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Microgametophyte population sizes and plant reproductive output in the insect-pollinated *Prunella grandiflora* (Lamiaceae)

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Summary

- Intraspecific variation in pollen deposition and number of pollen tubes per style is rarely quantified, but is essential for assessing the occurrence of pollen limitation and pollen competition and their evolutionary implications. Moreover, pollen deposition, pollen tube growth, and the fate of fertilized ovules are rarely distinguished in field studies. Here we present such a study in eight natural populations of *Prunella grandiflora*.
- We quantified microgametophyte population sizes and inferred pollen limitation when the number of fertilizable ovules exceeded pollen tubes, and assessed seed set and fate after open pollination.
- Two and three populations had on average significantly fewer pollen grains and pollen tubes per flower, respectively, than the fixed number of fertilizable ovules per fruit, while one population experienced significant pollen competition. Style length was positively correlated with the number of pollen tubes. While pollen availability was very variable, seed abortion was significantly less frequent in denser populations, and in one population the proportion of well-developed seeds was significantly, positively correlated with the number of pollen tubes in the style.
- Less pollen deposition, lower numbers of pollen tubes reaching the base of the style, lower pollen quality and therefore increased abortion of fertilized ovules can all reduce seed set in natural *P. grandiflora* stands. Substantial intraspecific variability implies that microgametophyte competition also occurs in this species. Finally, style morphology may affect pollen receipt.

Key words: fertilization, intraspecific variation, Lamiaceae, microgametophyte competition, pollen limitation, reproduction, seed abortion.

Introduction

As a result of their sessile habit, flowering plants have limited influence on pollen receipt, in terms of both the quantity and the quality (compatibility and relatedness) of pollen deposition. Large and genetically diverse pollen loads can increase offspring number and quality (Schlichting *et al.*, 1987, 1990; Winsor *et al.*, 1987; Karron & Marshall, 1990; Snow, 1990; Young & Young, 1992; Mitchell, 1997; Niesenbaum, 1999; Armbruster *et al.*, 2002; Paschke *et al.*, 2002; Armbruster & Rogers, 2004; Bernasconi *et al.*, 2004; Herrera, 2004; Vergnerie, 2006), while small pollen loads may reduce reproductive output and limit the opportunity for pre- and postzygotic selection. Despite the potentially important role of pollen availability in plant fitness and plant mating systems, only a few studies have investigated pollen availability at the level of individual flowers (e.g. Levin, 1990; Spira *et al.*, 1992; Herrera, 2002, 2004) or genetic diversity of paternal contributions at the level of individual fruits (S. Teixeira & G. Bernasconi, unpublished; Snow & Lewis, 1993; Bernasconi, 2003). However, unpredictability in the patterns of pollen deposition at the level of individual flowers may have important consequences for the evolution of plant reproductive strategies. Provided that there is a large variance among flowers in pollen receipt, the optimal number of ovules per flower is expected to exceed the average pollen load (Burd, 1995).

Pollen deposition may strongly depend on the spatial distribution of populations and the density of individuals within populations. Individuals in denser stands may have greater reproductive success because of the way in which pollinator foraging behavior responds to plant density and abundance (Thomson, 1981; Feinsinger *et al.*, 1991; Kunin, 1993, 1997). Reduced plant density and population size, which are often correlated (Gaston, 1994), can result in lower reproductive success (Aizen & Feinsinger, 1994; Groom, 1998; Aguilar & Galetto, 2004; Wagenius, 2004). This can be the direct consequence of (i) reduced pollinator visitation (Silander, 1978), because of lower reward profitability or lower patch attractiveness, or (ii) increased selfing and crossings between related neighbors (Klinkhamer & De Jong, 1989; Waser & Price, 1991; Kunin, 1993; Bosch & Waser, 1999; Duncan *et al.*, 2004). As a result, pollinator and pollen limitation can reduce not only the number but also the quality of offspring produced (Bertin, 1990; Burd, 1994; Byers, 1995; Brown & Kephart, 1999; Paschke *et al.*, 2002, 2005; Bernasconi, 2003; Fischer *et al.*, 2003; Armbruster & Rogers, 2004; Ashman *et al.*, 2004).

