Factors affecting relative seed fitness and female frequency in a gynodioecious species, *Silene acaulis*

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ABSTRACT

Sex-ratio variation is common among gynodioecious species. One argument predicts that when sex is determined by a combination of nuclear and cytoplasmic factors, the frequency of females will be determined by genetic rather than ecological factors. An alternative argument suggests that the relative seed fitness of the female and hermaphroditic morphs will control female frequency. Hence, sex-ratio variation can be influenced by any factor that affects relative seed fitness, including ecological factors such as variation in pollination or site quality. In this study, we investigated sex-ratio variation in the gynodioecious species *Silene acaulis*, which has nuclear-cytoplasmic sex determination. We determined whether the frequency of females in 10 sites on Niwot Ridge, in the Front Range of Colorado, was correlated with the quality of the site or the relative seed fitness of the two morphs. Furthermore, we determined whether the two morphs differed consistently in investment in flowers, ovules, seeds and fruits. We found significant variation in sex ratio, site quality and relative seed fitness of the two morphs across sites. Although ovule number was greater in flowers on females, seed number per fruit did not vary in a consistent manner between morphs. The morphs differed consistently only in their propensity to produce fruit, with hermaphrodites exhibiting highly variable, and relatively low, fruit set compared to females. Female frequency was not significantly correlated with site quality. However, the relative seed fitness of the morphs was significantly correlated with site quality and the frequency of females. These results suggest that ecological factors do play a role in determining female frequency in *S. acaulis* and, consequently, can impact breeding-system evolution in this long-lived species.

Keywords: fruit set, gynodioecy, sex-ratio variation, *Silene acaulis*.

INTRODUCTION

Gynodioecy is a breeding system in which hermaphrodites and females co-exist within a population (Darwin, 1877). This system is a combination of individuals exhibiting a multiple strategy (the hermaphrodites) and a genetic polymorphism (hermaphrodites and females). Because females produce only seeds, whereas hermaphrodites produce both seeds and pollen, females must compensate for the loss of male fitness if they are to persist.

Therefore, a mutation conferring male sterility (or femaleness) will be selected for and maintained in a population only if the seed fitness of females is greater than that of hermaphrodites, where fitness is measured as seed quantity, seed quality or both. Females require a two-fold advantage over hermaphrodites in cases in which the male-sterility gene is nuclear; however, only a slight advantage is necessary if this gene is cytoplasmic (Lewis, 1941).

Once females invade a population, the relative seed fitness of the two morphs and the genetics of sex determination also affect female frequency, as has been shown in several theoretical studies (Lloyd, 1976; Charlesworth, 1981; Delannay *et al.*, 1981; Frank, 1989; Maurice and Fleming, 1995; McCauley and Taylor, 1997). Sex-ratio variation among populations of gynodioecious species is a widespread phenomenon (see citations in Delph, 1990a; Manicacci *et al.*, 1998; Webb, 1999), and the factors thought to affect this variation range from purely genetic to ecological in nature. Based on theoretical models, sex ratios are expected to vary more when male sterility is cytoplasmic than when it is nuclear because of founder effects and dynamic equilibria (Charlesworth, 1981; Delannay *et al.*, 1981; Ross and Gregorius, 1985; Frank, 1989; Gouyon *et al.*, 1991). The role of founder effects in determining sex ratios has been shown to be important in *Thymus vulgaris*. This species exhibits cytoplasmic male sterility, and female frequency varies markedly among populations, declining with population age. This pattern suggests that appropriate nuclear-restorer alleles spread over time after the founding of new colonies by seeds from females (Dommée and Jacquard, 1985; Gouyon and Couvet, 1985; Couvet *et al.*, 1986; Belhassen *et al.*, 1989; Manicacci *et al.*, 1996). Furthermore, models have shown that dynamic sex-ratio equilibria can develop depending on the number of cytoplasmic male-sterility factors and nuclear restorers, the cost of the restorers, and the relative seed fitness of the morphs (as determined by sex-determining genes) (e.g. Gouyon *et al.*, 1991). Variation in these factors results in sex ratios that vary widely through time and that are independent of ecological factors.

However, ecological factors can also cause variation in the relative seed fitness of the morphs and hence affect population sex ratios. For example, higher rates of self-fertilization in hermaphrodites are expected to lead to higher female frequencies if selfed seed suffer from inbreeding depression (Lloyd, 1975). Differences in pollination, for example, might lead to variation in selfing rates among populations. The idea that variation in selfing rates has led to sex-ratio variation has been suggested for multi-species comparisons within the genera *Bidens* (Sun and Ganders, 1986) and *Hebe* (Delph, 1990b). Pollen limitation may also affect the relative seed fitness of the morphs (Lewis, 1941; Lloyd, 1974; Maurice and Fleming, 1995; McCauley and Taylor, 1997), as has been shown in natural populations of *Pachycereus pringlei* (Fleming *et al.*, 1994) and experimental populations of *Silene vulgaris* (McCauley and Brock, 1998). Females of these species exhibited frequency-dependent fruit set, whereas hermaphrodites did not. Hence, the relative seed fitness of the morphs varied with the frequency of females.

Another ecological factor affecting sex-ratio variation is environmental quality. Higher female frequencies will be favoured in poor sites if seed production of the hermaphrodites is more plastic than that of females (Delph, 1990a). In poor sites, hermaphrodites may reduce their fruit or seed set to use the resources for growth and maintenance, thereby enhancing their flower and pollen production in the following year. This reduces their relative seed fitness and allows a higher frequency of females to be maintained at equilibrium. This hypothesis has been shown to be consistent with sex-ratio variation among populations

of *Hebe strictissima* (Delph, 1990a) and *Fragaria virginiana* (Ashman, 1999), and the frequency of inconstant males in *Ochradenus baccatus* (Wolfe and Shmida, 1997). Moreover, in some genera with both monomorphic and dimorphic species, the dimorphic species occur in the poor (usually dry) sites (Hart, 1985; Barrett, 1992; Costich, 1995) and this pattern may be influenced by the same mechanism.

