°24′32.9″, 46°47′22.4″
Flims	2600	9°16′21.8″, 46°52′40.5″
	2060	9°13′42.1″, 46°53′5.7″
Mürren	2540	7°50′53.2″, 46°33′29.3″
	2180	7°51′49.8″, 46°34′4.7″
Säntis	2120	9°21′65.9″, 47°14′30.1″
	1860	9°21′13.7″, 47°14′17.9″
Vals	2450	9°14′24.0″, 46°36′20.9″
	2040	9°12′46.1″, 46°36′45.1″