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REPRODUCTIVE BIOLOGY AND DELAYED SELFING IN *VIOLA PUBESCENS* (VIOLACEAE), AN UNDERSTORY HERB WITH CHASMOGAMOUS AND CLEISTOGAMOUS FLOWERS

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Plant response to variation in the surrounding biotic and abiotic environment often involves mechanisms that promote reproductive assurance. The goal of this study was to determine whether production of chasmogamous (CH) and cleistogamous (CL) flowers as well as delayed CH selfing in the perennial herb *Viola pubescens* enhances reproductive output in a heterogeneous environment. A central Ohio population was monitored for several years to study its reproductive biology and to measure selfing rates in CH flowers. A temporal switch from CH to CL flowers corresponded with a reduction in light availability as the forest canopy formed. Although similar numbers of CH and CL flowers were produced per plant, CH flowers were nearly twice as likely to disperse seeds as CL flowers. Both floral types had similar numbers of seeds with comparable mass, and there was no difference in emergence of CH and CL seedlings. In addition to outcrossing, CH flowers were capable of delayed selfing if left unvisited by insect pollinators. This was consistent with the rate of selfing over a 2-yr period (0.60, 0.07) measured with allozymes. Both CH and CL flowers contributed to reproduction in *V. pubescens* as well as delayed CH selfing, thereby creating a mating system that may be an adaptation to pollinator or resource unpredictability within the flowering season.

Keywords: *Viola pubescens*, chasmogamy, cleistogamy, delayed selfing.

Introduction

Plant reproductive success is often influenced by variation in the surrounding biotic and abiotic environments. Reproduction can be affected by differences during the season in pollinator availability or seed predation (Schoen and Lloyd 1984) as well as changes in light, moisture, temperature, or nutrient availability (Uphof 1938). Plant response to these varying factors often involves mechanisms that increase fitness by optimizing reproductive output over different environments compared to only one environment. For example, delayed selfing allows seed production when pollinators are absent while retaining the ability for outcrossing when pollinators are present. Another mechanism is the production of chasmogamous (CH) and cleistogamous (CL) flowers, a relatively widespread condition occurring in 287 species in 56 angiosperm families (Lord 1981). Showy CH flowers are often assumed to be outcross-pollinated and typically appear when pollinators are present; they are also energetically costly (Schemske 1978; Waller 1979) and are usually produced when resources are highest. In contrast, the less costly CL flowers resemble small buds that are structurally modified for self-pollination. They usually appear when pollinators are absent or under limiting resource conditions. Schoen and Lloyd (1984; see also Lloyd 1984) were the first to establish a model showing that the CH/CL system would be favored in a heterogeneous parental environment if individuals could assess and respond appropriately to the variation.

Schoen and Lloyd (1984) suggested that the parental environment could vary in two ways with different effects on floral production. First, temporal variation in the abiotic or biotic environment could promote the sequential production of CH and CL flowers, a condition found in several species. CH flowers appear first in *Oxalis montana* (Jasieniuk and Lechowicz 1987), *Impatiens capensis* (Waller 1979; but see Simpson et al. 1985), and *Viola* (Evans 1956; Culver and Beattie 1978; Solbrig et al. 1988), while CL flowers appear first in *Ajuga iva* (Ruiz de Clavijo 1997), *Ononis minutissima*, *Ononis parviflora* (Darwin [1877] 1986), and *Ceratocapmos heterocarpa* (Ruiz de Clavijo and Jimenez 1993). Second, the environment can also vary spatially within a population or an individual, leading to the simultaneous production of CH and CL flowers. This is found in *Amphicarpum purshii* (McNamera and Quinn 1977), *Danthonia spicata* (Clay 1982), and *Glycine argyrea* (Brown et al. 1986). In addition, floral production can vary both temporally and spatially in some CH/CL species, such as *Impatiens biflora* and *Impatiens pallida* (Schemske 1978).

An assumption of Schoen and Lloyd's (1984) model is that the environment always changes in a constant, predictable way within the flowering season. In reality, this may not always hold in the biotic environment because pollinator abundance and composition in the early spring can vary from year to year (Tepedino et al. 1999), potentially disrupting the ability of plants to track the changing environment. Schoen and Lloyd (1984) suggest such pollinator unpredictability will favor complete cleistogamy, but an alternate solution is the development of delayed selfing in CH flowers. Delayed selfing has already been examined in non-CH/CL species with respect to polli-

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nator unpredictability (Kalisz et al. 1999) but, to my knowledge, not explicitly within a CH/CL species. By enabling CH flowers to produce seeds after pollinators have had an opportunity to visit, delayed selfing provides reproductive assurance (Lloyd 1992) and is always beneficial, i.e., it is better to produce some seeds than none at all. Although CH flowers are often assumed to be primarily cross-pollinated, CH self-pollination occurs in several CH/CL species through delayed or induced selfing (Schoen and Brown 1991; Bryan 1993; Ruiz de Clavijo 1997; Culley 2000) or other unknown means (Momose and Inoue 1993; Ruiz de Clavijo and Jimenez 1993; Porras and Álvarez 1999).

