

# Pollen limitation and cleistogamy in subalpine *Viola praemorsa*

Jessica Forrest and James D. Thomson

**Abstract:** Early-flowering species may be especially susceptible to occasional pollen limitation and, therefore, may benefit from a mixed-mating strategy that provides reproductive assurance. We studied cleistogamous (CL) and chasmogamous (CH) fruit set of spring-flowering *Viola praemorsa* Dougl. ex Lindl. along an elevational gradient in the Rocky Mountains, testing whether pollen limitation or allocation to CL reproduction covaried with timing of flowering onset, within and across sites. Contrary to predictions, we found no pollen limitation of reproduction at any site, and variation among sites in the pattern of allocation to cleistogamy was not related to growing season length. Differences in reproductive strategy between early- and late-flowering plants within sites were attributable to differences in plant size, with relative allocation to cleistogamy increasing with size. This pattern has been found in some other cleistogamous species, and may indicate a cost of large CH floral displays, perhaps associated with geitonogamy or herbivory. We found no experimental evidence for resource reallocation in response to CH reproductive output, although a weak negative relationship between CH and CL fruit set across a larger sample of unmanipulated plants suggests such a trade-off. The significance of cleistogamy may be clarified by studying how pollinator visitation, self-pollination, and herbivore damage vary temporally and with floral display size.

**Key words:** allometry, cleistogamy, plasticity, pollen limitation, Rocky Mountain Biological Laboratory, Violaceae.

**Résumé :** Les espèces à floraison hâtives peuvent être particulièrement sujettes à la limitation pollinique, et conséquemment pourraient bénéficier d'une stratégie mixte de fécondation assurant la reproduction. Les auteurs ont étudié la mise à fruit cléistogame (CL) et chasmogame (CH) du *Viola praemorsa* Dougl. ex Lindl. à floraison printanière, le long d'un gradient altitudinal dans les Montagnes Rocheuses, afin de vérifier si la limitation pollinique ou l'allocation à la reproduction CL covarient avec le moment de la mise à fleur, sur et entre les sites. Contrairement à ce qu'on attendait, les auteurs ne constatent aucune limitation pollinique de la reproduction, quel que soit le site, et la variation entre les sites du patron d'allocation à la cléistogamie ne montre aucune relation avec la durée de la saison de croissance. Ils attribuent les différences dans la stratégie de reproduction entre les plantes à floraison précoce et tardive à une différence dans la grosseur des plantes, l'allocation à la cléistogamie augmentant avec la dimension. On retrouve ce patron chez d'autres espèces cléistogames, qui pourrait indiquer un coût élevé de l'exposition florale CH volumineuse, associée à la géitonogamie ou l'herbivorie. Les auteurs n'ont obtenu aucune preuve expérimentale de la réallocation des ressources en réaction au coût reproductif CH, alors qu'une faible corrélation entre la mise à fruit CH et CL dans un échantillon plus vaste de plantes non-manipulées suggère un tel échange mutuel. On pourrait clarifier la signification de la cléistogamie en étudiant comment les visites des pollinisateurs, l'autofécondation et les dommages par les herbivores varient temporellement et avec la dimension des expositions florales.

**Mots-clés :** allométrie, cléistogamie, plasticité, limitation pollinique, Rocky Mountain Biological Laboratory, Violaceae.

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## Introduction

Spring-flowering plants that depend on insects for pollination may be especially vulnerable to occasional pollen-limitation of reproduction. Cold, unpredictable weather may prevent emergence of pollinators or limit their activity (Schemske et al. 1978; Kudo 2006). Simple flowers that allow autogamous self-pollination or xenogamous pollination by a range of visitors have been suggested as common strategies for early-spring plants to avoid reproductive failure in the event of pollinator shortages (Motten 1986). Because timing of flowering of early-season plants can be

particularly sensitive to variation in the cues that signal the onset of the growing season (Fitter and Fitter 2002; Dunne et al. 2003), while insects may be responding to different cues for emergence, these species may be especially at risk of flowering at a time when their pollinators are not yet active. An unusually warm spring, and correspondingly early flowering, have been implicated in reduced pollinator visitation and seed set in *Corydalis ambigua* Cham et Schlecht. (Kudo et al. 2004).

Violets (*Viola* spp., Violaceae) have zygomorphic flowers that tend to open in early spring throughout their range

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(Kochmer and Handel 1986), and pollinator visitation rates, at least in temperate-zone species, can be low (Darwin 1877; Beattie 1972; Culley 2000; 2002). Many *Viola* species display cleistogamy [“dimorphic cleistogamy”, according to Culley and Klooster’s (2007) classification], a mixed mating system in which plants can produce both open, chasmogamous (CH) flowers and closed, obligately self-pollinating, cleistogamous (CL) flowers within the same growing season (Lord 1981; Schoen and Lloyd 1984). In many violets, as with *Oxalis* spp. (Jasieniuk and Lechowicz 1987; Redbo-Torstensson and Berg 1995), CL flowers are produced later in the season than CH flowers. This seasonal cleistogamy probably provides reproductive assurance: CL flowers provide a fail-safe strategy for plants that face an uncertain pollinator environment (Richards 1997). However, all forms of delayed self-fertilization, whether in CL or CH flowers, provide this benefit, and additional factors are required to explain the maintenance of morphologically distinct CL flowers (Schoen and Lloyd 1984). Production of CL flowers after CH flowers might permit plants to compensate for failed fruit set of CH flowers by producing greater numbers of CL flowers and seed. Correlative evidence suggests that *Viola hirta* L. and *Oxalis acetosella* L. plants may be capable of such resource reallocation (Redbo-Torstensson and Berg 1995; Berg and Redbo-Torstensson 1998); however, it is not clear whether the observed trade-off represents a negative genetic correlation between CL and CH reproductive effort across plants or a plastic response to pollination success.

