

Effects of floral neighborhood on seed set and degree of outbreeding in a high-alpine cushion plant

Lea R. Wirth · Nickolas M. Waser · René Graf · Felix Gugerli · Urs Landergott · Andreas Erhardt · Hans Peter Linder · Rolf Holderegger

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Abstract Plants flowering together may influence each other's pollination and fecundity over a range of physical distances. Their effects on one another can be competitive, neutral, or facilitative. We manipulated the floral neighborhood of the high-alpine cushion plant *Eritrichium nanum* in the Swiss Alps and measured the effects of co-flowering neighbors on both the number of seeds produced and the degree of inbreeding and outbreeding in the offspring, as deduced from nuclear microsatellite markers. Seed set of *E. nanum* did not vary significantly with the presence or absence of two *Saxifraga* species growing as near neighbors, but it was higher in *E. nanum* cushions growing at low conspecific density than in those growing at high density. In addition, floral neighborhood had no detectable effect on the degree of selfing of *E. nanum*, but seeds from cushions growing at low conspecific density were more highly outbred than seeds from cushions at high density. Thus, there was no evidence of either competition

or facilitation between *E. nanum* and *Saxifraga* spp. as mediated by pollinators at the spatial scale of our experimental manipulation. In contrast, the greater fecundity of *E. nanum* cushions at low density was consistent with reduced intraspecific competition for pollinators and might also represent a beneficial effect of highly outbred seeds as brought about by more long-distance pollinator flights under low-density conditions.

Keywords Competition · Facilitation · Genetic paternity analysis · Inbreeding · Outbreeding · Realized mating · Scale effects

Introduction

Neighboring plants of the same or different species interact either positively or negatively by modifying each other's access to resources, including water, light, and nutrients. In addition, plants may interact over a wider range of physical distances through the activity of mobile animals, such as herbivores or pollinators. Insofar as the behavior of these animals changes with the properties of plant associations, individual plants can either increase or decrease components of the fitness of other plants growing within the range of distances moved by shared visitors.

Pollination provides both intuitive and non-intuitive examples of such effects (for reviews see Rathcke 1983; Waser 1983; Sargent and Ackerly 2008; Mitchell et al. 2009). The flowers of one plant may draw pollinators away from other plants, may leave the rate of pollinator visitation unchanged, or may enhance visitation for all plants involved. The first situation is likely to reduce the reproductive success of those plants receiving fewer visits, i.e., a competitive effect. Competition is even possible when

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L. R. Wirth · R. Graf · F. Gugerli · U. Landergott · R. Holderegger (✉)
WSL Swiss Federal Research Institute,
Zürcherstrasse 111, 8903 Birmensdorf, Switzerland
e-mail: rolf.holderegger@wsl.ch

N. M. Waser
School of Natural Resources and the Environment,
University of Arizona, Tucson, AZ 85721, USA

A. Erhardt
Integrative Biology, University of Basel,
St. Johanns-Vorstadt 10, 4056 Basel, Switzerland

H. P. Linder
Institute of Systematic Botany, University of Zurich,
Zollikerstrasse 107, 8008 Zurich, Switzerland

per-capita visitation is unchanged because the pollen of one species may be lost on stigmas of other species or foreign pollen may compromise the ability of a flower to receive or use conspecific pollen (e.g., Morales and Traveset 2008; Mitchell et al. 2009). However, if the association with other plants enhances visitation rate, several final outcomes are possible. The deleterious effects of interspecific pollen transfer just described may overwhelm any benefit of greater visitation (Kunin 1993; Sargent and Ackerly 2008; Mitchell et al. 2009). The outcome may also be neutral, and, finally, it may be positive in the form of the facilitation of male or female reproductive success.

How can the type of interaction among plants be evaluated? An accurate assessment requires that we measure fitness or its components, rather than aspects of pollinator visitation alone, since even an enhanced rate of visitation may have either positive or negative effects on fitness. The easiest component of fitness to measure is fecundity through female sexual function (i.e., seed or fruit set), but fecundity through male function (seed siring) might also be affected. In addition to quantitative effects on the numbers of seeds set or sired, aspects of offspring quality might also respond to plant interactions. These aspects of quality include the degree of inbreeding or outbreeding of the seeds produced and the genotypic diversity of seed families (Campbell 1985; Bell et al. 2005), both of which can influence offspring viability and their eventual fecundity (Price and Waser 1979; Antonovics and Ellstrand 1984; Schmitt and Gamble 1990).