The effect of a heterogeneous environment on floral production has been studied in several CH/CL species with the conclusion that factors such as light, moisture availability, or plant size can affect the relative number of CH or CL flowers produced (Waller 1980; Bell and Quinn 1987; Jasieniuk and Lechowicz 1987; Le Corff 1993; Berg and Redbo-Torstensson 1998). However, few of these studies subsequently examined CH and CL seed production or offspring performance to verify that both floral types contribute to a plant's overall fitness. When other studies compared the relative performance of CH and CL progeny without consideration of the environment (mainly in *Impatiens*), the two progeny types typically had similar fitness or expressed a slight CH fitness advantage, which is often expected because of the higher cost of CH flowers (Schemske 1978; Waller 1979; Waller 1984; Mitchell-Olds and Waller 1985; Antlfinger 1986; Schmitt and Ehrhardt 1987).

The downy yellow violet *Viola pubescens* (Violaceae) provides an ideal opportunity to study the applicability of the Schoen and Lloyd (1984) model to a natural population. Sequential production of CH and CL flowers has been reported in the species (Baird 1942), delayed CH selfing occurs within the genus (Culley 2000), and seasonal fluctuations in pollinator abundance have been recorded in the temperate deciduous forest where *V. pubescens* is found (Schemske et al. 1978; Motten 1986). The goal of this study was to investigate the reproductive biology of *V. pubescens* by addressing the following questions: (1) What is the CH and CL flowering phenology? (2) What are the relative contributions of CH and CL flowers to seed production? (3) Are there differences in CH and CL performance in early life history traits? (4) Are CH flowers capable of delayed selfing?

Material and Methods

Species and Site

Viola pubescens Aiton is a nonclonal perennial that overwinters as a rhizome and has a life span of at least 5 yr (T. Culley, personal observation). Due to recent taxonomic revisions, *V. pubescens* now includes two former species, *Viola pensylvanica* Michx. and *Viola eriocarpa* Schwein, as varieties (Ballard 1994 and references therein). In the early spring, this stemmed species produces bright yellow CH flowers that are pollinated by bees and other insects (Beattie 1974). CL flowers subsequently appear and resemble small buds produced in the axils of the uppermost leaves. CH capsules are formed on longer peduncles than CL capsules (T. Culley, personal observation), and seeds from both capsule types are ballistically dispersed up to 5.4 m as the capsule splits into three valves that dry and slowly squeeze shut (Culver and Beattie 1978). Both CH and CL seeds have elaisomes that attract ants and facilitate secondary seed dispersal (Culver and Beattie 1978).

Field experiments and observations were carried out during a 4-yr period (1996–1999) at Ohio Wesleyan University's Bohannan Scientific Preserve, a 40.5-ha area in Delaware and Morrow Counties, Ohio (lat. 40°21′N, long. 82°55′W). The preserve, a mature second-growth forest ca. 110–143 yr old, includes an overstory of beech, maple, oak, hickory, elm, ash, and sycamore. *Viola pubescens* is found throughout the preserve, especially in slightly disturbed sites on north-facing slopes.

Flowering Phenology

In April 1996, 40 newly emerged plants were randomly selected with the condition that they were located at least 2 m from one another. The numbers of CH and CL flowers were counted on each individual every 2–7 d during the season. In late fall, 46 additional plants were selected to increase the sample size for the following year. Since four individuals did not survive the following winter, flower number was monitored on only 82 plants during 1997.

The amount of photosynthetically active radiation (PAR) available to the plants was measured during 1997. A LI-1000 datalogger (LI-COR, Lincoln, Nebr.) with two quantum sensors (LI-190SA, LI-COR) was used to measure PAR during a 24-h period on five clear days during tree leafout (April 20, April 26, May 7, May 11, and May 21). The sensors were placed near two plants ca. 8 m apart. Clear days were chosen so that any decrease in radiation would result from leafing out of the forest canopy and not to changes in cloud cover. This method of selecting either completely cloudless or uniformly cloudy days for light measurements is not unusual (Schemske et al. 1978; Motten 1986).

Relative Success of CH and CL Flowers

During the 1997 season, fruit development was monitored on each of the 82 plants used to measure flowering phenology. During the course of the season, each flower and subsequent capsule was observed to determine whether it successfully dispersed seed. Those flowers that did not had either aborted or were damaged by herbivores. Three plants did not produce any flowers during 1997 and were excluded from further analyses.

Plants were grouped into one of three categories, depending on whether they produced both floral types (CH-CL: $n = 61$), only CH flowers (CH-only: $n = 7$), or only CL flowers (CL-only: $n = 11$). Plants in the first category were used to compare within an individual the total number of CH and CL flowers produced as well as percentages of CH and CL flowers that successfully dispersed seeds. Because of nonnormality of the data, nonparametric Wilcoxon signed-rank tests on paired data were used in the analysis (PROC UNIVARIATE; SAS Institute 1989). Plants with only one floral type (CH-only or CL-only) were compared to CH-CL plants using Wilcoxon rank sum tests (PROC NPAR1WAY; SAS Institute 1989) to determine whether they differed in the number of equivalent

flowers (CH or CL) and the percentage of like flowers that successfully dispersed seeds.