We investigated intraspecific variability in pollen deposition, pollen tube growth in the style, seed set and seed abortion in natural populations of *Prunella grandiflora* (Lamiaceae) which varied in density and size. *Prunella grandiflora*, as is typical of Lamiaceae, has a fixed number of ovules per flower, constraining seed set to a maximum of four nutlets per fruit in both females and hermaphrodites (i.e. females in this species do not produce more ovules per flower than hermaphrodites; Hess *et al.*, 1980). It is therefore ideally suited to the determination of pollinator and pollen limitation because it is possible to compare directly the number of ovules available for fertilization with the number of pollen grains on the stigma and the number of pollen tubes growing through the style of the flower, for both females and hermaphrodites. Moreover, unfertilized ovules, aborted seeds and developed seeds can be distinguished. Unfertilized ovules may result from incompatibility and selection during pollen-tube germination and growth, while aborted seeds may reflect inbreeding depression or maternal selection.

Materials and Methods

Study species and populations

The herbaceous, perennial plant *Prunella grandiflora* (L.) Scholler (Lamiaceae) is common in Europe, and grows on calcareous soil in nutrient-poor and dry grasslands and in sparse pine forests. The species is reported to be gynodioecious (Conert *et al.*, 1979). Male-sterile female flowers have a rudimentary androecium and a smaller corolla than hermaphroditic flowers (Conert *et al.*, 1979; Delph, 1996). Unfertilized ovules, developed seeds and aborted seeds are easily distinguishable (Fig. 1). The inflorescence is a false spike with six flowers each on one to 10 false whorls (Conert *et al.*, 1979). Under glasshouse conditions (16 h light, 22°C:8 h dark, 20°C; 75% humidity), most flowers open during the day. Unpollinated flowers remain open for 4–8 d. *Prunella grandiflora* is pollinated mainly by bumblebees, but also by bees, butterflies and hover flies (Conert *et al.*, 1979; D. J. Lang, pers. obs.).

Fig. 1 Aborted (*n* = 3) and well-developed (*n* = 1) seeds of *P. grandiflora*.

We searched for *P. grandiflora* populations in northern Switzerland based on information given in Fritsche & Kaltz (2000), the ZDSF/CRSF database (Zentrum des Datenverbundes der Schweizerflora/Centre du Réseau Suisse de Floristique; http://www.crsf.ch) and herbarium records (Institute for Systematic Botany, University of Zurich, Zurich, Switzerland). We selected populations that were neither grazed nor mown early in the season. We marked all flowering individuals with flags to estimate population area, which varied between 0.22 and 21.9 ha. To estimate population size, we first divided the occupied area into a 10 × 10 grid (with grid size varying proportionally with the area occupied) and then randomly selected a grid position at which we determined the abundance of *P. grandiflora* within a 0.5 × 0.5 m quadrat. We determined plant abundance in as many quadrats as were needed to cover 0.4% of the total surface of the population. This ensured a constant sampling effort, irrespective of the population area. We counted both vegetative and reproductive plants within quadrats during summer 2003. The populations varied about 100-fold in the estimated total number of vegetative and reproductive individuals and 15-fold in plant density, whereby larger populations also had higher plant densities (Table 1).

Pollen load and number of pollen tubes after natural pollination

In summer 2003, we collected one open flower from each of 30 randomly sampled individual plants in each of the

Table 1 Characteristics of *Prunella grandiflora* study populations, including location, elevation, size and population density estimates

Population	Coordinates (N/E)	Elevation (m a.s.l.)	Population size	No. of vegetative individuals	No. of reproductive (R) individuals	% R	Density (R m^{-2})
Andil^{g}	47°24′05″/7°25′30″	490	977	642	335	34	0.15
Villnachern^{g}	47°28′28″/8°09′37″	405	2500	–	–	–	–
Karismatt^{g}	47°25′38″/7°25′46″	420	3230	1720	1510	46	0.51
Buessberg^{g}	47°28′31″/8°20′10″	652	3500	–	–	–	–
Dättlikon^{g}	47°31′22″/8°38′05″	450	5640	4110	1530	27	0.64
Hesseberg^{pf}	47°29′54″/8°06′05″	490	198488	177132	21356	11	1.06
Nätteberg^{pf}	47°29′41″/8°05′50″	487	355420	312059	43361	12	1.98
Oltme^{g}	47°24′31″/7°25′36″	580	89880	64540	25340	28	2.26

The number and reproductive status of individuals were assessed in a constant proportion (0.4%) of the total surface occupied by the population, in randomly distributed 0.5 m × 0.5 m quadrats.