Plants growing under extreme conditions, for example at high elevations, may be especially likely to exhibit facilitation rather than competition in their use of water, light, and nutrients (Choler et al. 2001; Callaway et al. 2002; Kikvidze et al. 2005). High elevations might also foster facilitation among plants of the same or different species in the pollination of flowers. Insect flight at high elevations is restricted by periods of bad weather, and pollinator abundances may be low (Schröter 1926; Arroyo et al. 1982; Körner 2003) so that the number of insect visits received will often limit pollination and seed set. Because plant species often grow in discrete patches at high elevations (Kikvidze et al. 2005), larger aggregations of flowers might increase the number of pollinator visits to a patch by producing a larger visual or olfactory attraction signal. Of course, the final effect of pollinator attraction by co-flowering plants on components of their fitness also might be neutral or negative rather than facilitative, as described above.

Here we report on a manipulation of plant associations at high elevations in the Swiss Alps, where plants often grow as mats or cushions. We chose species that often grow in association and whose floral displays stand out against the surrounding rocks: *Eritrichium nanum*, with

bright-blue flowers, and *Saxifraga exarata* ssp. *exarata* and *S. bryoides*, with white or yellowish flowers (Zoller et al. 2002). We created three contrasting floral neighborhood treatments that allowed us to investigate interactions within and between species mediated through pollinators. We assessed these interactions in terms of seed set and the genetic quality of the seed produced, i.e., their degree of inbreeding or outbreeding of seed families.

Materials and methods

Study species and sites

The perennial cushion plant *Eritrichium nanum* (L.) Gaud. (Boraginaceae) mainly grows at elevations between 2,500 and 3,000 m a.s.l. in the European Alps (Gams 1975), which corresponds to the sparsely vegetated alpine-subnival zone. During June and July, cushions produce many blue hermaphroditic flowers that offer nectar at the base of short floral tubes. The species is tetraploid (Wirth et al. 2009) and self-compatible (Wirth et al. 2010a). Its fruits carry up to four nutlets, each containing one seed (Wirth et al. 2010b). The subalpine to alpine cushion plants *Saxifraga exarata* ssp. *exarata* Vill. and *S. bryoides* L. (Saxifragaceae) often grow in association with *E. nanum* and flower at the same time, but the white or yellowish flowers of the latter species offer openly accessible nectar (Zoller et al. 2002), and their fruits contain many seeds (Kaplan 1995). The three species share pollinators, which are mostly insects of the order Diptera (Zoller et al. 2002).

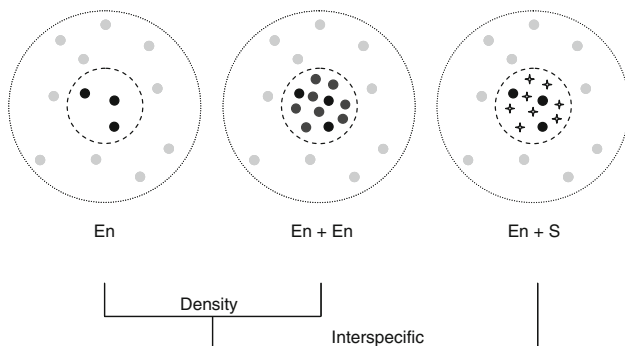
During the summer of 2006, we performed a study in the Upper Engadine and adjacent valleys of southeastern Switzerland. We chose seven sites separated from one another by at least 1 km. At each site we established a varying number of plots, as conditions allowed, in flat stabilized scree areas with naturally sparse vegetation. Overall, there were 60 plots situated at elevations between 2,750 and 3,055 m a.s.l. (Table 1). The plots at any given site were separated from one another by a minimum of 100 m of rocky outcrops, steep slopes, or rugged topography.