Differences in CH and CL Performance

To test for differences in performance of CH and CL progeny, mature seed capsules were collected randomly from at least 40 individuals during 1996 and 1997, and the following traits were measured: seed mass, number of seeds per capsule, and percentage seedling emergence. Some of the 1997 capsules originated from plants used to measure fruit production (see above). Seeds were collected after first covering mature CH and CL capsules with fine-mesh bags to prevent ballistic seed dispersal. The number of seeds was counted in each capsule, and all seeds were weighed individually. A regression was used to determine whether seed mass depended on seed number. A subset of seeds was removed for a selfing-rate study (see below). In November 1997, CH and CL seeds from both years (ca. 100 seeds per floral type and year; 429 seeds total) were planted in 5-cm community pots in groups of 10, with sibling seeds from the same capsule planted together. The pots were placed in a sandbox outside and covered with several layers of shade cloth and leaf litter to simulate natural conditions. Seeding emergence began in March 1998 and was scored over a one-and-a-half-month period until no new seedlings appeared.

A two-way mixed-model ANOVA (PROC GLM; SAS Institute 1989) with a random effect of year (1996 and 1997) and a fixed effect of flower type (CH and CL) was used to test separately for significant differences in the number of seeds per capsule, seed mass, and percentage seedling emergence. Seed mass data were log transformed, and percentage emergence data were arcsine–square root transformed. Because a difference in seed number between CH and CL flowers may result from a difference in ovule number, the number of ovules was counted in 20 CH and 10 available CL flowers. A *t*-test was used to test for a significant difference in ovule number between the two flower types.

Pollination of CH Flowers

A pollination experiment was conducted in 1999 in the field to determine whether CH flowers could autonomously self-pollinate as well as cross-pollinate. When grown inside a pollinator-free greenhouse, unmanipulated CH flowers frequently produced seeds, while emasculated CH flowers failed to form fruit, indicating that autogamy and not apomixis occurs in *V. pubescens* (T. Culley, personal observation).

The pollination experiment consisted of randomly selecting 160 plants that were emerging from under the leaf litter in early spring 1999. Forty plants were haphazardly placed into one of the following four treatments: (1) bagged: plants covered with pollinator-exclusion cages; (2) emasculated: CH buds emasculated by carefully removing the stamens with forceps; (3) hand-pollinated: CH buds emasculated and then hand-pollinated 2–3 d later with pollen from another individual at least 1 m away; and (4) control: plants left unmanipulated. In the first treatment, entire plants were bagged instead of individual flowers because the weight of a mesh bag on a CH flower frequently damaged the peduncle. Plants in all other treatments were left unbagged so they could be visited by pollinators. CH capsule development on emasculated plants indicates pollinator activity, while seed set on bagged plants is evidence of CH autogamy. The hand-pollinated treatment was used to ensure that the emasculation technique did not adversely affect seed set. Although it would be best from a statistical viewpoint for all treatments to be performed on each plant, this was not possible because of the limited number of flowers per plant (usually 2–5).

Flowers were monitored every 2–3 d to determine whether they developed into seed capsules and whether the capsules aborted, were damaged, or successfully dispersed seeds. A drought in late spring lasted ca. 3 wk and caused the abortion of some CH capsules. Remaining capsules were collected following dehiscence. For each capsule, the number of seeds per capsule and mean seed mass were recorded.

One-way ANOVAs were used to compare the effect of treatment on percentage fruit set, percentage successful seed set (i.e., percentage of flowers eventually dispersing seed), number of seeds per capsule, mean seed mass, and total number of seeds per plant. Because percentage seed set, fruit set, and total seed number data did not conform to ANOVA assumptions even after several different transformations, they were rank transformed. A one-way ANOVA performed with ranked data is analogous to a Kruskal-Wallis nonparametric test (Conover and Iman 1981). For each test, least squares means (LSMEANS; SAS Institute 1989) were used to determine significant differences among the four treatments.

Selfing Rates

The rate of self-pollination in CH flowers was measured with allozymes in 1996 and 1997 using seeds collected previously (see above). Enzyme extraction began by first treating the seeds to break dormancy. Seeds were soaked in concentrated sulfuric acid for 30 min, immediately rinsed with cold water, dried for 12 h, and stored in distilled water at 4°C for 3 d. Seeds were ground individually in Eppendorf tubes, using a small amount of sand and approximately one drop of extraction buffer (Morden et al. 1987). Starch-gel electrophoresis was conducted according to the methods of Culley and Wolfe (2001) to resolve the following three allozyme systems: aminopeptidase (AMP; EC 3.4.11.1), isocitrate dehydrogenase (IDH; EC 1.1.1.42), and glucose-6-phosphate isomerase (GPI; EC 5.3.1.9). One locus was resolved for each allozyme system (three loci total).

Selfing-rate estimates were calculated using MLTR (Ritland 1990). Within each year, seeds from different capsules collected from the same plant were grouped together to make up each maternal family. Only maternal families consisting of eight or more progeny were used in the analysis, resulting in a total of 36 families in 1996 and 13 families in 1997 (family number varied because of seed availability). Maternal genotypes were known from a previous electrophoretic survey (T. Culley, unpublished data) or were estimated using the most likely maternal genotype (Ritland 1990). Multilocus (t_m) and single-locus (t_s) outcrossing rates were estimated, and selfing rates were calculated as $(1 - t_m)$. A positive difference between t_m and t_s indicates biparental inbreeding. There were enough maternal families in 1996 to calculate the inbreeding coefficient of maternal parents averaged over loci (F or F_{is}).