*Viola praemorsa* Dougl. ex Lindl. is a widespread species in western North America, where it can grow at elevations up to 3500 m a.s.l. (Fabijan et al. 1987). Populations at high elevations experience shorter growing seasons and are likely to be visited by different pollinators; these factors might be expected to drive differences in reproductive success or breeding system (Kudo 2006). Self-fertilization is more likely to be favoured in environments where opportunities for cross-pollination are rare (Lloyd 1992). In *Clarkia xantiana* Gray, for example, a geographic gradient in pollinator abundance is matched by a gradient in autofertility and morphological traits associated with selfing (Moeller 2006). In subalpine *V. praemorsa*, CH seed production might be limited by pollinator availability for early-flowering populations or individuals, whereas production of CL fruit might be constrained at high elevations or in late-flowering individuals by the shorter growing season.

We studied *V. praemorsa* populations at sites along an elevation and phenology gradient near the upper limit of the species’ distribution, over which we expected variation in pollinator identity and abundance. Our main objective in this study was to determine whether pollen limitation of CH seed set covaried with timing of flowering onset, both within and across sites, and to test whether plants in more pollen-limited sites showed greater allocation to CL flowers or fruit (owing either to local adaptation or to a plastic response to CH flower failure). Such a relationship would support a reproductive assurance function of CL flowers. We also tested for plasticity in resource allocation to CL reproduction by manipulating CH pollination success. Finally, we evaluate other potential explanations for variation in reproductive strategies (duration of growing season, allometric relationships).

## Materials and methods

### Study species

*Viola praemorsa* Dougl. ex Lindl. subsp. *linguaeifolia* (Nutt. ex T. & G.) Baker is a perennial herb that occurs in mountainous areas of western North America at elevations of 1300–3500 m a.s.l. (Fabijan et al. 1987) and is common in subalpine meadows around the Rocky Mountain Biological Laboratory (RMBL), Crested Butte, Colorado. *Viola praemorsa* is a part of the taxonomically problematic *Viola nuttallii* Pursh. complex (Fabijan et al. 1987) and has been referred to as *V. nuttallii* in previous work at the RMBL (e.g., Thomson 1980; Turnbull and Culver 1983). The species produces chasmogamous (CH) flowers in early spring, ca. 2 weeks after snowmelt near the RMBL (usually late May – mid-June; D.W. Inouye, unpublished data, 1973–2007). The yellow, zygomorphic CH flowers are borne on ca. 6–13 cm peduncles and have a short (~2 mm) nectar spur. These flowers remain open for approximately 5 d and are visited in our study area by flies [chiefly *Platycheirus*, *Eupeodes* (Syrphidae) and *Systoechus* (Bombyliidae)], solitary bees [species of *Halictus*, *Evyllaenus* (Halictidae), and *Osmia* (Megachilidae)] and bumblebee queens (*Bombus bifarius* Cresson and *Bombus flavifrons* Cresson (Apidae)]. Cleistogamous (CL) flowers can be borne on shorter peduncles close to (or even buried in) the ground, but some plants will also produce CL flowers in the axils of leafy runners. The basal CL flowers mature relatively early in the season, and the capsules dehisce before or at approximately the same time as the CH capsules, typically in July near the RMBL (Turnbull and Culver 1983). Flowers on runners continue to be produced in new leaf axils as the runners extend, so maturation of these fruits can presumably extend for as long as the frost-free season (our study season in 2006 ended on 7 August, before maturation of all fruits was complete). Cleistogamous flowers typically contain 9 ovules (occasionally 10) and CH flowers typically contain 12 (occasionally as many as 16). Seeds of the two flower types are indistinguishable: both CL and CH seeds weigh approximately 3.2 mg and bear elaiosomes for ant dispersal (Turnbull and Culver 1983).

### Elevational gradient

We selected seven sites in the vicinity of the RMBL where *V. praemorsa* was abundant (>0.1 flowering individuals/m<sup>2</sup>) and established study plots measuring approximately 30 m × 30 m. The seven sites ranged in elevation from 2930 to 3510 m (Table 1), and our study plants began to bloom between 22 May at the lowest site and 27 June at the highest site. We used this gradient to test (i) whether pollen limitation and allocation to CL reproduction varied spatially, and (ii) whether that variation was associated with variation in timing of flowering onset. The date on which our study was initiated at each site (when most study plants had at least a first open flower) was used as an index of the date of flowering onset at that site, although certain individual plants might have flowered before that date. Sites with late flowering onset presumably experience an earlier end to the growing season, as well as a later start; however, we did not collect data on the exact length of the growing season at each site.

**Table 1.** Characteristics of the study sites.

Site	Elevation (m)	Aspect	Latitude (N)	Longitude (W)	Flowering date*
1	3010	SW	38°57'42"	106°59'04"	22 May
2	2970	—	38°57'49"	106°59'24"	27 May
3	2930	NE	38°58'39"	107°00'16"	2 June
4	3170	E	38°58'57"	107°00'50"	6 June
5	3260	S	38°58'38"	107°01'28"	15 June
6	3260	S	39°01'02"	107°03'13"	21 June
7	3510	SW	39°02'29"	107°02'54"	27 June
Pollinator exclusion experiment	2960	SW	38°58'50"	107°00'01"	29 May

\*"Flowering date" refers to the date on which study plants began to bloom.