Experimental treatments

We established three different floral neighborhood treatments with 20 replicates each (Fig. 1): (1) three *E. nanum* cushions alone (low conspecific density for *Eritrichium*, hereafter En); (2) three *E. nanum* cushions with additional [mean \pm standard error (SE) = 8 ± 1] *E. nanum* cushions (high conspecific density for *Eritrichium*, En + En); (3) three *E. nanum* cushions with *Saxifraga* neighbors (high interspecific density, En + S). All treatments were

Table 1 Location and altitude of the study sites and numbers of plots per site used for experiments to evaluate seed set and genetic characteristics of seeds of *E. nanum* in southeastern Switzerland

Study site	Longitude/latitude (E/N)	Altitudinal range (m a.s.l.)	Numbers of study plots	
			Seed set	Genetics
1	9°43'00"/46°31'42"	2,945–2,995	8	4
2	9°35'18"/46°27'74"	2,760–2,850	7	4
3	9°36'75"/46°25'61"	2,750–2,795	6	4
4	9°43'88"/46°30'00"	2,800–2,910	11	5
5	9°57'00"/46°31'77"	2,895–2,900	4	2
6	9°59'32"/46°28'64"	2,815–3,020	13	6
7	9°46'80"/46°30'42"	2,820–3,055	11	5
Total			60	30

**Fig. 1** Layout of floral neighborhood treatments. The inner dashed circles (radius 0.5 m) contain one of three distinct treatments: low conspecific density for *E. nanum* (*En*), high conspecific density (*En + En*), or high interspecific density for *E. nanum* and *Saxifraga* spp. (*En + S*). Black dots represent three focal *E. nanum* cushions, gray dots additional cushions within the inner circle, light-gray dots cushions within a ring of 1.5-m radius (dotted) around the inner circle, stars *Saxifraga* spp. Square brackets indicate the treatments that will be compared to test interspecific and intraspecific density effects on performance

established within a circle of 0.5-m radius in the center of a study plot, and the experimental treatments were maintained by repeatedly removing flowers from all other plants within this inner circle. We also trimmed flowers from the three focal cushions in each plot until we had produced similar floral displays of diameter 10–15 cm. As pollinators also respond to overall floral density at larger spatial scales (Kunin 1993; Bosch and Waser 1999, 2001), we removed the flowers of all other plant species (including *Saxifraga* sp.) within an additional ring of radius 1.5 m around each central 0.5-m-radius circle and also removed flowers on any surplus *E. nanum* so as to achieve a total of ten *E. nanum* cushions in the 1.5-m-radius ring (Fig. 1).

Seed number

In July and August 2006, we counted all flowers, collected all fruits, and counted all expanded nutlets for the three

focal *E. nanum* cushions in each plot, from which we calculated the mean number of seeds per flower.

Genetic quality of offspring

We used genetic analyses to explore treatment effects on aspects of offspring quality in *E. nanum*. We chose half of all plots in each treatment ($n = 10$ per treatment), since a power analysis indicated that results would not change at $\alpha = 5\%$ when all plots were included. From each plot used, we collected leaves from the three focal cushions, from neighboring cushions in treatment *En + En*, and from all other *E. nanum* cushions in the outer 1.5-m ring and dried the leaves in silica gel. We randomly selected 13–23 nutlets from the focal cushions and excised their embryos. Genomic DNA of embryos and leaves from adult *E. nanum* was extracted with the DNeasy 96 Plant kit (QIAGEN, Hilden, Germany). Each sample was genotyped at six polymorphic, codominant nuclear microsatellite markers with tetrasomic inheritance, as described in Wirth et al. (2009). Matings of focal *E. nanum* cushions were then inferred from paternity analysis (Sork et al. 1999) using a full-exclusion approach to identify fathers (Chase et al. 1996). In a first step, we classified offspring carrying only maternal alleles as selfed. It should be noted that this approach may overestimate selfing insofar as matings between close relatives might produce nutlets also containing only maternal alleles. The remaining outcrossed offspring were then assigned as products of (1) “close” matings, i.e., matings among focal cushions within the inner circle of radius 0.5 m; (2) “intermediate” matings, i.e., between focal cushions and cushions within the ring of radius 1.5 m, and (3) “distant” matings, i.e., between focal cushions and unknown fathers outside the study plot (Fig. 1). For each of these three classes of matings, we then calculated the number of different alleles across all marker loci that were not shared with the maternal plant. This value indicates the degree of outbreeding of offspring (sensu Waser and Williams 2001). For tetrasomic inheritance and six loci, the maximum possible number of

unshared alleles is 12. In addition, we calculated the expected heterozygosities (H_e) and fixation indices (F_{IS}) of outcrossed offspring for each plot using AUTOTET (Thrall and Young 2000). H_e is the expected percentage of loci being in heterozygous state, calculated from observed allele frequencies under the assumption of random mating. F_{IS} measures the proportional deviation of the observed heterozygosity in actual individuals from the expected heterozygosity and reflects deviations from random mating. F_{IS} values significantly larger than zero are indicative of inbreeding.