Understanding interpopulation variation in sex ratio within species, which can affect breeding-system evolution, clearly requires investigation of whether ecological factors contribute to this variation. We therefore documented how variation in flower, ovule, fruit and seed production contributed to variation in seed fitness for females and hermaphrodites of the gynodioecious tundra plant *Silene acaulis*, at 10 sites on Niwot Ridge in the Colorado Front Range. We also tested whether female frequency is correlated with environmental quality (as measured by site and plant quality) and with the relative seed fitness of the two morphs. We chose *S. acaulis* for this study because it exhibits interpopulation variation in both breeding system and sex ratio (see citations below). We chose Niwot Ridge because *S. acaulis* grows in abundance along a wide range of habitats and elevations, and the ridge has been characterized as having steep environmental gradients over short distances (Walker *et al.*, 1993; Bowman and Conant, 1994; Walker *et al.*, 1994). We predicted that, if environmental variation contributes to sex-ratio variation, then females should occur in higher frequencies in poor sites and in sites where their relative seed fitness was highest.

METHODS

Study species and sites

Silene acaulis is a long-lived evergreen cushion plant that is circumpolar in the Northern Hemisphere, occurring in arctic and alpine tundra. The flowers are pink and occur singly, and are raised slightly above the surface of the cushion. The breeding system of populations around the world varies, ranging from gynomonoecy to gynodioecy to dioecy (Müller, 1883; Shykoff, 1988; Phillip *et al.*, 1990; Gleason and Cronquist, 1991; Morris and Doak, 1998; Manicacci *et al.*, 1998). Populations of *S. acaulis* in central Colorado are of the variety *subacaulescens* and occur in the tundra above 3300 m. There are several pieces of evidence suggesting that sex determination in *S. acaulis* is nuclear-cytoplasmic: there is a strong maternal effect on progeny sex ratios, with female seed-parents more likely to segregate females than hermaphrodite seed-parents (L.F. Delph, unpublished data), the frequencies of females can be greater than 50% (Hermanutz and Innes, 1994) and *S. vulgaris* exhibits this form of sex determination (Charlesworth and Laporte, 1998).

At the Niwot Ridge study site, *S. acaulis* is gynodioecious and it is the primary cushion species in fellfields (exposed rocky summit areas) (Walker *et al.*, 1994). The most common pollinator of this species in the Colorado Front Range is *Bombus sylvicola*, although flowers are visited by a variety of flies, moths, ants and beetles. Niwot Ridge is an 8-km ridge that extends east from the Continental Divide in the Front Range of the Colorado Rocky Mountains (40°03′30″N, 105°35′00″W). A Long-Term Ecological Research (LTER) program site exists on Niwot Ridge and is managed by the University of Colorado Mountain Research Station and the US Forest Service as an ecological reserve. Climatic measurements made at the site of the Tundra Lab (3525 m) by the Mountain Research Station were retrieved from the LTER Data web site (http://culter.colorado.edu:1030/Niwot/Niwot_Ridge_LTER_data.html). During the study year (1991), the maximum daily

temperature for the period 15 June to 15 August was $13 \pm 0.3°C$ (mean $\pm$ standard error) and the minimum daily temperature was $4 \pm 0.3°C$. The mean precipitation per day was 2.7 ± 0.64 ml. No precipitation occurred on 39 of the 62 census days (see below) and there was a continuous 2-week period in late June with no precipitation. The 10 sites chosen for this study range in elevation from 3390 to 3870 m (see Table 1). Sites were named for ease of identification and are numbered sequentially according to elevation, with Site 1 being in the subalpine or 'krummholz' zone and Site 10 (Top, above the D-1 station) being near the uppermost point on the mountain at which soil occurs and plant growth can be sustained. Flowering did not occur sequentially according to elevation. Site 3 (South-Slope) was the first site to flower, with collection of sex-ratio data beginning on 22 June; Site 9 (Last) was the last site to flower, with collection of sex-ratio data beginning on 26 July.

Sex ratio

Sex ratios were determined by observing all *S. acaulis* individuals when in flower in 60-m^2 plots at each site. As not all plants began flowering at the same time within or between sites, individuals were marked with a numbered wire flag as they came into flower and repeated censuses were made until all plants that produced flower buds during the season had flowered. Small individuals, which were presumably young in age, did not always flower. Although *S. acaulis* was occasionally infected with the smut *Microbotryum violaceum*, which prevents one from determining sex by observing the flowers (Marr, 1997), no infections occurred within our plots. Individuals that produced only pistillate flowers were considered to be female, while individuals that made perfect flowers and zero or very few pistillate flowers were considered to be hermaphroditic. At all sites, most hermaphrodites produced only perfect flowers (L. Delph and S. Carroll, unpublished data).

Relative seed fitness

We collected 40–78 fully open flowers (each from a separate plant) from each of the 10 sites, for a total of 312 perfect flowers from hermaphrodites and 207 pistillate flowers from females. The number of ovules in each flower was counted under a dissecting microscope. Calyx and petal sizes were measured and the means were always greater for hermaphrodites than for females (L. Delph and S. Carroll, unpublished data).