At each site, we marked individual flowering plants with pin flags and aluminium tags nailed into the ground. In four of the study sites, we selected 40 plants per site. In the remaining three sites, we tagged 74 to 80 plants, which were divided into 2 "cohorts", composed of 40 "early-" and 34 to 40 "late-" flowering plants; the first flowers on plants in the early cohort opened at least a week before those on plants in the late cohort. This allowed us to test for differences in pollen limitation and other reproductive variables between early- and late-flowering individuals within a population, and to determine whether any relationships with flowering time observed across sites were duplicated within sites.

To test for pollen limitation of seed set, we hand-pollinated half of the marked plants in each site and cohort, and compared seed set (see below) between these pollen-supplemented plants (P) and the unmanipulated, control (C) plants (all plants were open to insect visitation). Plants were chosen in pairs that appeared similar in overall size and number of open flowers; each member of the pair was randomly assigned a treatment (P or C). For hand-pollinations, we used forceps to remove fresh, dry pollen from undehisced or recently dehisced anthers of newly opened flowers from outside the study plot and to apply it to stigmas of experimental plants. We visited each site every 2–3 d, allowing us to pollinate most flowers twice; however, a few flowers wilted before they could be pollinated a second time. [In many taxa, floral longevity is reduced by pollination (van Doorn 1997), suggesting that these wilted flowers may have already been successfully pollinated]. We counted the number of leaves on each plant as an index of plant size.

### Pollinator exclusion experiment

On 26 May, at an eighth study site, we began an experiment to test whether *V. praemorsa* can display a plastic response to variation in CH pollination rates by reallocating resources to or from CL reproduction. To establish variation in CH reproduction, we selected 38 plants with CH buds, covered each with a bag of 1 mm mesh to exclude pollinators, and assigned half to a hand-pollination treatment. Unusually cold weather during the flowering period caused most of the flowers to remain closed on one of the days on which they should have been pollinated, so the majority of flowers in the P treatment were pollinated only once (technique as above). Flowers on control plants were unmanipulated. Bags were removed one week after all CH flowers had completely wilted (on 15 June). CL capsules and seeds were counted (see below) as response variables.

### Fruit and seed set

Sites were revisited 2–3 times as seed capsules began to mature. Most capsules were collected before they dehisced, allowing us to obtain an exact count of seeds. Some capsules were not completely mature when collected, so any developing ovule approximately equal in length to a mature (brown) seed was counted as a seed. However, some capsules dehisced before we could collect them, and many additional capsules were partially or entirely eaten. In these cases, we were able to estimate the number of fruits of each type by counting turgid peduncles. Where possible, we collected valves from capsules that had dehisced or been damaged. The number of seed attachment points (funiculi) was a good predictor of the number of matured seeds ( $r^2 = 0.99$ ,  $N = 31$  plants), so we used it to estimate the number of seeds in fruits for which we only had valves. The number of seeds per fruit was estimated by multiplying the mean number of funiculi per valve by the number of valves per capsule (3). The total number of seeds produced by one flower type on one plant was a product of the estimated or observed mean number of seeds per fruit and the estimated or observed number of fruits per plant. Thus, for the 260 plants that set fruit in the elevational gradient sites, values for CH seed set are precise counts for 129 plants, estimates for 97 plants, and lacking entirely for 34 plants. We counted only CL seeds for the pollinator exclusion experiment and only CH seeds from elevation gradient sites.

Data analyses involving the numbers of CL fruit or seeds face a problem because some plants were still producing CL flowers in late summer when our project had to be terminated, and numbers of CL fruit produced by that point would be an underestimate of total CL reproductive output. Consequently, for some analyses, we use the number of "surviving CL flowers" as an alternative to the number of CL fruits actually matured. "Surviving CL flowers" includes any CL flowers that had set fruit by the end of the season along with unwilted CL flowers remaining on plants and any flowers that were accidentally broken off during the season, making the assumption that no additional flowers would be produced. Although this assumption affected many plants that had not finished fruiting by early August [131 plants from the elevation gradient (35% of the total) and 17 (49%) from the pollinator exclusion experiment], the error introduced should be minor because the number of remaining flowers was small relative to the numbers of fruits already matured (mean = 3.4 versus 13.1 for affected plants in the elevation gradient; 4.6 versus 17.9 for those in the pollinator exclusion experiment).

### Data analysis

To detect pollen limitation at each site, we tested for a difference between pollen-supplemented and control treatments in the proportion of plants setting CH fruit (using contingency tables), the mean number of seeds per CH capsule for each plant (using *t* tests), and the total number of CH seeds per plant (using Kruskal–Wallis tests, because the large number of zero values made parametric tests inappropriate). Because we found no effect of supplemental pollination on reproductive variables, we pooled control and manipulated plants in subsequent analyses.

Because the number of CH flowers per plant took on only a limited number of values (range: 1–9), we treated this as a categorical variable for most analyses. We used  $\chi^2$  tests to evaluate differences among sites in the proportion of CH flowers setting fruit and in the numbers of CH flowers per plant, and used ordinal logistic regression to test for a relationship between plant size (leaf number) and number of CH flowers. For the latter two analyses, plants were categorized as having 1, 2, or  $\geq 3$  flowers to avoid categories with small sample sizes or small expected values. Leaf number, CL flower number, and seed number took on a broader range of values and so were treated as continuous variables and were  $\log_e$ -transformed to meet assumptions of ANOVA and regression.