Statistical analyses

We used general linear models in SPSS ver. 11.0 (SPSS, Chicago, IL) to test for an effect of the three neighborhood treatments (fixed factor) on (1) seed set and (2) selfing rate of focal *E. nanum* cushions, (3) H_e and (4) F_{IS} of outcrossed *E. nanum* offspring, and (5) percentage of outcrossed offspring from close versus intermediate and distant matings. Models included study site as a random effect. In no case was the interaction between treatment and study site significant, so the interaction was dropped

from the models. The treatment effect was further subdivided into planned orthogonal contrasts of (1) interspecific (En + S) versus conspecific (En and En + En) *E. nanum* neighborhoods and (2) low- versus high-density neighborhoods (En vs. En + En). We also used one-way analysis of variance (ANOVA) with planned orthogonal contrasts of (1) close versus intermediate and distant matings and (2) intermediate versus distant matings to examine whether the three mating distances influenced the percentage of unshared alleles. In no analysis did residuals from the models deviate from normality.

Results

Effects of floral neighborhood treatment on seed set

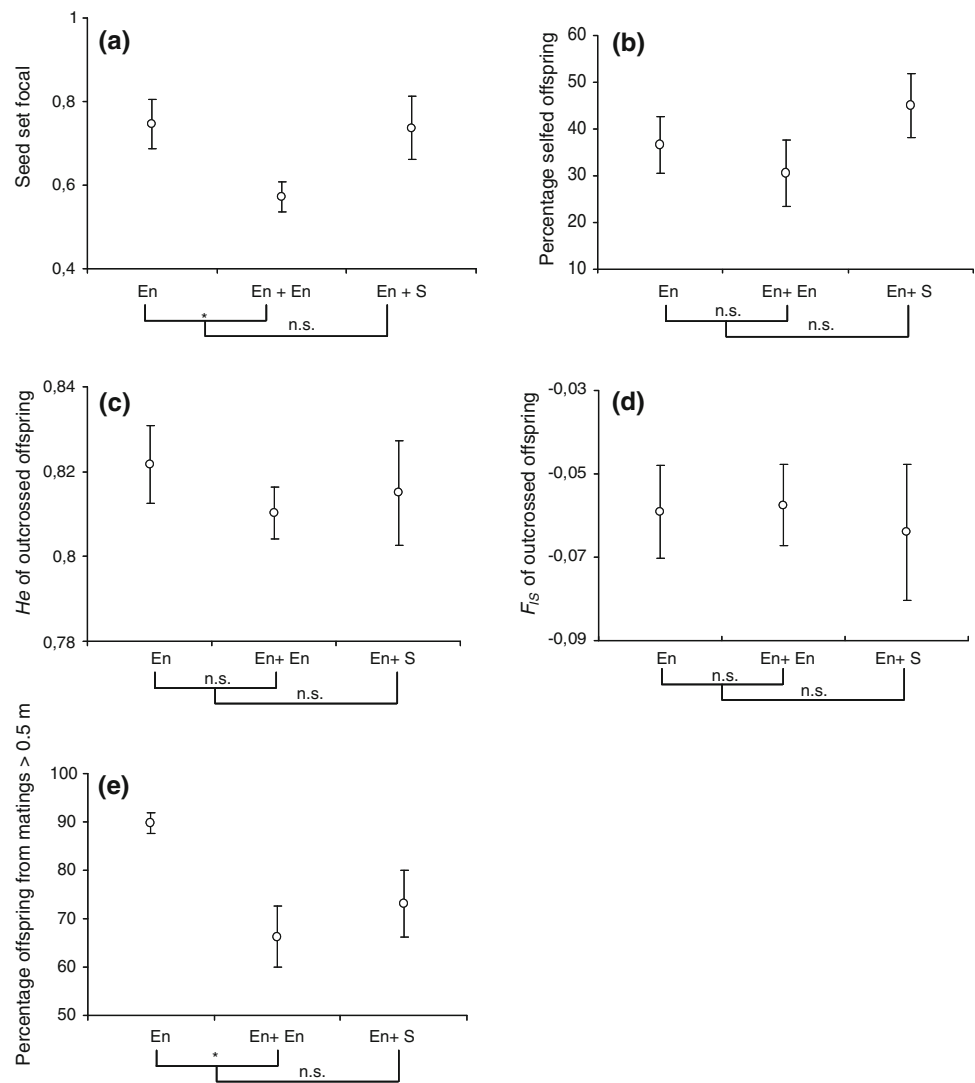
The mean seed set per flower of focal *E. nanum* cushions over all treatments was 0.69. Treatment had a marginally significant overall effect ($P = 0.056$) on the seed set of focal *E. nanum* cushions (Table 2), mainly due to a reduction from an average of 0.75 seeds per flower in treatment En to an average of 0.57 in treatment En + En