To determine the relative seed fitness of females and hermaphrodites, fruit set and seed number per fruit were determined for eight and nine sites, respectively. Neither fruit nor seed data were collected at Site 4 (Saddle) because flowering was interrupted when the plants were covered with snow for several days during the peak of flowering at this site. Fruit set was determined for a total of 387 individuals (232 hermaphrodites and 155 females) across eight sites (i.e. 17–35 individuals of each sex per site). Individuals were chosen arbitrarily within plots, such that plants from a range of sizes were included. The total number of flowers produced by a plant, the number of flowers eaten and the number of flowers that developed into fruit were determined for each plant, by repeatedly censusing individual plants as they flowered and fruited. Fruit set was calculated as the number of flowers that developed into fruit divided by the number of flowers that opened on a plant (subtracting those that were eaten). Seed number per fruit was determined by collecting fruit that were nearly mature, but which had not yet dehisced, and counting the number of maturing seeds in each. Aborted seeds were easily distinguished from maturing seeds at this

stage. Seed number per fruit was determined for a total of 411 plants across nine sites. Between 2 and 20 fruit from 15–32 plants of each sex were collected per site; hence seed number per fruit was determined for over 5500 fruit.

The relative seed fitness of hermaphrodites was calculated for eight sites as the number of seeds per flower produced by hermaphrodites divided by the number of seeds per flower produced by females. The number of seeds per flower was calculated by multiplying fruit set by the number of seeds per fruit. Hence, our calculation of relative seed fitness takes into account the quantity, but not the quality, of seeds produced.

Site and plant quality

We quantified site and plant quality in a number of ways. Our measures of site quality included density and percentage cover of *S. acaulis*, and percentage cover of all plants in 60-m^2 plots at each site. Percentage cover was determined by running three, parallel transects through each plot and determining whether 100 points (one every 10 cm) intercepted bare soil, rock, *S. acaulis* or other plant species. Total vegetative cover was interpreted as the capacity of each site to support plant growth.

In addition, the percentage of carbon, nitrogen and the carbon isotope composition ($\delta^{13}C$) of *S. acaulis* leaf tissue was determined by collecting and drying green leaf tissue for 14–21 individuals of each sex per site in mid-August (a total of 278 plants). This tissue was produced during the June–August growing season of that year. A portion of the leaf samples was ground to 100 mesh and analysed for the percentage of carbon and nitrogen using GC-combustion (Carlo Erba, model NA-1500 carbon-nitrogen analyser, Milan, Italy), at Cornell University in the laboratory of T. Dawson. Differences in the percentage of nitrogen could indicate differences in carbon fixation among sites or sexes, to the extent that they indicate differences in the photosynthetic capacity of the leaves (if secondary compounds did not differ; Field and Mooney, 1986). Another portion of the field-collected samples was analysed for $\delta^{13}C$ with a Finnigan delta S isotope ratio mass spectrometer, as discussed in Ehleringer and Osmond (1989), at the University of Utah Stable Isotope Facility for Environmental Research. Carbon isotope ratios are influenced by environmental conditions (Ehleringer and Osmond, 1989), and they are thought to be useful in assessing environmental quality at the time of growth of sampled tissue. They have also been used as an indication of water-use efficiency in plants (Farquhar and Richards, 1984; Ehleringer and Osmond, 1989), which is the ratio of the amount of carbon fixed through photosynthesis to the amount of water lost through transpiration, thus measuring the efficiency of carbon fixation in terms of water costs. This index is, therefore, an integrated measure representing the balance between plant gas exchange and water-relations, and is potentially indicative of plant performance under water stress. Alpine sites such as the fellfields on Niwot Ridge are known for their consistent dryness, whereas wet meadows have a tendency to show a pattern of decreasing soil moisture through the growing season (Walker *et al.*, 1994). Of particular relevance to our study, water-use efficiency has been found to differ between the sexes of dioecious species (Dawson and Geber, 1999).

Plant vigour or quality was measured by determining the relationship between flower number and plant size. Plant size may be a good indicator of the age of a plant, but it is likely to be a poor indicator of vigour, as individual plants have been estimated to live for 300 years or more (Morris and Doak, 1998) and typically expand in size as they age. Plant size was estimated as length × width of the cushions for 14–23 individuals of each sex per

site (i.e. 287 plants in total). This information, combined with flower number per plant, allowed us to assess differences in reproductive effort under different environmental conditions.

Statistical analyses

All statistical analyses were performed either by hand or with SYSTAT 5.2 or SPSS 6.1 (statistical packages for Macintosh computers). A heterogeneity *G*-test (Sokal and Rohlf, 1981) was performed to determine whether there was significant heterogeneity in sex ratio among populations. A variety of measures were compared between the sexes and among the sites using mixed-model, two-way analyses of variance. Variables were transformed when necessary to meet statistical assumptions (e.g. fruit set data were arcsin(square-root) trans-formed). In these cases, the data are presented as backtransformed data, which can result in asymmetric standard errors. The interaction term (sex × site) was lumped with the error term when it was non-significant at or above the $P = 0.25$ level (Sokal and Rohlf, 1981). Scheffé's *a posteriori* tests were performed to determine significant differences among the various sites when the site effect was shown to be significant. One-way analyses of variance combining sex and site into one variable were also used to allow for orthogonal contrasts between the sexes at each site.

To reduce the influence of correlations among the dependent variables representing site and plant quality, and the number of statistical tests, principal-components analysis was used to reduce sets of variables to independent axes of variation. All axes with eigenvalues greater than 1.0 were used for further analysis. The eight variables – number of *S. acaulis* within each plot, cover of *S. acaulis* (%), total vegetative cover (%), carbon and nitrogen (%) in *S. acaulis* leaf tissue, $\delta^{13}C$, number of flowers per plant size (cm^2) and elevation – were reduced to two principal components. Factor scores were saved and the correlations between them and female frequency and relative seed fitness were then determined (one-tailed tests). No rotation was applied to the matrix presented, as rotating the matrix neither made the factors more interpretable, nor did it alter the outcome of the correlation analysis.

RESULTS

Sex ratio

The site values for elevation, the frequency of females and sex ratio are given in Table 1. The elevation range between the lowest and highest sites was 480 m. The frequency of females varied from 23 to 39%, with a mean (± 1 standard error) of 30 ± 2.2% and a coefficient of variation (CV) of 23%. Consequently, sex ratios (H : F) varied from 1.56 to 3.44. The among-population variation in sex ratio was significantly heterogeneous ($G_H = 60$, d.f. = 9, $P < 0.001$).