Results

Flowering Phenology

CH and CL flowers were produced at different times during the season with no overlap and this was consistent over a 2-yr period (fig. 1). After the plants emerged from under the leaf litter in early April, CH flowers appeared and peaked in frequency within 2-3 wk. Production of CH flowers declined as the surrounding trees leafed out and had ended by the time the canopy was fully developed. At this point, CL flowers began to appear and were continuously produced until plant senescence in late autumn. This distinct switch point between CH and CL floral production was observed in 1996 and 1997 (fig. 1) as well as 1998 and 1999 (data not reported). Individuals also varied in flower production; of the 82 plants that emerged in 1997, 3 remained vegetative, 11 produced only CH flowers, 7 produced only CL flowers, and 61 plants produced both CH and CL flowers. Of the latter, 11 (18%) of individuals had equal numbers of CH and CL flowers, 20 (33%) produced more CH flowers, and 30 (49%) had fewer CH flowers. Overall, a significant difference was not detected between the number of CH and CL flowers produced per plant (Wilcoxon signed-rank test: $W = -3$, $P = 0.545$), but this was largely due to wide variation in both CH and CL flower production across individuals.

Light availability also differed between the times when CH and CL flowers were produced. The maximum amount of PAR was highest during CH flower production (mean for two sensors: 1534 μmol m^{-2} s^{-1}), declining to low levels before CL flowers appeared (102 μmol m^{-2} s^{-1}; fig. 1).

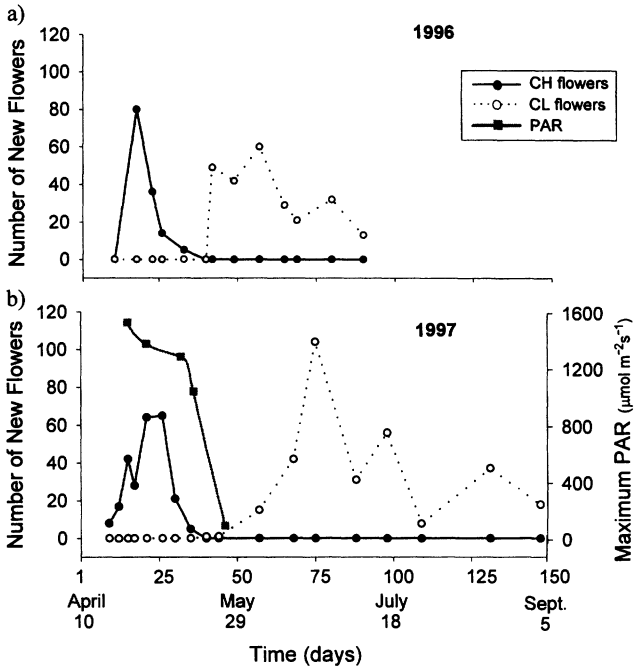

Fig. 1 The flowering phenology of *Viola pubescens* in (*a*) 1996 and (*b*) 1997. Filled circles represent the number of CH flowers and open circles signify the number of CL flowers in the group of marked individuals. Forty plants were observed in 1996, and an additional 46 plants were added for the 1997 season. Squares represent photosynthetically active radiation (PAR) in 1997 (mean daily maximum for two sensors).

Relative Success of CH and CL Flowers

A total of 256 CH flowers and 296 CL flowers was produced by the 82 individuals in 1997. Because most flowers or developing fruits were damaged by herbivores or aborted naturally, only 22% of the CH flowers successfully dispersed seeds, compared with 11% of CL flowers. Of those flowers that successfully set seed, 64% were CH flowers and 36% were CL flowers.

Within the 61 plants that produced both floral types, there was no difference in the number of CH and CL flowers produced (Wilcoxon signed-rank test: $W = -5$, $P = 0.203$), but CH flowers were nearly twice as likely to form capsules and disperse seeds as CL flowers (Wilcoxon signed-rank test: $W = 12$, $P < 0.001$; table 1). Thus at least in 1997, CH flowers were probably responsible for a greater proportion of the seeds produced within the population than CL flowers. Of those plants that only produced one floral type, there was no increase in flower number or seed production as compared with CH-CL plants (table 1). In fact, CH-only plants produced marginally significantly fewer CH flowers than CH-CL plants (Wilcoxon rank-sum test: $S = 280$, $P = 0.053$), with no difference in the percentage of CH flowers that formed seeds (Wilcoxon rank-sum test: $S = 345$, $P = 0.357$). CL-only plants produced significantly fewer CL flowers than CH-CL plants (Wilcoxon rank-sum test: $S = 135.5$, $P = 0.030$), with a lower but nonsignificant difference in the percentage of CL flowers that dispersed seeds (Wilcoxon rank-sum test: $S = 220.5$, $P = 0.645$). In addition, many plants that produced only one flower type in 1997 produced the opposite flower type in other years. Therefore, production of only one floral type or of a given ratio of CH and CL flowers within an individual cannot be considered a permanent condition.