Habitat types: ^{g}, grassland; ^{pf}, sparse pine forest.

–, no data available.

a.s.l., above sea level.

eight populations (total 240 flowers). To address within- and among-population variation in flower morphology, and the potential correlation of pistil length with pollen capture and pollen tube growth (Aizen *et al.*, 1990), we measured the length of the anther thecae and the length of the pistil to the nearest μm with a measuring table (Completron CX; A. Steinmeyer, Albstadt, Germany).

The flowers were incubated for 2 d in formaldehyde–acetic acid solution (FAA) prepared from Solution A (12 ml formaldehyde and 88 ml 95% ethanol) and Solution B (10 ml glacial acetic acid and 90 ml distilled water) mixed 1 : 1 before use (Johansen, 1940). Thereafter, the flowers were stored in 70% ethanol at room temperature. We counted pollen grains on the stigmata and pollen tubes (microgametophytes) in the style by adapting published protocols (Martin, 1959; Glaettli *et al.*, 2006). Flowers were dissected and the pistil softened (in 4 N NaOH for 2 h at room temperature), rinsed in distilled water, stained (in 1% aniline blue and phosphate buffer, pH 7.8, for 2 h) and rinsed again. We placed each pistil on a microslide (surrounded by Parafilm® M (Pechiney Plastic Packaging, Chicago, IL, USA) as a spacer) with two drops of 1% aniline blue solution and immediately counted pollen grains on the stigma and pollen tubes at the base of the style near the ovary using an Axioskop 2 Mot microscope (Zeiss) equipped with epifluorescence (HBO 50 W burner; excitation filter BP362/150, dichroic mirror FT395, barrier filter LP397; Plan Neofluar/Fluar objective; 10 × 10 magnification). Each set of callose plugs linearly aligned at periodic distances in the stigma–ovary direction was counted as one pollen tube; pollen tubes were counted below the stigma and at the style to ovary junction. Fixation of flowers may have led to loss of some pollen grains on the stigma; however, in our study this does not seem to have been a problem, as the number of pollen grains on the stigma and the number of pollen tubes in the style were positively correlated (see the Results section).

Field estimates of microgametophyte population sizes may depend on floral age and time needed for pollen tube growth inside the pistil. We ensured that the flowers were at the beginning of wilting when collected, by selecting those whose corollas showed signs of phenol oxidation (over all populations, 19.6% of the corolla surface was wrinkled and brown). With floral ageing, the stigmatic lobes open and curl. We recorded curling of the stigmatic lobes in five classes (1, closed lobes, to 5, open, curled lobes). The percentage of the corolla surface that was wrinkled and brown and the degree of curling of the stigmatic lobes were positively correlated in all populations (Spearman's $r_s = 0.17–0.58$), and in six out of eight populations this was significant or marginally significant ($P < 0.05$). Neither value was significantly correlated with stigmatic pollen load or with the number of pollen tubes near the ovary in any of the populations. In a preliminary experiment, we examined *in vivo* pollen tube growth 2, 4, 8, 16 and 24 h after hand-pollination with outcross pollen in 39 flowers in the glasshouse. In 33 (85%) of 39 flowers, the median number of pollen tubes near the ovary exceeded the number of ovules available for fertilization 2 h after pollination. Given that we collected flowers presenting signs of senescence, and that such a short time window is sufficient for pollen tubes to reach the ovules, we assume that, despite some potential variation in floral age, the pollen tube counts at the base of the style in our field-collected flowers probably reflect most of the pollen deposition events that are relevant for fertilization.