Flower number, ovule number, seed number and fruit set

The ratio of flower number to plant size averaged 0.33 ± 0.03 cm^2 for females and 0.35 cm^2 (+0.03, −0.02) for hermaphrodites (backtransformed data, from log[number of flowers/ plant area]). A two-way analysis of variance (ANOVA) revealed a significant sex × site

Table 1. Characteristics of the 10 sites on Niwot Ridge used in the study

Site	Elevation (m)	Frequency of females (%)	Sex ratio (H : F)
1. Subalpine	3390	33.5	1.99
2. Trailer	3450	22.5	3.44
3. South-Slope	3470	39.1	1.56
4. Saddle	3540	37.7	1.65
5. Dry	3660	22.5	3.44
6. Main Flat	3670	26.3	2.81
7. Rockgarden	3675	23.2	3.32
8. Watershed View	3705	36.9	1.71
9. Last	3765	23.1	3.32
10. Top	3870	34.4	1.91

interaction ($F_{8,269} = 2.60$, $P = 0.009$), so one-way analyses of variance with orthogonal contrasts were performed to reveal differences between the sexes and differences among sites. Site 1 (Subalpine) had values for females that were significantly greater than those for hermaphrodites ($P = 0.003$), whereas Site 9 (Last) had values that were significantly greater for hermaphrodites than those for females ($P = 0.001$; see Fig. 1). In addition, plants in Sites 7 (Rockgarden) and 9 (Last) had significantly more flowers per unit area than plants in Site 5 (Dry).
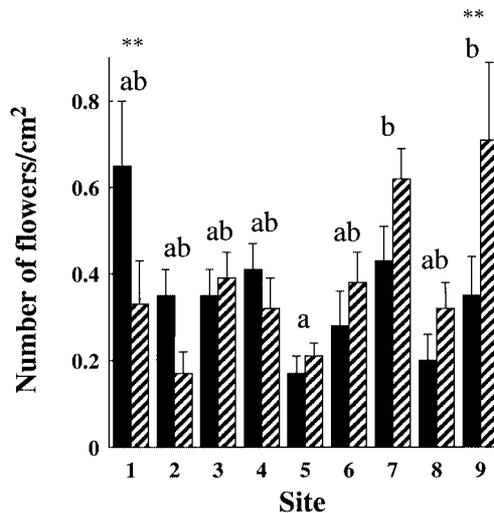

Fig. 1. Variation among sites and morphs in the number of flowers produced per cm² of plant. Means (± 1 standard error) shown are backtransformed from log(number of flowers per cm²). The standard errors shown are the average of the asymmetric positive and negative standard errors that result from backtransformation. Sites with similar letters above the means are not significantly different from each other. Asterisks above the means indicate significant differences between females (■) and hermaphrodites (▨) within a site (**$P < 0.01$).

The variance in ovule number was greater for perfect flowers on hermaphrodites than for pistillate flowers on females (163 *vs* 35, $F_{205,301} = 4.6$, $P < 0.001$). Averaged over all 10 sites, pistillate flowers on females contained significantly more ovules per flower (31 ± 0.4) than perfect flowers on hermaphrodites (26 ± 0.4; $F_{1,495} = 7.7$, $P = 0.006$) and there were significant differences among sites ($F_{9,495} = 10.7$, $P < 0.001$; two-way ANOVA on ranked data). By combining sex and site into one categorical variable, we were able to determine whether the sexes differed in their ovule number per flower at each site using a one-way ANOVA. Ovule number was greater in pistillate flowers on females than perfect flowers on hermaphrodites at all 10 sites, and these differences were significant at five of the 10 sites: Sites 2 (Trailer; $P = 0.036$), 4 (Saddle; $P = 0.002$), 8 (Watershed View; $P = 0.042$), 9 (Last; $P = 0.023$) and 10 (Top; $P = 0.013$) (Fig. 2; all $P > 0.081$ at the other five sites).

Across nine sites, mean number of seeds per fruit was significantly correlated with mean number of ovules per flower for females ($r = 0.76$, $P = 0.02$) but not for hermaphrodites ($r = 0.15$, $P = 0.68$; Fig. 3). The number of seeds per fruit was 13.4 ± 0.73 for fruit from females versus 12.5 ± 0.71 for fruit from hermaphrodites, a non-significant difference (two-way ANOVA, $F_{1,8} = 1.38$, $P = 0.27$). There was a significant sex $\times$ site interaction for the number of seeds per fruit ($F_{8,393} = 2.71$, $P = 0.006$). Using a categorical variable that combined sex and site into one variable, a one-way ANOVA revealed that, although females produced more seeds per fruit than hermaphrodites at seven of the nine sites (see Fig. 4a), this difference was significant only at Site 7 (Rockgarden; $P = 0.006$) and marginally so at Site 2 (Trailer; $P = 0.067$). Moreover, hermaphrodites produced significantly more seeds per fruit than females at Site 9 (Last; $P = 0.01$).