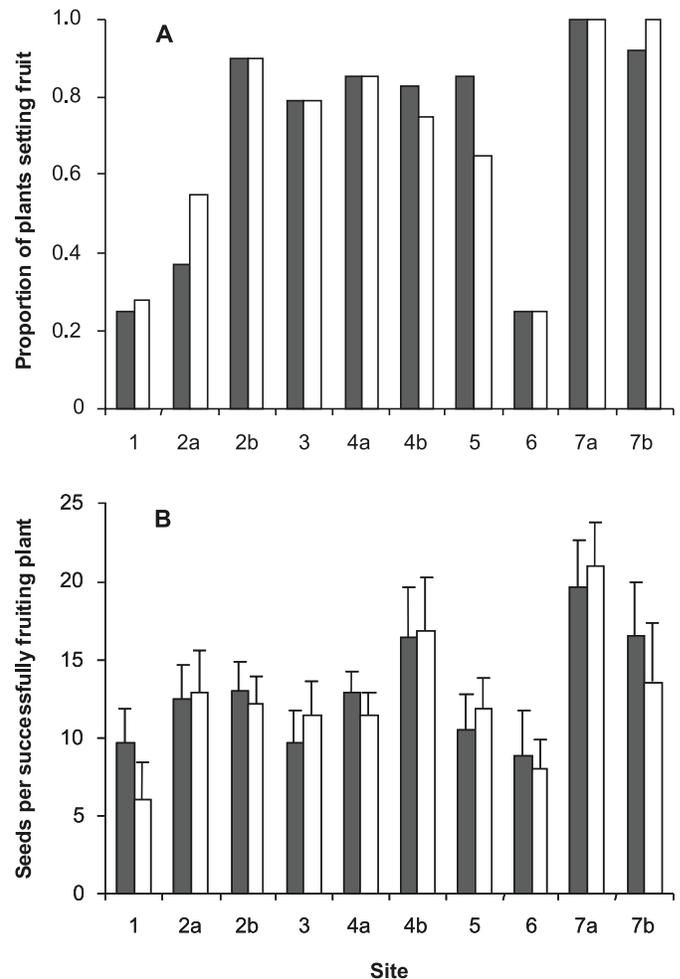
To analyse relative allocation to CL reproduction, we calculated the proportion of all flowers that were cleistogamous, and arcsine-square-root transformed these data prior to analysis. Variation among sites and cohorts in patterns of allocation to CH vs. CL reproduction was tested using mixed-model ANOVAs (with site a random factor and cohort a fixed factor) and linear mixed models [including plant size (number of leaves) as a covariate]. We tested for allometry in reproductive allocation by calculating slopes of  $\log_e$ – $\log_e$  regressions of CL and CH flower number; slopes differing significantly from unity indicate allometry (Niklas 1994) (for this test, number of CH flowers was necessarily treated as a continuous variable). For consistency, we present results based on ordinary least-squares regression, but the same conclusions regarding allometry are obtained using reduced major axis regression.

We tested for a relationship across sites between date of first flowering and reproductive variables, using the mean values of leaf number, CH and CL flower number, and proportion CL flowers across all plants within each site or cohort.

The effect of CH flower or fruit production on CL flower and fruit production was evaluated first using observational data from plants at all sites and, second, using data from the pollinator exclusion experiment. The observational data were analysed using ANCOVA, with number of CH flowers (1, 2, or  $\geq 3$ ) or fruits (0, 1, 2, or  $\geq 3$ ) as predictor variables and plant size (leaf number) as the covariate. For the experimental data, we first tested for a treatment effect on the proportion of CH plants setting fruit using contingency tables (to ensure that the treatment had produced the anticipated difference in CH reproductive output). We then tested for a treatment effect on CL fruit and seed set using ANCOVA, with leaf number as a covariate.

Analyses of covariance were checked for homogeneity of slopes by verifying that the factor  $\times$  covariate interaction was not significant. Models including site number as a pre-

**Fig. 1.** (A) Proportion of plants setting fruit and (B) estimated number of seeds per fruiting plant, for each site and cohort (early, a; late, b). Filled bars represent pollen-supplemented plants and open bars represent unmanipulated plants. There are no significant differences between treatments for any site or cohort (all Fisher's exact tests (A) and Kruskal–Wallis tests (B)  $P > 0.05$ ). Error bars are  $\pm 1$  SE. There are no error bars in (A) because values are proportions (there is no estimate of variance).



dictor were run using PROC MIXED in SAS release version 8.02 (SAS Institute Inc., Cary, N.C.), using restricted maximum likelihood estimation. Significance of fixed effects was tested using the corresponding interaction MS and df for the *F*-ratio denominator. Significance of random effects was tested using likelihood ratios of models with and without the random term. All other analyses were done using JMP IN version 5.1.2 (SAS Institute Inc.).

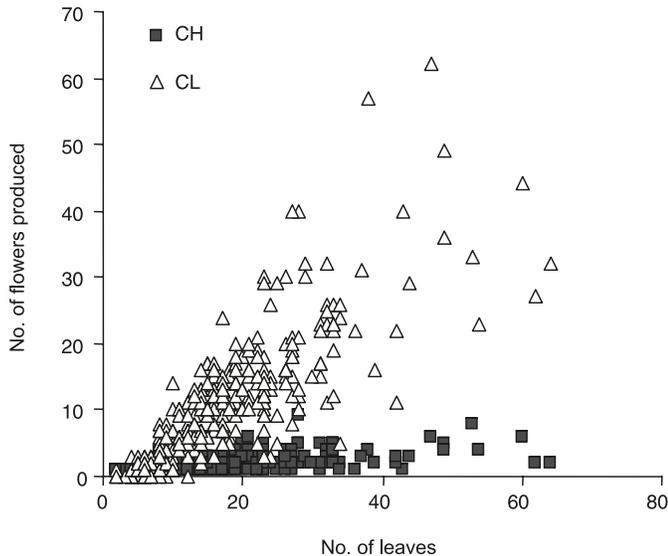
## Results

### Elevational gradient

#### *Fruit set and pollen limitation*

Sites varied significantly in the proportion of CH plants setting fruit (Pearson  $\chi^2 = 87.6$ ,  $df = 6$ ,  $P < 0.0001$ , Fig. 1A) and in the number of CH seeds per capsule (ANOVA,  $F_{[6,219]} = 5.27$ ,  $P < 0.0001$ ). Date of flowering onset for each site, or for each cohort within sites, was posi-