Floral insects

To address the potential influence of floral insects (pollen feeders and herbivores) on microgametophyte densities within flowers, we counted and characterized (order, family, genus, developmental stage and functional group) the insects

Table 2 Microgametophyte population sizes (pollen load on the stigma and number of pollen tubes in the style, near the ovary), pollen limitation (percentage of pollen-limited flowers, i.e. flowers having fewer than four pollen tubes near the ovary), and anther and style length in *Prunella grandiflora* populations

Population	Pollen load	t_{pg}	p_{pg}	Pollen tubes	t_{pt}	p_{pt}	% limited	Style length (cm)	Anther length (cm)
Andil	5.48 ± 6.86	0.99	0.336	3.03 ± 3.93	–1.35	0.190	70	1.83 ± 0.27	2.17 ± 0.20
Villnachern	1.88 ± 4.37	–2.47	0.021	0.40 ± 0.68	–29.23	0.001	100	1.84 ± 0.28	2.12 ± 0.20
Karismatt	2.78 ± 3.15	–1.86	0.077	4.77 ± 4.24	0.99	0.330	43	1.82 ± 0.22	1.94 ± 0.22
Buessberg	2.44 ± 2.98	–2.71	0.012	2.03 ± 2.68	–4.01	0.001	80	1.69 ± 0.20	2.07 ± 0.23
Daettlikon	6.32 ± 6.87	1.79	0.085	3.27 ± 3.33	–1.21	0.240	60	1.89 ± 0.23	2.35 ± 0.22
Hesseberg	3.48 ± 3.83	–0.70	0.490	2.90 ± 2.60	–2.31	0.028	57	1.77 ± 0.28	2.05 ± 0.31
Naetteberg	5.52 ± 4.52	1.68	0.106	4.63 ± 4.19	0.83	0.414	47	1.80 ± 0.17	2.14 ± 0.25
Oltme	7.00 ± 5.83	2.67	0.013	4.60 ± 3.40	0.97	0.342	40	1.69 ± 0.20	2.07 ± 0.24

Means are given ± standard deviation.

t_{pg} and t_{pt}, one-sample *t*-test values for the null hypothesis that mean stigmatic loads (pg) and pollen tube numbers (pt), respectively, equal 4; p_{pg} and p_{pt}, two-sided *P*-values. Note that significant departure indicates pollen limitation except for Oltme, where there was a significant excess of pollen grains on the stigma. Populations are ordered by increasing population size (see Table 1).

that were present within all the field-collected flowers (total of 240 flowers).

Reproductive output after open pollination

In all but two populations (Buessberg and Villnachern) between 6 and 23 July 2003 we collected 43–74 open-pollinated infructescences from randomly sampled, separate individuals. In each infructescence we counted fruits and seeds per fruit, distinguishing fully developed seeds, unfertilized ovules, and aborted seeds, and recording scars of already dispersed seeds. The proportions of developed and aborted seeds and of unfertilized ovules were calculated for the entire infructescence, using as the denominator (or total) the number of fertilizable ovules (i.e. number of fruits times 4) minus the number of already dispersed seeds. In the results, these proportions are expressed as the average number of developed, aborted or unfertilized ovules per fruit; that is, for a given individual, the sum of the average numbers of developed seeds/fruit, aborted seeds/fruit and unfertilized ovules/fruit equals 4, the maximum seed set per fruit in *P. grandiflora.* Seed mass was determined for a) the total of aborted and developed seeds; and b) for four well-developed seeds (Mettler MTS balance, Mettler-Toledo, Nänikon, Switzerland). To estimate reproductive success in open-pollinated inflorescences, we used data for six populations. 'Population' was entered as a random factor in univariate analysis of variance (ANOVA) to compare reproductive success and ovule fate among populations.

Results

As *P. grandiflora* has a fixed maximum seed set of four nutlets per flower, pollen availability and limitation at the level of individual flowers can be inferred from counting pollen grains on the stigma and pollen tubes in the style. Our census of pollen deposited on the stigma and of pollen tubes growing inside recipient flowers (i.e. a census of 'microgametophyte population sizes') at the beginning of wilting revealed substantial variation within and among populations in the numbers of pollen grains and pollen tubes (Table 2). The proportion of individual flowers with fewer than four pollen tubes near the ovary ranged from 40 to 100%, and varied significantly among populations (likelihood-ratio χ^2 test: $G = 47.5$, degrees of freedom (d.f.) = 7, $P < 0.001$). We found that three of eight populations were significantly pollen-limited, i.e. had on average significantly fewer than four pollen grains near the ovary, and in two of those populations there were also significantly fewer than four pollen grains on the stigma. By contrast, in one large population (Oltme), pollen loads on the stigma exceeded 4, indicating significant pollen competition (one-sample *t*-tests; Table 2). Accordingly, the number of pollen tubes near the ovary varied significantly among populations (analysis of covariance (ANCOVA) with population as the random factor and style length as covariate: $F_{7,231} = 6.66$, $P < 0.001$). There was also evidence for a role of style morphology in explaining variation in the number of microgametophytes reaching the ovary (ANCOVA, $F_{1,231} = 10.31$, $P = 0.002$), with longer styles having significantly more pollen tubes.