Averaged across eight sites, there was a significant effect of sex on fruit set ($87 \pm 0.02\%$ on females versus $46 \pm 0.04\%$ on hermaphrodites, backtransformed means $\pm$ standard
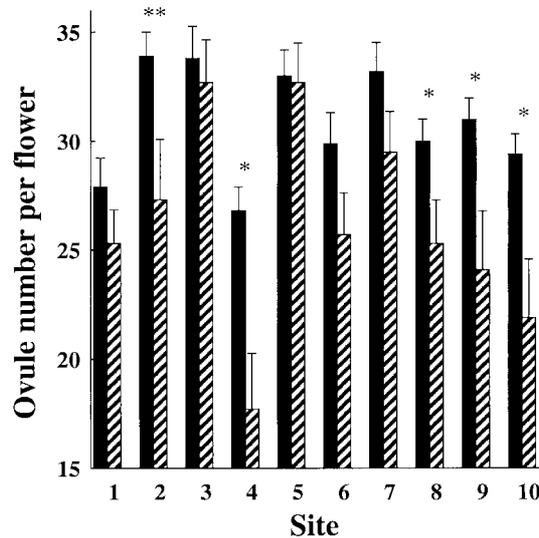

Fig. 2. Variation among sites and morphs in the number of ovules produced per flower (means ± 1 standard error). Asterisks above the means indicate significant differences between pistillate flowers on females (■) and perfect flowers on hermaphrodites (▨) within a site (*$P < 0.05$, **$P < 0.01$).
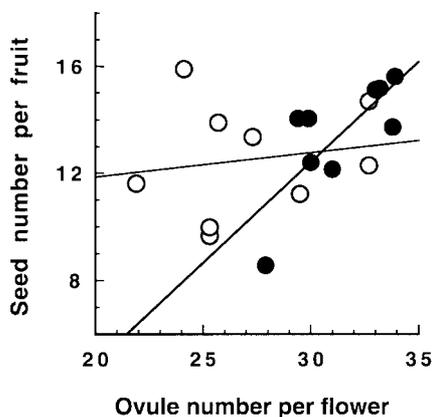
Fig. 3. Relationship between the mean number of seeds produced per fruit for a given site and the mean number of ovules per flower for the same site. The regression is significant for females (●; $R^2 = 0.57$, $P = 0.02$) but not for hermaphrodites (○; $R^2 = 0.02$, $P = 0.68$).
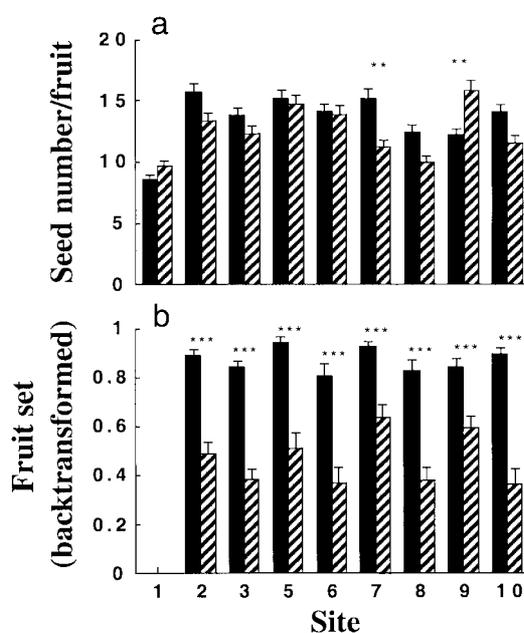

Fig. 4. Variation among sites and morphs in (a) the number of seeds produced per fruit and (b) the percentage fruit set (means ± 1 standard error). Means of fruit set shown are backtransformed from arcsin(square-root(percentage fruit set)). Asterisks above the means indicate significant differences between females (■) and hermaphrodites (▨) within a site (**$P < 0.01$, ***$P < 0.001$).

error; two-way ANOVA, $F_{1,7} = 179.6$, $P < 0.001$). Neither the main effect of site nor the sex × site interaction was significant ($F_{7,371} = 2.8$, $P = 0.10$ and $F_{7,371} = 1.37$, $P = 0.22$, respectively). A one-way ANOVA using a variable combining sex and site revealed that females set significantly more fruit than hermaphrodites at all eight sites (all $P < 0.001$) (Fig. 4b).

Site and plant quality

The number of *S. acaulis* in the 60-m^2 plots varied from 98 to 513, with a mean of 299 ± 50.5 and a coefficient of variation (CV) of 54% (Fig. 5). This translates into a range of 1.6–8.6 plants per m^2. The percentage cover of *S. acaulis* within the plots varied from 0.7 to 7%, averaging 3.5 ± 0.59% (CV = 54%), and the total vegetative cover (%) varied from 44 to 80%, averaging 59.4 ± 3.72% (CV = 20%) (Fig. 5). Percentage cover of *S. acaulis* was highest in Site 9 (Last).

The percentage of carbon in leaf tissue of *S. acaulis* individuals varied among sites from 41 to 45%, averaging 43 ± 0.4%. The percentage of carbon did not vary significantly according to sex (43.1 ± 0.39% for females and 42.8 ± 0.46% for hermaphrodites; $F_{1,268} = 2.5$, $P = 0.11$), but there were significant differences among the nine sites measured ($F_{8,268} = 17.6$, $P < 0.001$) (Fig. 6). Scheffé's test revealed that plants from Sites 7 (Rockgarden) and 10 (Top) had a significantly higher percentage of carbon than plants from all other sites except Site 9 (Last), and plants from Site 9 had significantly greater amounts than those from Sites 5 (Dry) and 8 (Watershed).
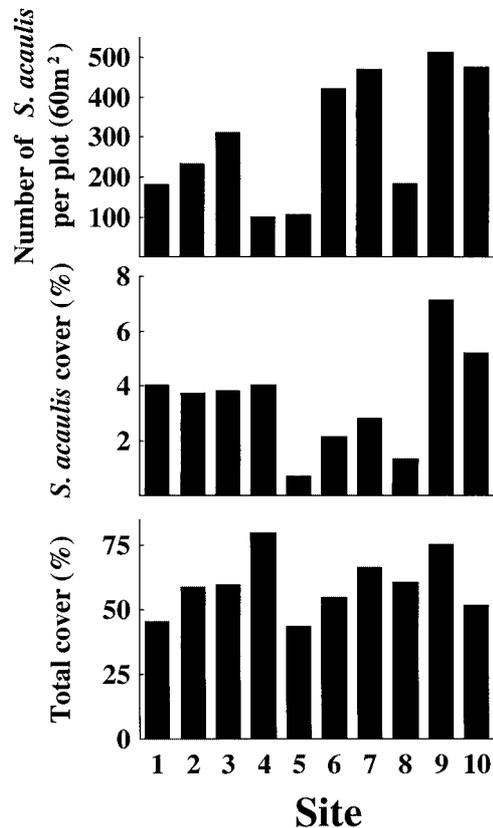