Table 2 Results of general linear models and contrasts in testing the effects of treatments and location on seed set, selfing rate, expected heterozygosity (H_e), fixation index (F_{IS}), and percentage of outcrossed offspring from intermediate plus distant matings for focal *E. nanum* cushions

Parameters	Statistics	P
Seed set <i>E. nanum</i>		
Treatment	$F_{2,51} = 3.055$	0.056
Study site	$F_{6,51} = 1.091$	0.380
En, En + En vs. En + S	0.076 (−0.070, 0.223)	0.301
En vs. En + En	−0.189 (−0.358, −0.020)	0.030
Percentage of selfed <i>E. nanum</i> offspring		
Treatment	$F_{2,21} = 1.162$	0.332
Study site	$F_{6,21} = 0.525$	0.525
En, En + En vs. En + S	11.538 (−6.056, 29.132)	0.187
En vs. En + En	−6.729 (−27.524, 14.067)	0.508
H_e of outcrossed <i>E. nanum</i> offspring		
Treatment	$F_{2,21} = 0.053$	0.949
Study site	$F_{6,21} = 1.302$	0.299
En, En + En vs. En + S	−0.002 (−0.044, 0.048)	0.932
En vs. En + En	0.011 (−0.013, 0.038)	0.405
F_{IS} of outcrossed <i>E. nanum</i> offspring		
Treatment	$F_{2,21} = 0.299$	0.745
Study site	$F_{6,21} = 1.487$	0.231
En, En + En vs. En + S	−0.011 (−0.059, 0.082)	0.718
En vs. En + En	−0.002 (−0.034, 0.037)	0.930
Percentage of outcrossed <i>E. nanum</i> offspring from intermediate plus distant matings		
Treatment	$F_{2,21} = 2.718$	0.089
Study site	$F_{6,21} = 1.611$	0.194
En, En + En vs. En + S	−3.487 (−17.177, 10.203)	0.602
En vs. En + En	−17.693 (−33.874, −1.511)	0.034

For contrasts, the contrast value and the lower and upper bounds of the 95% confidence interval (CI) are given (in parenthesis). Treatment: En = (low conspecific density for *Eritrichium* (three *E. nanum* cushions alone); En + En = high conspecific density for *Eritrichium* (three *E. nanum* cushions with additional *E. nanum* cushions); En + S = high interspecific density (three *E. nanum* cushions with *Saxifraga* neighbors)

Fig. 2 Effects of floral neighborhood on seed set of focal *E. nanum* (a), percentage of selfed offspring of focal *E. nanum* (b), heterozygosity (H_e) (c) and fixation index (F_{IS}) (d) of outcrossed *E. nanum* offspring, and percentage of outcrossed offspring from intermediate and distant matings (e, i.e., those >0.5 m). Values are given as means (open circles) \pm standard errors (SE) (whiskers). For the contrasts (square brackets) shown below each x-axis, asterisks indicate means that are significantly different ($P < 0.05$), ns not significant



(Table 2, Fig. 2a). Study site had no effect on seed set (Table 2).

Effects of floral neighborhood on the genetic quality of offspring

A total of 524 adult *E. nanum* cushions and 572 embryos were genotyped and 209 distinct microsatellite alleles detected. Thirty-nine percent of all offspring resulted from self-fertilization, with outcrossing accounting for the remaining 61% of offspring. Across treatments, 33% of the outcrossed offspring were sired by a father within the plots, while 67% were sired by fathers outside the plots. Of the outcrossed offspring sired by fathers within the study plots, 63% were sired by fathers from within the inner circle (close matings at distances <0.5 m) and 37% were sired by fathers from within the 1.5-m ring of each plot (intermediate matings <1.5 m).