The number of pollen grains on the stigma and the number of pollen tubes at the base of the style were positively correlated (Pearson's correlation: $r = 0.458$, $n = 204$, $P < 0.001$). Neither measure of pollen limitation was significantly correlated with the density of reproductive individuals across populations (Pearson's $r = 0.124$ and 0.123, respectively, $n = 6$, $P > 0.10$; Table 2).

The proportion of developed seeds out of all fertilized ovules (developed and aborted seeds) after open pollination

Table 3 Reproductive output in natural populations of *Prunella grandiflora* after open pollination

Population	Number of fruits	Number of developed seeds	Total seed mass[1]	Developed seed mass[2]	Developed seeds/fruit[3]	Aborted seeds/fruit[3]	Unfertilized seeds/fruit[3]	*N*
Andil	21.4 ± 9.8	32.0 ± 15.9	21.7 ± 14.1	0.66 ± 0.24	1.90 ± 0.73	0.59 ± 0.34	1.51 ± 0.86	71
Karismatt	25.6 ± 7.3	36.7 ± 19.6	28.0 ± 13.9	0.74 ± 0.22	1.51 ± 0.67	0.73 ± 0.38	1.75 ± 0.80	48
Dättlikon	25.2 ± 5.3	28.2 ± 13.1	20.2 ± 9.8	0.72 ± 0.29	1.36 ± 0.68	0.61 ± 0.31	2.02 ± 0.87	44
Hesseberg	21.0 ± 4.5	24.7 ± 14.2	18.7 ± 12.7	0.73 ± 0.26	1.33 ± 0.77	0.52 ± 0.35	2.16 ± 0.95	48
Nätteberg	22.1 ± 4.4	28.6 ± 15.5	20.5 ± 11.9	0.72 ± 0.23	1.36 ± 0.68	0.51 ± 0.34	2.11 ± 0.84	43
Oltme	24.7 ± 5.4	20.2 ± 11.4	14.1 ± 9.0	0.63 ± 0.19	0.94 ± 0.55	0.38 ± 0.29	2.69 ± 0.69	74
Total	23.3 ± 6.87	28.0 ± 15.8	20.1 ± 12.7	0.69 ± 0.24	1.24 ± 0.65	0.49 ± 0.32	1.89 ± 0.91	328

Means are given ± standard deviation. *N*, number of plants. Populations are ordered by increasing population size (see Table 1).

[1] Mass (mg) of developed and aborted seeds. Total seed mass does not include already-dispersed seeds.

[2] Average mass of one well-developed seed (mg) measured on four developed seeds.

[3] Calculated by correcting for already dispersed seeds, i.e. as (X/(available ovules – dispersed seeds) × 4).

Table 4 Insects recorded in flowers of *Prunella grandiflora* (30 flowers in each of eight populations), by developmental stage and functional group

Order	Family or suborder	Stage	Ecology	*n*	n_p
Collembola		Imago	Pollen feeder[1]	1	1
Hemiptera	Homoptera (Aphidina)	Imago	Sap-sucking	15	3
	Heteroptera	Larva	Sap-sucking[1]	1	1
Thysanoptera	Aeolothripidae (Aeolothrips)	Imago	Sap-sucking	1	1
	Aeolothripidae (Melanthrips)	Imago	Sap-sucking	121	6
	Phlaeothripidae (Haplothrips)	Imago	Sap-sucking	1	1
	Thripidae (Frankliniella cf)	Imago	Sap-sucking	133	6
		Larva		5	3
Coleoptera	Nitidulidae (Meligethes)	Imago	Pollen feeder[1]	6	4
	Nitidulidae cf (Meligethes cf)	Larva	Anther feeder[1]	7	3
	Staphylinidae	Imago	Thrips predator[1]	5	1
Diptera	Cecidomyiidae	Imago	Gall former[2]	3	3
Hymenoptera	Chalcioidea	Imago	Parasitoid	1	1
	Formicidae (Formicinae; Lasius cf)	Imago	Omnivorous	5	4
	Formicidae (Myrmecinae)	Imago	Omnivorous	1	1

n, total number of individuals; n_p, number of populations in which each insect group was recorded.