Differences in CH and CL Performance

There were no significant differences between CH and CL floral types in the number of seeds per capsule (ANOVA: df = 1,1, $F = 0.03$, $P = 0.89$), seed mass (ANOVA: df = 1,1, $F = 3.51$, $P = 0.31$), or percentage seedling emergence (ANOVA: df = 1,1, $F = 0.15$, $P = 0.77$). Seed mass did not depend on seed number per capsule in either 1996 ($r^2 = 0.015$, $n = 52$, $P = 0.38$) or 1997 ($r^2 = 0.022$, $n = 46$, $P = 0.32$). A mean of 8–11 seeds formed in each capsule type, despite a large number of ovules per flower that differed between floral types (24.3 in CH and 31.8 in CL flowers; *t*-test: $t = 2.01$, $P = 0.07$). Both CH and CL seeds weighed ca. 3 mg and emergence rates of both seedling types were only 16%–21% (table 2). Although untested, ungerminated seeds may still have been viable since stored seeds retain viability for at least 3 yr (T. Culley, personal observation). There were also no significant differences between years with regard to number of seeds per capsule (ANOVA: df = 1,93, $F < 0.01$, $P = 0.97$), mean seed mass (ANOVA: df = 1,93, $F = 0.40$, $P = 0.53$), or percentage seedling emergence (ANOVA: df = 1,53, $F = 0.45$, $P = 0.50$).

Table 1

Comparison of CH and CL Parameters Averaged on a per Plant Basis (Mean ± SE) for Individuals That Produced Both CH and CL Flowers, Only CH Flowers, or Only CL Flowers (CL-Only Plants)

Average number per plant	CH	CL
CH/CL plants (n = 61):		
Total flowers	3.75 ± 0.30	4.64 ± 0.59
Successful flowers	0.87 ± 0.13 (22.8%)	0.51 ± 0.16 (9.0%)
CH-only plants (n= 11):		
Total flowers	2.45 ± 0.45	...
Successful flowers	0.45 ± 0.21 (14.4%)	...
CL-only plants (n = 7):		
Total flowers	...	1.86 ± 0.34
Successful flowers	...	0.14 ± 0.14 (7.1%)

Note. Data are from the 1997 season. Successful flowers are those that formed capsules that dispersed seeds. The remaining flowers and their developing capsules either aborted or were damaged by herbivores. Numbers within parentheses are the percentage of total flowers that were successful per plant for each group of plants.

Pollination of CH Flowers

Of the 40 plants in each treatment, some plants were removed from the analysis because they did not produce any CH flowers, and one bagged individual was removed because a moth was found in its cage. This left 38 plants in each of the four treatments. All plants produced similar numbers of CH flowers (3–5), and fruit set was in excess of 50% (table 3). The majority of flowers that did not form fruit were produced at the end of the CH flowering season as tree leafout occurred; these flowers all aborted within a few days. Several CH capsules (24.6%) were damaged by deer, snails, or lepidopteran larvae. Other CH capsules (13.3%) aborted following a midseason drought that began in mid-May and lasted several weeks.

Outcrossing occurred in the CH flowers, as indicated by fruit set in emasculated flowers (58%; table 3). Although pollinators were not observed during many hours of fieldwork in 1996–1998, many floral visitors were seen in 1999. These included bee flies (*Bombylius major*, Bombyliidae, Diptera), skipper butterflies (*Erynnis juvenalis*, Hesperiidae, Lepidoptera), bumblebees (Apidae, Hymenoptera), carpenter bees (Anthophoridae, Hymenoptera), and four species of halictid bees (Halictidae, Hymenoptera).

Although outcrossed emasculated flowers frequently formed fruits, they did not result in substantial seed production. Compared with individuals in the control treatment, emasculated plants had significantly lower fruit set, seed set, and fewer seeds per capsule and per plant. Emasculated plants produced seeds of higher mass, but this may reflect a negative correlation between seed number and mean seed mass per capsule (r^2 = −0.237, df = 37, P = 0.002). The emasculation treatment itself was not completely responsible for this decline in reproductive output because plants with emasculated hand-pollinated flowers had similar percentages of successful capsules and numbers of seeds per plant as control plants (table 3). Because hand-pollinated flowers were not capable of self-pollination since they were emasculated, these results cannot be used to measure pollen limitation.

CH flowers were able to self-pollinate in the absence of pollinators, as indicated by fruit set that occurred in bagged plants (59%; table 3). However, self-pollination did not always guarantee complete seed set because when compared with the control, bagged individuals had significantly fewer CH flowers that formed fruits (58.7% vs. 88.3%) with fewer capsules successfully dispersing seeds (5.0% vs. 12.5%). This translated into fewer seeds produced per plant (0.71 vs. 3.29) for bagged individuals as compared with the control.

Although CL flowers were not the focus of this experiment, the performance of CH flowers could subsequently affect that of CL flowers if resources are limiting. However, there were

Table 2

Characteristics of CH and CL Floral Types in the Number of Seeds per Capsule, Mean Seed Mass, and Percentage of Seedling Emergence

	1996		1997	
Character	CH	CL	CH	CL
Number of seeds per caspule	11.30 (43, 0.81)	8.25 (8, 1.91)	8.74 (35, 0.90)	10.91 (11, 1.32)
Individual seed mass (mg)	3.08 (43, 0.08)	2.93 (8, 0.23)	3.40 (35, 0.11)	2.84 (11, 0.21)
Percentage emergence	19.91 (18, 5.30)	15.94 (13, 4.51)	14.54 (14, 6.83)	20.85 (12, 8.99)

Note. Numbers in parentheses are the sample size (number of capsules for seed number, and seed mass data and number of pots for seedling emergence data) and the SE for each mean. Percentage seedling emergence for both years was measured in spring 1998. None of the differences between cross types or years was significant (see text).