Fig. 5. Site-quality measures for the 10 sites on Niwot Ridge (means ± 1 standard error). From top to bottom these are the number of *S. acaulis* plants per 60-m^2 plot, the percentage cover of *S. acaulis* and the percentage cover by all vegetation.
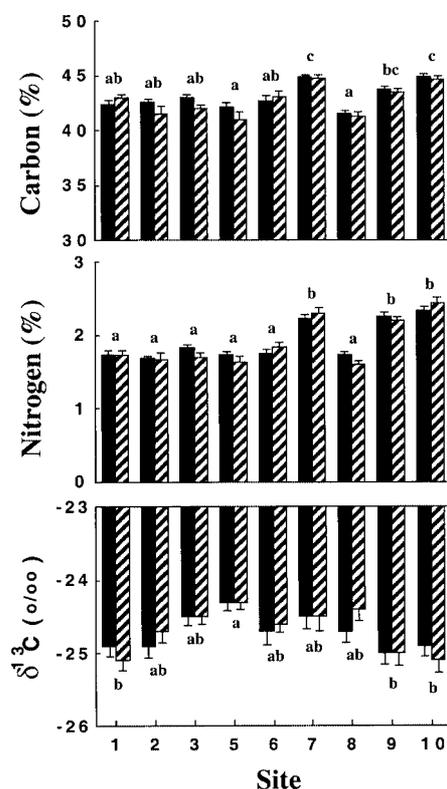
Fig. 6. Site-quality measures for the 10 sites on Niwot Ridge (means ± 1 standard error). From top to bottom these are the percentage of carbon, the percentage of nitrogen and the carbon isotope composition (δ^{13}) of leaf tissue of *S. acaulis*. Sites with similar letters above the means are not significantly different from each other (■ = females, ▨ = hermaphrodites).

The percentage of nitrogen in leaf tissue showed a similar pattern, with values among sites ranging from 1.7 to 2.4% (mean of 1.9 ± 0.10%). There was no difference between the sexes in the percentage of nitrogen contained in leaf tissue (1.9 ± 0.09% for females and 1.9 ± 0.11% for hermaphrodites; $F_{1,268} = 0.3$, $P = 0.61$), but there were significant differences among the nine sites measured ($F_{8,268} = 45.9$, $P < 0.001$) (Fig. 6). Plants from the same sites that were relatively high in carbon were also relatively high in nitrogen. Plants from Sites 7 (Rockgarden), 9 (Last) and 10 (Top) contained a significantly greater percentage of nitrogen than plants from the other six sites (Scheffé's test).

The δ^{13}C of leaf tissue varied from −24.3 to −25.3 among sites, averaging −24.7 ± 0.11. The sexes did not differ in their δ^{13}C, with a mean of −24.7 ± 0.08 for females and −24.7 ± 0.10 for hermaphrodites ($F_{1,268} = 0.003$, $P = 0.96$). There were, however, significant differences among sites ($F_{8,268} = 6.0$, $P < 0.001$) (see Fig. 6). Plants from Site 5 (Dry) had δ^{13}C values that were significantly less negative than those from Sites 1 (Subalpine), 9 (Last) and 10 (Top) (note that less negative values of δ^{13}C potentially indicate drier sites or higher water-use efficiency). Site 5 (Dry) was also the site with the lowest number of *S. acaulis* of

the nine sites for which physiological measures were taken, and was the site with the lowest percentage cover by *S. acaulis* and the highest sex ratio (Table 1 and Fig. 5).

Correlation between the percentage of females and site/plant quality

Data reduction by principal components analysis of the eight site and plant-quality variables produced two principal components with eigenvalues > 1, together accounting for 81% of the variation in site or plant-quality variables (Table 2). The first principal component (PC1) explained 62% of the variance and was positively correlated with all of the variables except $\delta^{13}C$, with which it was negatively correlated. The highest correlations were with high-quality plant variables and high nutrient variables, whereas the correlation with elevation was quantitatively lower. We interpret this component to represent high-quality sites or plants. The second principal component (PC2) explained 19% of the variance. PC2 was highly positively correlated with elevation and negatively correlated with percent cover by *S. acaulis*. In contrast to PC1, PC2 was positively correlated with $\delta^{13}C$. The other variables were only weakly correlated with PC2, so we interpret this component to be an indicator of relatively poor sites or plants. The percentage of females across sites was not significantly correlated with either of the principal components (Table 2).

Correlations with relative seed fitness

Averaged across eight sites, females produced, on average, 1.92 times as many seeds per flower as hermaphrodites (11.7 ± 0.55 *vs* 6.1 ± 0.63), a significant difference (paired *t*-test, $t_7 = 7.25$, $P < 0.001$). As can be seen from Fig. 4, relatively low fruit set on hermaphrodites,

Table 2. Factor loadings for a principal components (PC1, PC2) analysis of the eight site and plant-quality measures taken at Niwot Ridge, and Pearson correlation coefficients of each principal component with sex ratio