**Fig. 2.** Numbers of cleistogamous (CL; open triangles) and chasmogamous (CH; filled squares) flowers per plant as a function of plant size (number of leaves). No plants with zero CH flowers were included in the study.  $N = 372$  plants.



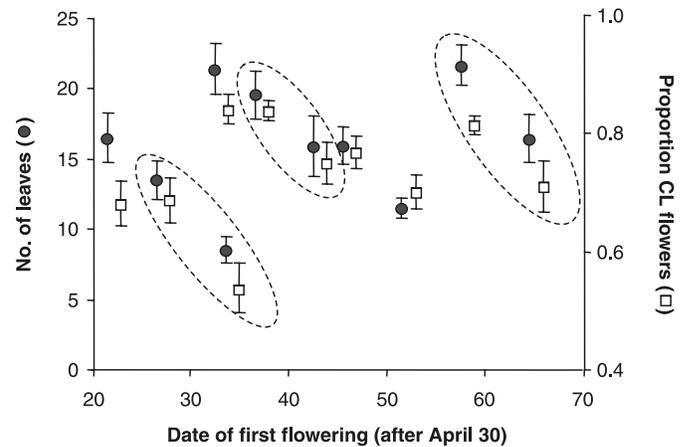
tively, but not significantly, related to variation in CH reproductive success (linear regression using mean values for each site or cohort,  $r^2 = 0.21$ ,  $F_{[1,8]} = 2.15$ ,  $P = 0.18$  for fruit set;  $r^2 = 0.30$ ,  $F_{[1,8]} = 3.36$ ,  $P = 0.10$  for seeds per capsule). We found no effect of pollen supplementation on fruit set at any site, regardless of whether plants bloomed early or late (Fisher's exact tests on proportion of plants setting fruit, all  $P > 0.2$ ). Similarly, there was no positive treatment effect on the number of seeds per CH capsule ( $t$  tests, all  $P > 0.1$ , except at one site where pollinated plants had significantly fewer seeds per capsule,  $P = 0.013$ ) or total CH seeds per plant (Kruskal–Wallis tests, all  $P > 0.2$ ; Fig. 1B). Therefore, CH reproduction was not pollen limited at any site. Considering plants from all sites, the likelihood of setting one or more CH fruit did not increase with increasing plant size (logistic regression, likelihood-ratio  $\chi^2 = 2.17$ ,  $df = 1$ ,  $P = 0.14$ ), although plants that set CH fruit tended to be slightly larger.

### Flower production

Across all sites, numbers of CH and CL flowers increased with increasing plant size [ordinal logistic regression of number of CH flowers on  $\log_e(\text{leaf number})$ , likelihood-ratio  $\chi^2 = 97.2$ ,  $df = 1$ ,  $P < 0.0001$ ; linear regression of  $\log_e(\text{CL flower number} + 1)$  on  $\log_e(\text{leaf number})$ ,  $r^2 = 0.75$ ,  $F_{[1,370]} = 1125.4$ ,  $P < 0.0001$ ; Fig. 2]. However, slopes of log–log regressions of numbers of CL or CH flowers versus number of leaves differ significantly from unity [ $1.25 \pm 0.04$  (mean  $\pm$  SE) for CL flowers;  $0.43 \pm 0.04$ ,  $r^2 = 0.25$  for CH flowers], indicating allometry in reproductive allocation. The proportion of flowers that were cleistogamous was strongly and positively related to plant size (linear regression,  $r^2 = 0.40$ ,  $F_{[1,370]} = 246.5$ ,  $P < 0.0001$ ).

There were significant differences among sites in the number of leaves per plant (ANOVA,  $F_{[6,365]} = 11.7$ ,  $P < 0.0001$ ), number of CH flowers per plant (contingency analysis, Pearson  $\chi^2 = 41.0$ ,  $df = 12$ ,  $P < 0.0001$ ), number of CL flowers per plant (ANOVA,  $F_{[6,380]} = 12.0$ ,  $P < 0.0001$ ), and

**Fig. 3.** Mean number of leaves per plant (filled circles) and mean proportion CL flowers (open squares) for each site or cohort, shown as a function of the date of flowering onset. Symbols are offset for clarity. Error bars are  $\pm 1$  SE. The dashed ovals group together the early and late cohorts for a given site.