Table 3

Performance of CH Flowers per Plant during 1999

Treatment	Cross type	Number of flowers	Fruit set (%)	Successful seed set (%)	Seeds per capsule	Mean seed mass	Seeds per plant
Bagged	Self	4.47^{A} (0.45)	58.66^{A} (6.42)	5.00^{A} (3.06)	11.75^{A} (2.87)	3.00^{A} (0.17)	0.71^{A} (0.52)
Emasculated	Outcross	3.97^{A} (0.31)	58.46^{A} (5.59)	3.78^{A} (1.48)	5.17^{B} (0.70)	3.76^{B} (0.10)	0.82^{A} (0.36)
Hand-pollinated	Outcross	3.89^{A} (0.27)	68.68^{A} (4.76)	10.90^{B} (2.64)	6.50^{B} (0.81)	3.08^{A} (0.12)	2.05^{B} (0.56)
Control	Unknown[a]	3.71^{A} (0.30)	88.33^{B} (2.99)	12.50^{B} (3.08)	8.93^{A} (1.01)	2.97^{A} (0.18)	3.29^{B} (0.86)

Note. $n = 38$. Shown are the number of flowers, percentage fruit set, percentage successful seed set (i.e., flowers that successfully dispersed seeds), number of seeds per capsule, and mean seed mass (averaged per capsule). Numbers in parentheses are the SE of each mean. Superscripts of different capital letters denote significant differences among treatments ($P < 0.05$) using least square means.

[a] Presumed combination of outcross pollination and delayed selfing.

no significant differences in fruit set or seed set of CL flowers among the four treatments (data not shown), even though differences were detected with CH flowers. On unmanipulated control plants, CH and CL flowers had similar levels of fruit set (CH: 88.3%, CL: 80.4%; t-test: $t = 1.48$, df = 67, $P > 0.05$) and seed set (CH: 12.5%, CL: 8.6%; t-test: $t = 0.98$, df = 67, $P > 0.10$), which is not entirely consistent with previous results but could be due to smaller sample sizes. One unexpected result was that CL flowers, which are generally thought to be highly efficient in self-pollination, produced fewer seeds than available ovules (8–11 seeds vs. ca. 32 ovules). Although selective abortion cannot be discounted, it is likely that space limitations within the capsule allowed only the fastest-growing seeds to develop, thereby enhancing offspring performance and perhaps minimizing early acting inbreeding depression.

Selfing Rates

The rate of self-pollination in CH flowers was significantly higher in 1996 ($s = 0.60$) than in 1997 ($s = 0.07$; Z-test: $Z = 7.42$, $P < 0.001$; table 4). The selfing-rate estimate (s) from 1996 was actually greater than the predicted estimate based on the inbreeding coefficient, assuming mating-system equilibrium ($s = 1 - t$, where $t = [1 - F]/[1 + F]$; Jain 1979). This was true if F was obtained from this study ($F = 0.28$; $s = 0.44$) or from a previous, more extensive allozyme investigation of the population ($F = 0.26$; $s = 0.41$; Culley and Wolfe 2001). These inbreeding coefficients suggest that moderately inbred plants exist in the population. Biparental inbreeding was not substantial in either year because the difference between t_m and t_s was close to 0 (table 4).

Discussion

The reproductive biology of *Viola pubescens* is consistent with Schoen and Lloyd's (1984) model for maintenance of CH and CL flowers within a heterogeneous environment. As expected, there was a temporal separation of CH and CL floral production (fig. 1). CH flowers were produced under high light levels in the early spring when pollinators were most active, and CL flowers appeared under low light levels after the overstory trees had leafed out and pollinators had virtually disappeared. Although energetic costs of showy CH flowers are likely to be substantial (Schemske 1978; Waller 1979), they apparently did not affect CH reproduction. There was no difference in CH and CL performance in seed number per capsule, mean seed mass, or seedling emergence rates. However, CH flowers were responsible for a greater proportion of seeds produced in 1997, resulting from the greater success of CH fruits in dispersing seeds. Although this indicates a CH advantage, results are based on a single season in one population, and it is unknown whether CH/CL reproductive success varies in other years or populations.

One requirement for the maintenance of CH and CL flower production in Schoen and Lloyd's (1984) model is a heterogeneous parental environment. In this study, both abiotic and biotic environments were highly variable. First, the light environment changed during the flowering season with light levels declining to 7% of initial levels before tree leaf out. High light availability in the early spring may lead to increased photosynthetic rates, resulting in more resources for production of the costly CH flowers (Schemske 1978; Waller 1979). CL flowers, which lack petals and other attractive features, may subsequently appear under low light levels because they are less costly to produce. However, other abiotic factors such as daylength, nutrient or moisture availability, and light levels the previous year may also be important as these influence floral production in several other understory herbs (Mayers and Lord 1983; Dahlem and Boerner 1987; Gara and Muenchow 1990; Le Corff 1993).