Site or plant-quality variable	PC1	PC2
Number of *S. acaulis* per 60-m^2 plot	0.93	0.17
Percent cover of *S. acaulis*	0.75	−0.59
Total percent cover by vegetation	0.82	0.16
Carbon in leaves of *S. acaulis* (%)	0.87	0.13
Nitrogen in leaves of *S. acaulis* (%)	0.92	0.31
$\delta^{13}C$ (‰)	−0.60	0.66
Number of flowers per plant area	0.86	−0.16
Elevation	0.38	0.75
% of total variance	61.70	19.20
Eigenvalue	4.94	1.54
Pearson correlation coefficient (*P*) (one-tailed test) of:	PC1	PC2
with female frequency (*n* = 8)	−0.29 (0.24)	−0.22 (0.30)
with relative seed fitness (*n* = 7)	0.67 (0.05)	−0.27 (0.28)

rather than lower seed number per fruit, was the main cause of this difference. Females produced more seeds per flower than hermaphrodites at all eight sites (Table 3). Consequently, the relative seed fitness of the hermaphrodites (#seeds per flower on hermaphrodites/#seeds per flower on females) was less than 1.0 at all eight sites, and averaged 0.53 ± 0.06. Across sites, the relative seed fitness of hermaphrodites was significantly negatively correlated with the frequency of females (Spearman's $r = -0.68$, $P = 0.03$, $n = 8$, one-tailed test). In addition, relative seed fitness was significantly correlated with PC1 (Table 2). This indicates that the relative seed fitness of hermaphrodites varied with site quality, being higher in the high-quality sites than poor-quality sites.

DISCUSSION

We predicted that, if ecological factors contribute to sex-ratio variation, then females of *S. acaulis* should occur in higher frequencies in poor sites and in sites where their relative seed fitness was highest. For these predictions to be testable, we needed variation among sites in sex ratio, site quality and relative seed fitness. The 10 sites we examined on Niwot Ridge exhibited significant variation in all three variables, and we found support for one of our predictions: female frequency was higher in sites in which the hermaphrodites had relatively low seed fitness. Below, we discuss the patterns of variation in these three variables and the relationships among them.

Stages of adjustment in investment in seeds

Plants should be able to serially adjust their investment in female function by adjusting flower production, ovary development, seed number and fruit maturation (Lloyd, 1980; Stephenson, 1981). Because hermaphrodites and females of gynodioecious species differ in terms of how they acquire their fitness, they may also vary in terms of the number of stages at which investment in female function is adjusted and the magnitude of the investment at each stage. Gynodioecy, in general, spans a continuum from populations in which hermaphrodites have high seed production and in which female frequency is low to

Table 3. The number of seeds produced per flower for hermaphrodites and females, and the relative seed fitness of hermaphrodites for *S. acaulis* plants from eight sites on Niwot Ridge

Site	Hermaphrodites	Females	Relative seed fitness
2. Trailer	6.4	13.4	0.48
3. South-Slope	4.8	11.4	0.43
5. Dry	7.5	13.2	0.56
6. Main Flat	5.2	10.6	0.49
7. Rockgarden	6.9	13.4	0.51
8. Watershed View	4.0	9.8	0.41
9. Last	9.3	9.8	0.95
10. Top	4.5	12.1	0.37
Mean	6.1	11.7	0.53

populations in which hermaphrodites have low seed production and in which female frequency is high (Lloyd, 1976; Delph and Lloyd, 1991). The higher the female frequency, the more the hermaphrodites will function as males and the greater will be the selection for mutations that increase their pollen production at the cost of a decrease in their seed production (Lloyd, 1976). Such selection can take the breeding system all the way to dioecy, with the pollen-producing morph turning into a pure male that does not produce any seeds (Charlesworth and Charlesworth, 1978). Given that the breeding system of *S. acaulis* is purported to vary from co-sexuality all the way to dioecy, determining the stages at which seed production is adjusted in the two morphs will help explain how breeding-system evolution occurs in this species.

   One striking result of our study is that the percentage of flowers that are matured into fruit is the *main* factor causing differences in seed production between the two morphs. Females have uniformly high fruit set, whereas fruit set on the hermaphrodites is lower and more variable. Studies of other gynodioecious species have shown relatively low fruit set on hermaphrodites (see reviews in Ashman, 1999; Webb, 1999) and this is consistent with the idea that flowers on hermaphrodites are acting more as pollen producers than as pollen receivers (Sutherland and Delph, 1984). In contrast to fruit set, there were no consistent differences between the morphs in the number of flowers produced or in the number of seeds produced per fruit. Females sometimes showed greater values for these two variables and sometimes hermaphrodites did, but for most sites the two morphs did not differ significantly. The fact that seed number per fruit was not higher in females is especially interesting, given that ovule number per flower was higher for females than hermaphrodites in all 10 sites, significantly so in five. For example, in Site 9 (Last), although flowers on hermaphrodites contained significantly fewer ovules than flowers on females, they contained significantly more seeds. Across all sites, we found that seed production in females was determined more by ovule number per flower than that in hermaphrodites. Hence, hermaphrodites either vary their seed production per fruit in response to factors other than ovule number more so than females, or there is greater variation among flowers within a plant on hermaphrodites. Nevertheless, seed-fitness differences between hermaphrodites and females exist mainly because of differences in their propensity to mature flowers into fruit.

Relationship between site quality, relative seed fitness and female frequency

We found significant variation in the frequency of females across our sites, ranging from 23 to 39%. Sex-ratio variation has also been found at other locations where *Silene acaulis subacaulescens* and other subspecies of *S. acaulis* occur. For example, in the nearest population to ours that has been investigated (Pennsylvania Mountain, CO), the percentage of females ranges from 23 to 43% across sites (*S. a. subacaulescens*, Marr, 1997; see also Shykoff, 1988). Similarly, female frequency ranged from 20 to 54% in Greenland (Phillip *et al.*, 1990; subspecies not indicated), from 42 to 59% in northern Sweden (Alatalo and Molau, 1995; subspecies not indicated) and from 37 to 61% in *Silene acaulis cenisia* in the French Alps (Maurice *et al.*, 1998). In contrast, *Silene acaulis exscapa* often exhibits significantly female-biased sex ratios, as reported for populations on Baffin Island in Canada (50–80% females; Hermanutz and Innes, 1994) and the French Alps (38–71% females; Maurice *et al.*, 1998). These populations of *S. a. exscapa* also contained a relatively high frequency of individuals that appeared to be strictly male, with flowers containing short styles that did not set fruit. A high frequency of such individuals also occurs in

Finland, at a population near the Kevo Subarctic Research Institute (P. Mutikainen and L. Delph, personal observation). Hence, *S. a. exscapa* appears to be closer to the dioecy end of the gynodioecy continuum than *S. a. subacaulescens* (see also Maurice *et al.*, 1998). These comparisons highlight that the breeding system of *S. acaulis* is highly variable across populations and subspecies.