[1] Probable feeding habit; [2] adults reported to feed on pollen.

varied significantly among populations ($F_{5,321} = 4.9$, $P < 0.001$; Table 3). A higher proportions of seeds developed well in plants that grew in denser populations (Pearson's $r = 0.812$, $n = 6$, $P = 0.05$). However, one sparse population (Andil) also had a relatively high proportion of developed seeds. The number of pollen tubes near the ovary, i.e. the severity of pollen limitation, was significantly correlated with the fate of ovules in one population (Hesseberg). After correcting for flower number per plant, the number of pollen tubes near the ovary was also positively correlated with the residuals of the number of developed seeds per fruit ($r = 0.495$, $P = 0.014$). This suggests that high pollen loads increase the probability of obtaining compatible pollen.

Within populations, one-third (71 of 240) of the investigated flowers were visited or inhabited by floral insects (306 insect specimens in total). Sap-sucking thrips were the most frequent and abundant insects (Table 4). Some insects were in their larval stage, namely pollen beetles (*Meligethes* cf. *umbrosus* group) and gall midges (Diptera: Cecidiomyiidae). There was a suggestion that floral insects (i.e. small insects that inhabit flowers) may have acted as pollinators, with a weak, nonsignificant tendency for these insects to be found more often in flowers with more pollen tubes near the ovary: they were found in 26% of flowers (39 of 149) with less than four pollen tubes near the ovary and in 35% of flowers (32 of 91) with four or more pollen tubes near the ovary (Fisher's exact test: $p_1 = 0.091$). This tendency was also observed when considering solely the presence of *Melanthrips* (present in 15% of pollen-limited flowers and 23% of flowers with four or more pollen tubes near the ovary; $P = 0.096$). The pollen-feeding beetle *Meligethes*, which may negatively affect pollination success (Krupnick & Weis, 1999), was rare and mainly restricted to Karismatt. The presence of this pollen-feeder may explain why in this population (apparent) stigmatic pollen loads were on average smaller than the number of pollen tubes near the ovary (Table 2).

Discussion

Plants, particularly in sparse populations, may suffer reduced reproductive success because of pollen limitation (few pollinator visits and/or low-quality pollen). However, plants or flowers receiving abundant and genetically variable pollen may produce offspring that have higher viability and vigour through postpollination selection. We took advantage of the fact that *P. grandiflora* has a fixed maximum seed set of four nutlets per flower to investigate pollen availability in eight natural populations that varied in the number and density of reproductive individuals, by examining individual flowers at the beginning of wilting. Three of the populations had significantly fewer pollen tubes near the ovary than would have been needed to fertilize all ovules. However, pollen availability varied significantly among populations, and one population had stigmatic pollen loads significantly exceeding 4, i.e. experienced pollen competition. At the same time, we observed large within-population variation, suggesting that stochasticity in pollen deposition is important in natural populations of this species (Burd, 1995). Overall, variation in pollen limitation was large among individuals but pollen limitation was not significantly more severe in sparse populations. However, plants in sparse populations suffered reduced reproductive success through an increase in seed abortion among fertilized ovules. Although this may result from variation in pollen quality, in the absence of ecological perturbations, reduced seed set may not be demographically as critical, because perennial species such as *P. grandiflora* may be less sensitive to seed set than to other vital rates (Ashman *et al.*, 2004; Knight *et al.*, 2005).