The parental environment was also heterogeneous in terms of pollinator activity. During 1999, pollinators were only observed in early spring visiting CH flowers and never while CH flowers were produced. In elevated light environments during early spring, CH flowers can easily be detected and air temperatures are high enough (>12°C; Motten 1986) for maximum pollinator activity (Schemske et al. 1978). For example, skipper butterflies are more active in sunny locations or during noncloudy days when they can elevate their temperatures by basking (Pivnick and McNeil 1987). Consequently, it is not surprising that flowering peaks in several spring ephemeral species occur before tree leafout during the first period of weather consistently suitable for insect pollinators (Schemske et al. 1978). After the forest canopy forms, temperatures may be too low for suitable insect thermoregulation, and insects may be restricted to sunflecks on the understory floor (Schultz 1998). In this case, self-pollinated CL flowers would be favored in shaded environments because they do not depend on insect pollinators.

Pollinator activity in the early spring was also indicated by seed production in emasculated CH flowers (table 3). Although

Table 4

Selfing-Rate Estimates for 1996 and 1997

Year	Number of families	Number of progeny	s	t_m	t_s	$t_m - t_s$	F
1996	36	493–654	0.60	0.40 (0.09)	0.40 (0.07)	−0.003 (0.02)	0.28 (0.10)
1997	13	124–190	0.07	0.93 (0.05)	0.86 (0.06)	0.07 (0.03)	...

Note. Shown for both years are the multilocus outcrossing rate (t_m), the single-locus outcrossing rate (t_s), and the difference ($t_m - t_s$) indicating biparental inbreeding. The selfing rate (s) was calculated as ($1 - t_m$). Each family consisted of all progeny from a single maternal parent. Family size was sufficiently large enough in 1996 for calculations of the parental inbreeding coefficient (F). Numbers shown in parentheses are SDs based on 500 bootstraps conducted among families. The number of progeny used in the analysis varied because not all progeny were scored for all three loci.

seed set in these flowers was approximately one-third of that in control flowers (ca. 4% vs. 12.5%), the emasculation technique was not directly responsible for this reduction because emasculated flowers that were hand-pollinated produced seeds on levels consistent with control plants (table 3). Rather, emasculation may have indirectly affected seed set because *Viola* pollinators, most of which visit CH flowers for both nectar and pollen (Beattie 1972), may have been able to discriminate against emasculated flowers. Alternatively, low seed set may have resulted from pollen limitation because delayed selfing was not possible. A higher number of ovules than seeds in CH flowers (ca. 24 ovules vs. 8–11 seeds per flower) indicates the potential for pollen limitation in this system. Unfortunately, it could not be tested because an unemasculated/hand-pollinated treatment was not included in the study.

A requirement of Schoen and Lloyd's (1984) model is that plants must be able to assess and respond appropriately to changes in the environment. Although not measured in this study, assessment may involve a seasonal cue, such as a reduction in maximum light availability or a change in temperature or daylength. Uphof (1938) reviewed phenological studies of *Viola* and concluded that changes in light, temperature, nutrient availability, and even herbivory rates can trigger production of either CH or CL flowers. In addition, changes in daylength were responsible for flower production in *Viola odorata* (Mayers and Lord 1983) and *Viola palustris* (Evans 1956). Although the exact response mechanism is unknown in *V. pubescens*, observations of flowering phenology suggest that it involves a change in light availability (fig. 1). Plant size, which influences CH floral production in some CH/CL species (Waller 1979; Diaz and Macnair 1998), was not directly measured in *V. pubescens* but was probably of negligible effect because most individuals were approximately the same size.

The abrupt shift between CH and CL flower production in *V. pubescens* indicates a tight association between the ability of an individual to track and respond to environmental changes. In other CH/CL species, the transition is more gradual and often involves production of intermediate forms (Lord 1981; Ruiz de Clavijo and Jimenez 1993) or an overlap of CH and CL flowering times (Schemske 1978; Waller 1979; Simpson et al. 1985). CH and CL floral production in some species may also alternate more than once during the season (Le Corff 1993; Porras and Álvarez 1999). To my knowledge, only abrupt transitions have been documented in other *Viola* species (Solbrig et al. 1988; Redbo-Torstensson and Berg 1995), *Oxalis montana* (Jasieniuk and Lechowicz 1987) and *Oxalis acetosella* (Redbo-Torstensson and Berg 1995), all of which are perennial. The lack of abrupt transitions in annual species may reflect their difficulty in tracking seasonal changes in an unpredictable environment.

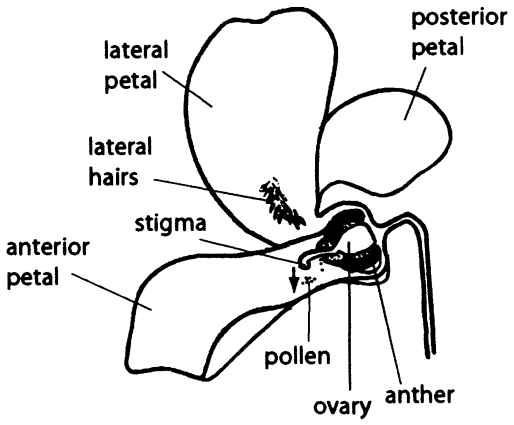

Fig. 2 Diagram of a cutaway view of an older chasmogamous flower of *Viola pubescens* in which delayed selfing is about to occur. The petals have reflexed backward, and the stigma is about to move downward in direction of the arrow to contact pollen that has fallen out of the anthers and is resting on the anterior petal.