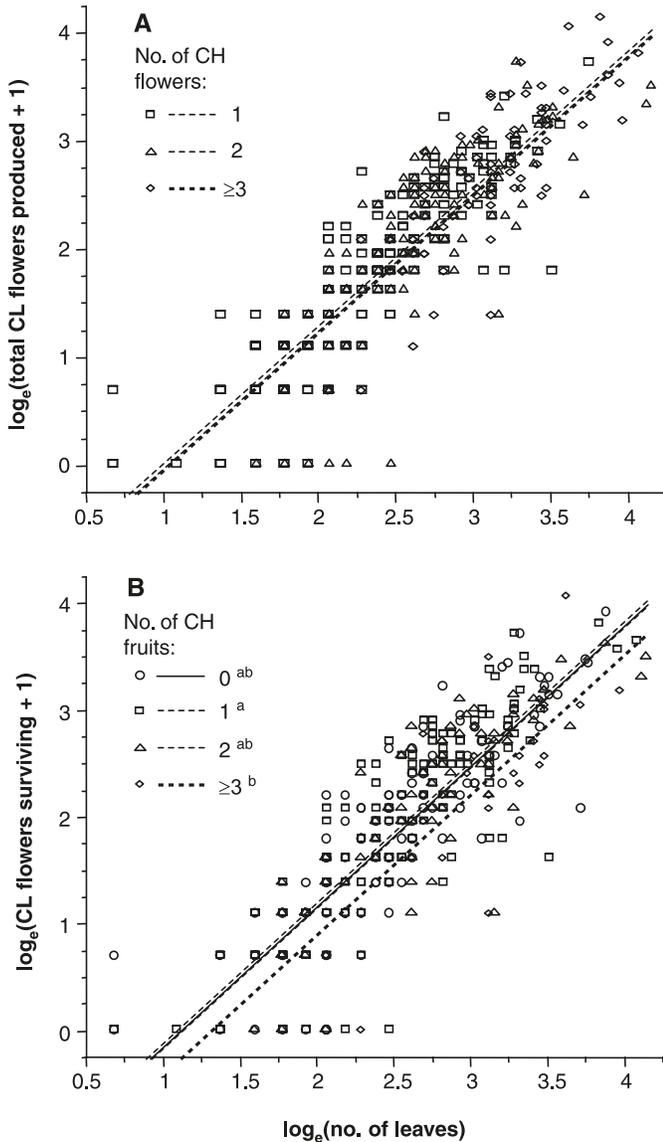


proportion of CL flowers (ANOVA,  $F_{[6,380]} = 7.52$ ,  $P < 0.0001$ ), but none of these variables was related to date of flowering onset at the site (linear regressions using site means, all  $P > 0.5$ ; Fig. 3). Furthermore, site differences in the number and proportion of CL flowers did not remain significant after accounting for variation in plant size (linear mixed models, likelihood-ratio  $\chi^2 < 2.0$ ,  $df = 1$ ,  $P > 0.10$  for both models; Fig. 3); however, this result must be interpreted with caution because sites vary significantly in leaf number, the predictor variable.

Within sites, late-flowering plants were smaller than early-flowering plants (mixed-model ANOVA; cohort  $F_{[1,2]} = 26.0$ ,  $P = 0.04$ ) and tended to have a smaller proportion of CL flowers (mixed-model ANOVA; cohort  $F_{[1,2]} = 16.0$ ,  $P = 0.06$ ; Fig. 3). A model incorporating leaf number showed only a nonsignificant residual tendency for later-flowering plants to have a lower proportion of CL flowers (linear mixed model, cohort  $F_{[1,2]} = 7.21$ ,  $P = 0.12$ ).

Considering plants from all sites, the total number of CL flowers produced by a plant was not related to the number of CH flowers produced, after accounting for plant size (ANCOVA,  $F_{[2,368]} = 0.60$ ,  $P = 0.55$ ; Fig. 4A). Plants producing more CH fruits tended to produce fewer CL flowers (ANCOVA,  $F_{[3,367]} = 2.36$ ,  $P = 0.07$ ), and plants that matured three or more CH fruits had fewer surviving CL flowers (i.e., those potentially producing fruit) for their size than plants maturing fewer CH fruits (ANCOVA,  $F_{[1,366]} = 2.97$ ,  $P = 0.03$ ; Fig. 4B). Specifically, plants that matured three or more CH fruits had, on average, 1.3 fewer surviving CL flowers than plants with fewer CH fruits (from back-transformation of  $y$ -intercepts; Fig. 4B). This effect was not significant at the 0.05 level when the analysis was repeated using the number of CL fruit actually produced by the end of the study (ANCOVA,  $F_{[3,367]} = 2.25$ ,  $P = 0.08$ ), suggesting that the production of additional CH fruits may come primarily at the expense of late-season CL fruit or flowers. Indeed, plants producing three or more CH fruits were significantly less likely than other plants to have CL flowers remaining at the end of our study, after accounting

**Fig. 4.** (A) Numbers of cleistogamous (CL) flowers per plant as a function of plant size for plants producing different numbers of chasmogamous (CH) flowers. Lines for different categories of CH flower production do not have significantly different intercepts (ANCOVA,  $P > 0.05$ ). (B) Potential CL fruit production as a function of plant size for plants maturing different numbers of CH fruits. Lines for different categories of CH fruit production having significantly different intercepts are indicated by different super-script letters (Tukey's HSD,  $P < 0.05$ ).  $N = 371$  plants.