In addition to variation in sex ratio, we found significant variation in site and plant quality across our sites. Moreover, except for elevation, the site and plant-quality variables covaried with each other, as shown with PC1 (Table 2), which loaded strongly with all of the variables except elevation. This suggests that some sites on Niwot Ridge are better than other sites for *S. acaulis*, but that they do not follow a gradient with elevation. For example, Site 5 (Dry) and Site 8 (Watershed View) appear to be relatively poor sites. They exhibited relatively low density and percentage cover of *S. acaulis*, and Site 5 had the lowest percentage cover by vegetation. Furthermore, these sites ranked low in terms of the percentage of carbon and nitrogen in the leaves of *S. acaulis*, and Site 5 had the most positive $\delta^{13}C$ values of all sites (indicating dryness). Both Sites 5 and 8 also had the two lowest mean number of flowers on *S. acaulis* plants. In comparison, Site 9 (Last) appears to be a relatively high-quality site. It ranked among the highest for all of the site and plant-quality measures, except elevation. The fact that there are significant differences in site quality should enhance our ability to detect a correlation with the two principal component factors characterizing site quality, PC1 and PC2, and the percentage of females. In spite of this, the correlations were not significant. Removing elevation from the set of site-quality variables did not change these results; however, it increased the percentage of the total variance explained by the principal components (from 81 to 86%). Given these results, the lack of a relationship between the principal component factors for quality and female frequency does not appear to be the result of a lack of clear differences in the quality of the sites.

These results are in contrast with results of a study of *Hebe strictissima* in New Zealand, in which females were found to be more prevalent in poor sites (Delph, 1990a). In *H. strictissima*, the fruit set of the hermaphrodites was highly plastic, ranging from less than 30% to greater than 90% across eight sites on Banks Peninsula. In comparison, fruit set of *S. acaulis* in our sites ranged from 38 to 61%. Consequently, although fruit set on hermaphrodites varies in *S. acaulis* and appears to vary with environmental factors (see below), it may not have varied sufficiently among sites on Niwot Ridge to significantly increase the percentage of females in poor sites. In addition, although the number of sites investigated was similar for the two studies, site quality may not have varied as much among sites on Niwot Ridge as it did among sites on Banks Peninsula, and this could have contributed to us not finding a significant relationship in this study.

Our results also contrast with those of Phillip *et al.* (1990), who looked at female frequency in *S. acaulis* in five sites in Greenland. They did not measure site quality *per se*, but noted that they 'arranged the sites subjectively in order of increasing severity and there appears to be an increase in the proportion of females along this gradient'. Many differences exist between our sites and the sites in Greenland, including elevation, pollinators (the Greenland sites lack bumblebees), soil type, climate and possibly subspecies, making it difficult to conclude why our results differ from those of Phillip *et al.* In addition, they did not measure fruit set, so comparisons of the extent of variation in fruit set between their study and ours cannot be made. However, it raises the possibility that a meta-population analysis of the pattern might show more support for greater female frequency in poor sites (see also Manicacci *et al.*, 1998).

In spite of the non-significant relationship between female frequency and site quality on Niwot Ridge, we did find a significant relationship between female frequency and the relative seed fitness of the two morphs. Sites in which the hermaphrodites had relatively low seed fitness had a higher percentage of females than sites in which hermaphrodites had higher seed fitness. This outcome fits the prediction of Lloyd's (1976) model, which states that the relative seed fitness of the two morphs should affect the frequency of females in the population. Similar results have been found in multi-population studies of the gynodioecious species *Hebe strictissima* (Delph, 1990a), *Ochradenus baccatus* (Wolfe and Shmida, 1997) and *Fragaria virginiana* (Ashman, 1999), and in multi-species comparisons of New Zealand apioid Umbelliferae (Webb, 1981) and *Thymus* (Manicacci *et al.*, 1998). Our results suggest that ecological factors do affect female frequency to some extent in *S. acaulis*, as the alternative explanation would be that all of the variation in hermaphrodite seed fitness among sites was due to genetic differences. This is unlikely given that the relative seed fitness of the two morphs varied significantly with site quality, and this variation was mostly caused by variation in fruit set on the hermaphrodites. In addition, gene flow for this species appears to be high on mountains in the Front Range of Colorado, reducing the likelihood of genetic differentiation among hermaphrodites at the various sites ($F_{ST} = 0.007$; Gehring and Delph, 1999).

CONCLUSIONS

Overall, our results indicate that hermaphrodites reduce their seed production in poor-quality sites and increase it in high-quality sites more so than females, which impacts on the frequency of females. Hence, our study joins a growing list of studies supporting the hypothesis that ecological factors contribute to the frequency of females in populations and can, therefore, impact breeding-system evolution. Our results are especially interesting given that male sterility is inherited via the cytoplasm in *S. acaulis*, as this is the type of genetic sex determination in which variation in female frequency is not expected to 'correspond to any adaptive or ecological cause' (Gouyon *et al.*, 1991). Our results suggest that we should not dismiss the possibility that both ecological and genetic factors can contribute to female frequency, even with nuclear-cytoplasmic gynodioecy.

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