Selfing in CH Flowers

One assumption of Schoen and Lloyd's (1984) model is that the environment changes in a constant way within every flowering season, but in reality, annual fluctuations in pollinator abundance in the early spring may lead to selection for complete cleistogamy or delayed selfing in CH flowers. In this study, pollinator variation and delayed selfing were both evident. Insect pollinators were only seen during one out of 4 yr, although they probably went undetected in some years (see below). When pollinators were excluded, delayed selfing occurred in older CH flowers as the stigma slowly bent down and contacted pollen that had fallen out of the anthers and was resting on the anterior petal (fig. 2). This mechanism, which was previously unknown in *V. pubescens*, has been detected in *Viola canadensis* (Culley 2000) but is not widespread in the genus (Knuth 1908; Beattie 1969; Banasinska and Kuta 1996).

Concurrent with these observations of CH self-pollination,

the amount of CH selfing in *V. pubescens* ranged from 0.60 in 1996 to 0.07 in 1997. These selfing rates should be only considered maximum estimates because the analyses included only one population and the minimum number of required loci (Ritland and Jain 1981). The high selfing rate the first year was well within the values reported for other CH/CL species (0.46–0.96, with one exception of 0.03; Clay 1982; Mitchell-Olds and Waller 1985; Brown et al. 1986; Waller and Knight 1989; Cole and Biesboer 1992; Stewart 1994; Lu 2000). In *V. pubescens*, the high selfing rate was probably due to delayed selfing rather than geitonogamy or biparental inbreeding because most plants rarely had two or more compatible flowers open at once, and levels of biparental inbreeding were low. In other CH/CL species, geitonogamy and biparental inbreeding (Waller and Knight 1989; Stewart 1994; Lu 2000) or pollinator-facilitated selfing (Cole and Biesboer 1992) may be more influential in affecting selfing rates. These studies show that CH flowers in some CH/CL species have substantial selfing rates, and it may be inaccurate to broadly assume that CH flowers are primarily cross-pollinated.

The large difference in selfing rates between years may have been caused by two factors. The most likely reason is annual fluctuations in pollinator activity (Kalisz et al. 1999), especially if higher visitation rates in 1997 led to an increase in cross-pollination (i.e., a decrease in delayed selfing). Although pollinators were not observed during 1996 and 1997, visits the second year may have gone undetected, especially since only one visit is necessary for full seed set (Beattie 1971). Pollinator activity may have increased in 1997 because of higher average monthly temperatures and less cloudy skies than in 1996 (T. Culley, unpublished data). Selfing rates may also have differed because of the number of families used in the analyses. Due to seed availability, only 13 maternal families could be analyzed in 1997, compared with 36 families the previous year. Individuals may differ in their rates of selfing due to proximity to potential mates (e.g., on the edge vs. the interior of a population) such that rates in a small sample of individuals may vary by chance alone. However, nine individuals sampled in both years expressed similar selfing rates (0.61 in 1996 and 0.02 in 1997) as in the larger sample.

Although the electrophoretic analysis revealed that self-pollination occurred in *V. pubescens*, selfed flowers on bagged individuals in the pollination experiment had lower seed set than control or hand-pollinated flowers (table 3). This reduction in seed set may reflect the modified environment of the pollinator-exclusion cages, but cage removal when pollinators were inactive was not practical because it would have repeatedly disturbed the plants. In addition, seed set in selfed CH flowers may have been lower than normal because of a drought early in 1999 that caused the abortion of several developing fruits. In a preliminary experiment in 1998 with only 10 bagged individuals, there was 100% fruit set, compared with 59% in 1999.

The delayed selfing mechanism is one advantage *V. pubescens* has over other spring wildflowers. Delayed selfing is especially important because flowering in the early spring is a high-risk option in terms of pollinator availability (Schemske et al. 1978; Motten 1986). *Viola pubescens* also flowers at a similar time as other spring ephemerals (e.g., *Claytonia virginica*) and must compete for many of the same pollinators (T. Culley, personal observation). Delayed selfing may eventually become more important if pollinator abundance declines as habitat fragmentation continues to increase. In contrast, delayed selfing would not be advantageous in a species that has reliable pollinator visitation. This may explain why it is not found in *Impatiens*, which is mid- to late-summer flowering and fairly regularly pollinated (Schemske 1978).

Conclusion

The CH/CL system and delayed selfing in CH flowers are both advantageous in *Viola pubescens* because they optimize reproductive output across different pollinator and resource environments, thereby increasing plant fitness relative to an individual that can only reproduce in one environment. The CH/CL system provides reproductive assurance throughout the entire season as pollinator availability and resources change. CH flowers enable seeds to be produced in the early spring when pollinators are present and resources are highest, while subsequent CL flowers facilitate seed production in the absence of pollinators and abundant resources. *Viola pubescens* has a further advantage over other CH/CL species because its delayed selfing mechanism allows CH seed production early in the season despite infrequent pollinators. These results are consistent with Schoen and Lloyd's (1984) model of environmental heterogeneity. The ability of *V. pubescens* to track and respond to changing abiotic or biotic environments is an example of why the CH/CL system in *Viola* "is a sexual system of great evolutionary versatility" (Beattie 1971, p. 360), and it may be one reason why the species is so common throughout the deciduous forests of midwestern North America.

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