Treatment had no detectable effect on the percentage of selfed offspring or on the heterozygosity (H_e) or fixation indices (F_{IS}) of outcrossed *E. nanum* offspring (Table 2, Fig. 2b–d). However, there was an effect on the degree of outbreeding among the outcrossed offspring. Although the percentage of offspring derived from close matings (<0.5 m) versus intermediate plus distant matings (>0.5 m) did not vary significantly across the three floral neighborhood treatments tested together ($P = 0.089$, Table 2), the orthogonal contrast between treatments En and En + En revealed a significant difference, with the latter treatment only producing about 75% as many seed sired by intermediate and distant matings than treatment En (Table 2, Fig. 2e). These matings should confer a higher probability that the offspring carry alleles across loci that are unshared with the maternal parent. Indeed, the percentage of unshared alleles was higher in offspring of intermediate or distant matings than in those from close matings

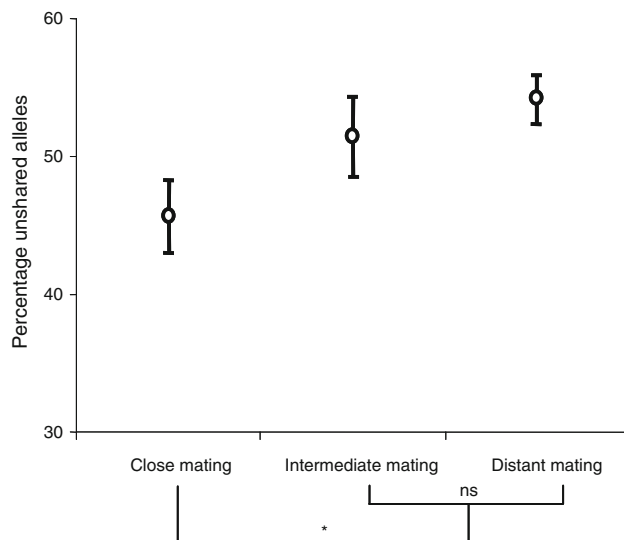


Fig. 3 Mean percentage (\pm SE) of unshared alleles in outcrossed *E. nanum* offspring for three classes of matings described in the text. For the contrasts shown below the *x*-axis, the *asterisk* indicates means that are significantly different ($P < 0.05$); *ns* not significant

($P = 0.016$; Fig. 3). Compared to the higher density En + En treatment, therefore, the En treatment yielded both a higher proportion of offspring from more distant sires and a greater degree of outbreeding in those offspring.

Discussion

Effects of floral neighborhood on seed set

From the experiment described here we conclude that the presence of *Saxifraga* spp. in the immediate floral neighborhood had neither a facilitative nor a competitive effect on *E. nanum* mediated through insect pollination, at least in terms of seed set, a measure of fecundity through the female sex function. We are aware of only two similar experimental studies from other arctic or alpine habitats. Armbruster and McGuire (1991) and McGuire and Armbruster (1991) used small arrays of potted plants to manipulate the floral neighborhoods of two pairs of native species in Alaska and detected no interspecific effects on seed set. The interplant spacing used in these two studies was 0.5 m, which resembles our spatial scale, and their result of neutrality agrees with our result.

In contrast, more numerous experiments performed at lower elevations and latitudes have reported reproductive effects of plant associations that range from interspecific competition to neutrality to facilitation (e.g., Waser 1983; Laverty 1992; Moeller 2004; Ghazoul 2006; Sargent and Ackerly 2008; Mitchell et al. 2009). This greater range of outcomes may derive from a greater variety among studies in terms of properties of the flowers (e.g., of type of

rewards, floral architecture, and morphology of sex parts), of the pollinators (e.g., taxon identity, sensory and cognitive abilities, and flight ranges), and of the climatic and landscape context (which might influence how pollinators move about and their choices of flowers). It also might reflect a greater variety of spatial scales used in the previous experiments. In general, any given effect of plant association—whether competition, neutrality, or facilitation—might be detectable at one spatial scale but not necessarily at a larger or smaller scale (Geber and Moeller 2006).

A recognition of spatial scale certainly also applies to our own results. Although we detected no facilitation of *E. nanum* by neighboring *Saxifraga*, for example, the overall presence of *Saxifraga* in an area might still facilitate *E. nanum* by attracting or supporting additional pollinator species or individuals. The support of additional pollinators might furthermore have a temporal dimension (Waser and Real 1979) in addition to a spatial one. Currently, there are too few published studies to enable any generalized statements on how interactions among plant species that attract some of the same pollinators might depend on spatial scale (and none of the studies that we are aware of have considered temporal scale in the sense of Waser and Real 1979). We thus caution against any premature attempt to estimate the overall relative prevalence of facilitation, neutrality, or competition among plants as mediated through pollination.