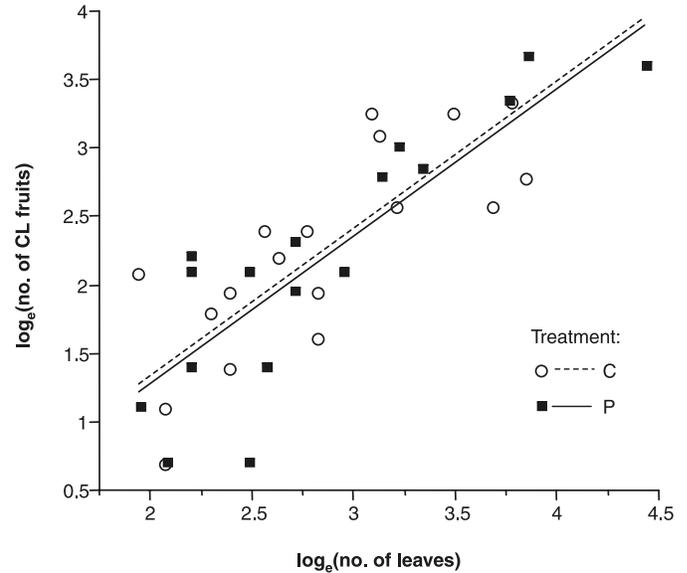


for size (multiple logistic regression, likelihood-ratio  $\chi^2 = 9.15$ ,  $df = 3$ ,  $P = 0.03$ ). Again, however, these results must be interpreted with caution because of the significant relationship between plant size and number of CH fruits (ordinal logistic regression, likelihood-ratio  $\chi^2 = 15.7$ ,  $df = 1$ ,  $P < 0.0001$ ).

#### Pollinator exclusion experiment

Hand-pollination significantly increased CH fruit set in bagged plants (2/19 control plants and 10/17 pollinated plants setting fruit; Fisher's exact test,  $P = 0.004$ ), suggesting that (i) our pollination treatments were effective, and

**Fig. 5.** Numbers of cleistogamous (CL) fruits produced by control (C) and hand-pollinated (P) plants in the pollinator exclusion experiment, as a function of plant size. The two lines do not have significantly different intercepts (ANCOVA,  $P > 0.05$ ).  $N = 35$  plants.



(ii) autogamous pollination of CH flowers is rare in *V. praemorsa* (the two control plants setting fruit produced only one capsule each, out of a total of 10 flowers). Hand-pollination had no effect on CL fruit or seed set (Fig. 5; ANCOVA on number of CL fruit, with leaf number as covariate, treatment  $F_{[1,32]} = 0.128$ ,  $P = 0.72$ ; ANCOVA on estimated CL seeds, with leaf number as covariate, treatment  $F_{[1,32]} = 0.471$ ,  $P = 0.50$ ). Repeating this analysis using the potential number of CL seeds produced (assuming maturation and average seed production for all CL flowers remaining at the end of the season) gave qualitatively identical results.

#### Discussion

Despite considerable variation in the date of flowering onset across sites, we found no variation in pollen limitation of *V. praemorsa*. Chasmogamous fruit set was low at some sites, but pollen supplementation produced no increase in seed production, suggesting that resources or weather conditions were instead limiting reproductive output. Low fruit set at the earliest site (Site 1) and among early plants at the second site (2a) may have been due to early-season frost, something that has been implicated in reproductive failure of other subalpine species and that may be increasing in frequency with climate change (Inouye 2000; Inouye et al. 2002). Site 6, where we also observed low fruit set, may simply be a poor-quality site for this species; plants at this site were relatively small, on average, and produced relatively few CH flowers (although we did not find an association between plant size and probability of setting fruit).

It is possible that a problem with our hand-pollination methods could have resulted in the lack of response to pollen supplementation (Young and Young 1992; Thomson 2001); however, the fact that hand-pollination did increase fruit set in the pollinator exclusion experiment suggests that

our technique was at least moderately effective. Instead, it seems that plants were receiving sufficient insect visitation at all sites, regardless of timing of flowering. The absence of pollen limitation is somewhat surprising, given the low levels of pollinator visitation that have been noted in violets (see Introduction); however, few studies have previously tested for pollen limitation in *Viola* (see Culley 2002 for an exception). We observed 81 insect visits to *V. praemorsa* during 61 h of pollinator surveys at our study sites, of which approximately one-third (by the bombyliid fly *Systoechus*) appeared unlikely to result in pollen transfer. It is unclear whether 2006 was an unusually successful year for the species at the RMBL: long-term data on flowering phenology indicate that 2006 was an early year in terms of flowering onset (D.W. Inouye, unpublished data, 1973–2007), but there are no equivalent data on trends in early-season insect activity, pollination rates, or fruit set.

We found no evidence that CH:CL ratios were related to length of the growing season (or timing of flowering onset). CH flowers in early-flowering sites may have suffered from early-season frost damage, but there is no indication that plants at these sites differ in their relative allocation to CL reproduction as an alternative strategy. Although there was significant variation between and within sites in relative allocation to cleistogamy, much of this variation could be explained by differences in plant size and the allometric pattern of investment in cleistogamy. This study focused on a subset of the elevational distribution of *V. praemorsa* (which can occur as low as 1300 m a.s.l.), and it is possible that a significant gradient in reproductive strategy might be apparent over a broader elevational range. On the other hand, our study encompassed substantial variation in the timing of flowering onset (approximately one month); and a 600 m gradient is certainly sufficient to produce changes in both pollinator communities (e.g., Pyke 1982) and plant reproductive traits (Freeman et al. 2003). We may have failed to detect a correlation between flowering onset and reproductive strategy simply because of our small number of study sites. Alternatively, if pollen-limitation does not vary across the elevational gradient, as we found, there may be less reason to expect variation in reproductive variables.

Given that pollinators were not limiting seed set of CH flowers, at least in our study area in 2006, we lack positive evidence that cleistogamy serves to provide reproductive assurance in the event of pollinator shortages. Because cleistogamy is a phylogenetically widespread condition in *Viola* (Ballard et al. 1998; Culley and Klooster 2007), it would be inappropriate to base broad conclusions about its adaptive significance on one year's study of one species. However, cleistogamy has been lost in several violet lineages, indicating that the trait is evolutionarily labile, and its maintenance in other species requires some explanation (Culley and Klooster 2007). Although we were unable to demonstrate that cleistogamy provides reproductive assurance, we cannot rule out the possibility that CL flowers provide a back-up strategy for *V. praemorsa* in years in which CH fruit set fails, whether as a result of poor weather conditions or pollen limitation.