Prunella grandiflora is gynodioecious, and variation in the proportion of hermaphrodites may be an additional mechanism potentially explaining the observed among-population variation in pollen availability at the level of individual flowers. Indeed, when hermaphrodites are rare, which may happen more frequently in small populations as a result of demographic and genetic stochasticity (Shaffer, 1981; Kery *et al.*, 2003; Paland & Schmid, 2003), pollen limitation and inbreeding depression are expected to be greater (although some of these effects may be compensated by greater gene flow distances; Antonovics & Levin, 1980). In the gynodioecious *Fragaria virginiana*, experimentally increasing the frequency of the pollen-bearing morph decreased the degree of pollen limitation of females (Ashman & Diefenderfer, 2001). Also, females and hermaphrodites may differ in their probability of obtaining pollen (Lewis, 1942). However, a meta-analysis found no significant evidence for sex-differential pollen limitation at the individual flower level in 32 gynodioecious species (Shykoff *et al.*, 2003; see also Lopez-Villavicencio *et al.*, 2003). Nevertheless, it would be interesting to experimentally investigate the joint effects of sex ratios and plant density, as well as habitat quality and resource availability, on pollen distribution patterns in future studies. Finally, self-compatible hermaphrodites may assure reproduction through autogamous pollen deposition when pollinator visitation is low (Kalisz & Vogler, 2003). *Prunella grandiflora* from these populations had low fruit and seed set following autogamous pollen deposition (approximately 1/10 of the fruit set after open pollination; D. J. Lang & G. Bernasconi, unpublished data), suggesting that reproductive assurance may play a limited role in this species.

Whether pollen reaches the stigma and whether it reaches the ovary may depend on floral morphology, in particular style length. For example, Nishihiro *et al.* (2000) reported a positive relationship between stigma height and seed set in short-styled *Primula sieboldii.* Alternatively, a longer style may also lead to greater prezygotic selection (Bernasconi *et al.*, 2004). We found that the number of pollen tubes that reached the ovary increased when the style was long. This may result from a higher probability of receiving pollen in longer styled flowers because of better positioning of receptive surfaces with respect to pollinating insects, or with respect to the anthers for autogamous pollen deposition, or because of increased attractiveness to pollinators – directly, or through correlation with other floral traits. We examined flowers with anthers presenting pollen; however, we did not test whether pollen was sterile and cannot therefore infer whether some of the variation in style length was associated with male sterility. We also examined the role of floral insects and found that they may play an additional but very limited role in ensuring pollination.

A few studies directly investigated pollen tubes growing inside the style or estimated pollen deposition at the level of individual flowers, and also reported substantial variability (Levin, 1990; Plitmann & Levin, 1996; Winsor *et al.*, 2000; Herrera, 2002, 2004; Nemeth & Smith-Huerta, 2003). Like those of these previous studies, our results confirm that variability in pollen availability and deposition can be large, both within and among populations (Burd, 1995; Knight *et al.*, 2005). This suggests that reproduction is limited by pollen availability or quality in some of these natural populations, while others (and individual flowers within populations) experience pollen competition. It would be particularly interesting to investigate experimentally how the number of pollen donors contributing to pollen loads (i.e. genetic diversity within pollen loads and among the resulting progency) contributes to increase offspring diversity and fitness (Bernasconi *et al.*, 2003) and whether this effect is modified by population characteristics such as size and density (Vergnerie, 2006).

Plants that grew in denser populations produced a higher proportion of well-developed seeds and, accordingly, fewer aborted seeds. The only sparse population (Andil) that had a relatively high proportion of developed seeds interestingly also showed high stigmatic pollen loads and was not pollen-limited. At the level of individual plants, the number of pollen tubes near the ovary was significantly correlated with the fate of ovules in Hesseberg. In plants with more available pollen (as tested in one random flower per inflorescence), a higher proportion of ovules developed well in the whole inflorescence, and a smaller proportion remained unfertilized, suggesting that high pollen loads increase the probability of obtaining compatible pollen. Alternatively, some individuals may be genetically superior and more likely to obtain pollen and to produce viable seeds, for instance if fitter individuals also produced higher or otherwise more attractive inflorescences.

In conclusion, comparison of stigmatic loads and the number of pollen tubes within styles with the number of fertilizable ovules in natural populations of *P. grandiflora* revealed that style morphology was associated with the number of pollen tubes, and that these measures of pollen limitation varied greatly within and among populations. Plant density was associated with reproductive output: plants that grew in denser populations had a higher proportion of fertilized ovules giving well-developed (rather than aborted) seeds, and in one population this was correlated with the number of pollen tubes in the style. This indicates that reproduction in this species declines in sparse populations and suggests that this, at least in some cases, depends on deposition of compatible pollen.

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