In contrast to the lack of detectable interspecific effects, the fecundity of *E. nanum* did respond to conspecific density. Whereas many studies have reported higher seed or fruit set at higher conspecific densities (e.g., Sih and Baltus 1987; Feinsinger et al. 1991; Kunin 1993; Roll et al. 1997; Bosch and Waser 2001; Feldman 2006, 2008; Zorn-Arnold and Howe 2007), and others have detected no effect of density (Caruso 1999; Moeller 2004), we found that seed set of *E. nanum* was higher at lower conspecific density. Working with a biennial gentian, Spigler and Chang (2008) similarly reported a decline in fruit set with increasing conspecific density at spacings of <1 m. Intriguingly, they saw the opposite result with spacings of 1–4 m. These researchers suggested that negative density effects among near neighbors might arise from resource competition. Conceivably, resource competition, rather than intraspecific competition for pollination services, might also explain our result, since our plants grew in situ and could have interacted via shared resources other than pollinators. On the other hand, if this form of resource competition was occurring, it might also have been expected between neighboring *Saxifraga* and *E. nanum*. Conspecific density effects that are unambiguously mediated through pollination certainly are possible, and these have been demonstrated in the past through manipulations of potted plants,

which cannot interact via resource competition (e.g., Bosch and Waser 2001).

Effects of floral neighborhood on the genetic quality of offspring

The genotyping of offspring allowed us to investigate additional influences of floral neighborhood. Once again, the presence of *Saxifraga* had no detectable effect on the proportion of selfed *E. nanum* offspring or the expected heterozygosity and fixation indices of outcrossed nutlets. On the other hand, the number of matings with distant sires as well as degree of outbreeding of outcrossed offspring increased significantly at low compared to high conspecific density. A growing body of literature suggests that viability and fecundity are sensitive to the degree of outbreeding, i.e., the continuum of genetic dissimilarity between parents (Waser and Williams 2001; Leimu et al. 2006). The effects of greater outbreeding on offspring viability are often positive (Teixeira et al. 2009), in which case *E. nanum* may benefit from low conspecific density by this effect as well as by higher seed set. It also must be noted that effects of greater outbreeding beyond some point can be negative (i.e., outbreeding depression; Waser 1993; Waser et al. 2000). The final test of such fitness effects is to grow offspring under similar, preferably natural conditions, but this experimental design is often impractical, as it was here for *E. nanum*, due to low seed germination (<3%, L. Wirth, unpublished). However, it is possible that the higher seed set of *E. nanum* at lower conspecific density is itself an effect, at least in part, of greater outbreeding of the nutlets, which increases their viability or the degree to which they are provisioned by the maternal parent as they are maturing (Waser 1993; Bernasconi et al. 2004).

Possible mechanisms of conspecific density effects in *E. nanum*

No interspecific interaction between *Saxifraga* spp. and *E. nanum* was detected in our high-elevation sites—neither the facilitation that we thought might occur because of a combined floral display nor competition, which also was possible. We recognize that this result applies only to the spatial scale we studied and that it underscores how much there still is to learn about the responses of insect pollinators (in particular, the dipterans that are the main pollinators of *E. nanum* and *Saxifraga*; Zoller et al. 2002) to visual and olfactory floral cues (Raguso 2004; Chittka and Raine 2006). Indeed, the same can be said about how pollinator behavior might have led to the observed effects of lower conspecific density on seed set and on genotypic makeup of seed offspring in *E. nanum*. The higher seed set at low density suggests reduced competition for pollinator

visits at low density. The higher average degree of outbreeding of outcrossed nutlets at low density in turn corresponded to the fact that a greater fraction of these offspring had fathers from outside the plots. The causal interpretation is that kinship declines with physical distance (Heywood 1991; Glaetli et al. 2006; Williams 2007) so that longer distance pollinations yield more outbred seeds. Alpine dipterans do have the potential to fly across the considerable distances that often separate patches of alpine vegetation (Schröter 1926). However, not all flower-to-flower flights will be long, especially once a pollinator has entered a patch (Waser 1982). That a smaller percentage of seeds was fathered from beyond the immediate floral neighborhood at higher densities, either of *E. nanum* alone or mixed with *Saxifraga*, could suggest that more pollinator flights in dense patches were between neighbors, which in turn tend to be related.

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