Whether there is any plasticity in allocation to cleistogamy also remains unclear. Plants in our pollinator exclusion experiment showed no ability to adaptively reallocate resources

to CL structures if CH reproduction failed. On the other hand, data from the >300 plants in the elevational gradient study suggested plants that had successfully matured three or more CH fruits tended to mature fewer CL fruits. This argues for some trade-off between CL and CH reproduction, but perhaps one that is manifested only at high levels of CH fruit set (note that only 2 plants set more than 2 fruits each in our pollinator exclusion experiment). This apparent trade-off was stronger at the fruit stage than at the flower stage (particularly when we included late-season CL flowers in our estimate of CL fruit set), suggesting that (i) any negative genetic correlation between CL and CH flowering was masked by an overriding effect of plant size, and (ii) costs of CH fruit production are expressed in a reduction in the number of CL flowers or fruit produced relatively late in the season. Many perennial alpine plants initiate bud formation in the preceding season (Bliss 1971), so that a plastic response of flower production to early-season pollination rates would be unlikely. In *V. praemorsa*, basal CL buds may be produced before the beginning of the flowering season, but those growing in upper leaf axils are presumably not formed until much later.

A negative association between CH fruit production and CL flower production has been observed in *O. acetosella* and *V. hirta* (Redbo-Torstensson and Berg 1995; Berg and Redbo-Torstensson 1998), although the mechanism behind these apparent trade-offs (whether plastic responses to pollination, as claimed by the authors, or more fixed individual differences in mating system) is unclear. An experimental manipulation of CH fruit set in *V. pubescens* (Culley 2002) found no effect on CL seed set, although the experiment was not designed to test such a trade-off and, like ours, may have generated insufficient variation in CH fruit set to detect an effect. Manipulations provide a much stronger test for plasticity in resource allocation than simple correlations (cf. Reznick 1992); it remains possible that a similar experiment with larger sample size and greater variation in CH fruit set would lend support to the observational results.

Studies of some other cleistogamous species have shown that CH flowers and fruits are more susceptible than CL structures to herbivory (Trapp and Hendrix 1988; Redbo-Torstensson and Berg 1995). We did not collect data on herbivory, but it seems likely that the same pattern obtains in *V. praemorsa*: CL flowers are far less conspicuous than CH flowers, and CL fruits often develop belowground. It has been suggested that the ecological significance of self-fertilization in some plants may be associated more with selection imposed by herbivores than by pollinators, both of which are likely to be more attracted by outcrossing flower types (Eckert et al. 2006). Even in the absence of herbivores, CH flowers are likely to be significantly more costly to produce than CL flowers (Schemske 1978). Our finding of increased allocation to CL reproduction with increasing plant size may reflect diminishing returns on investment in additional CH flowers as plant size increases, both because of an increased likelihood of geitonogamous self-pollination and pollen discounting (Stewart 1994; Harder and Barrett 1995) and, perhaps, herbivore attraction (Gómez 2003; Sandring et al. 2007), with increasing floral display size. Indeed, a model incorporating the inbreeding cost of geitonogamy predicts

the maintenance of a stable mixed mating system including both CL and CH flowers and a relative increase in cleistogamy with increasing total reproductive effort (Masuda et al. 2001). Such a pattern has also been documented in another violet, *V. mirabilis* (Mattila and Salonen 1995), as well as in *Oxalis* spp. (Oxalidaceae) (Jasieniuk and Lechowicz 1987; Berg and Redbo-Torstensson 1998) and the grass *Danthonia spicata* (Clay 1982). In contrast, studies of other cleistogamous genera [*Impatiens* (Balsaminaceae), *Amphicarpea* (Fabaceae), *Emex* (Poaceae), and *Collomia* (Polemoniaceae)] have found the opposite pattern, with increased relative allocation to CH flowers with increasing plant size or resource levels (Waller 1980; Weiss 1980; Wilken 1982; Schnee and Waller 1986; Trapp and Hendrix 1988), and a study of *Calathea micans* (Klotzsch) Körn. (Marantaceae) found no relationship between percent cleistogamy and plant size (Le Corff 1993). In species for which cleistogamy increases with increasing size, the costs of large CH floral displays presumably outweigh the benefits. The opposite may be true for plants that show decreased CL reproduction with increasing size; however, life history and plant architecture likely also play a role, as discussed by Jasieniuk & Lechowicz (1987): species that show the latter pattern are typically annuals that must reach a certain size before production of terminal CH structures is possible.

In summary, we found no link between timing of flowering onset and pollen limitation or mating system in *V. praemorsa*. Between-site differences in plant size and flower number may be related to other unmeasured factors such as edaphic conditions or rates of herbivory. The strong relationship between plant size and investment in cleistogamy suggests that a better understanding of how plant size affects rates of geitonogamy and herbivory, and how these factors vary spatially and temporally, would help to clarify the significance of the variation in reproductive strategy. We did not find strong evidence of plasticity in the reproductive strategy of *V. praemorsa*: Maturation of large numbers of CL fruit is not contingent upon poor CH fruit set; however, production of large numbers of CH capsules may slightly reduce CL reproductive output. Further experiments will be necessary to determine the extent to which this species is capable of plastic resource reallocation, both within and across growing seasons.

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