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EXPERIMENTAL ANALYSIS OF PROTOGYNY IN *AQUILEGIA CANADENSIS* (RANUNCULACEAE)[1]

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Dichogamy is very common in flowering plants and is widely thought to reduce pollen-pistil interference, especially self-pollination. Yet, the functional significance of dichogamy has rarely been studied. We investigated the nature and functioning of dichogamy in eastern Ontario populations of *Aquilegia canadensis*, a highly selfing columbine previously described as protogynous. We then manipulated flowers to determine whether increased protogyny would reduce self-fertilization. Contrary to previous reports, A. canadensis is not dichogamous. Controlled pollinations in a greenhouse showed that pollen tubes generally begin to develop after anther dehiscence. Although stigmas can collect pollen early in floral development, naturally pollinated flowers collected from four populations had few pollen grains on stigmas and almost no pollen tubes in styles until after anther dehiscence. Limited pollen deposition before anther dehiscence was also associated with low nectar availability and limited sepal expansion. Because inbreeding depression is strong in this species, selection may favor increased protogyny if it reduces selfing. We tested this hypothesis by comparing the level of selfing in flowers rendered protogynous by the removal of the first 19 (of 39) anthers to develop, with nonprotogynous control flowers. Contrary to expectations, protogyny did not reduce selfing. Our results emphasize the importance of detailed field observations and manipulative experiments in understanding the nature and functional significance of dichogamy.

Key words: *Aquilegia canadensis*; columbine; dichogamy; floral morphology; nectar; plant mating systems; pollen tubes; pollination; protogyny; self-fertilization.

Because plants are sessile, they rely on a variety of biotic and abiotic pollen vectors (animals, wind, and water) to mediate gamete transfer and use an array of physiological and morphological mechanisms to regulate mating. Temporal separation of male and female function within flowers (intrafloral dichogamy) is one of the most widespread morphological mechanisms and is found in >75% of cosexual angiosperm species (Bertin and Newman, 1993). There are two forms of dichogamy: protandry, where anthers dehisce before stigmas become receptive, and protogyny, where receptive stigmas are presented before anther dehiscence (Lloyd and Webb, 1986; Bertin and Newman, 1993). There is wide variation in the degree to which the two sexual phases are separated among species and sometimes within species (Bertin and Newman, 1993).

In general, dichogamy is thought to have evolved to reduce interference between pollen import and export (Wyatt, 1983; Lloyd and Webb, 1986; Bertin and Newman, 1993). A particularly important form of interference is self-pollination. In species that lack physiological self-incompatibility, the contamination of stigmas with self-pollen can lead to self-fertilization, which may reduce fitness if selfed offspring suffer inbreeding depression. Dichogamy is widely expected to limit self-fertilization in self-compatible taxa (Proctor and Yeo, 1972; Faegri and van der Pijl, 1979; Richards, 1986; Cruden, 1988; Barrett and Eckert, 1990). Protogyny, in particular, is viewed as an anti-selfing mechanism because it provides opportunities for the receipt of outcross pollen before self-pollen is shed, and it is more common in self-compatible than self-incompatible taxa (Lloyd and Webb, 1986; Bertin, 1993). However, the effect of either form of dichogamy on the mating system has rarely been investigated. A few correlative studies have detected positive covariation between the level of self-fertilization and degree of dichogamy among populations (Schoen, 1982; Holtsford and Ellstrand, 1992) or between individuals within populations (Brunet and Eckert, 1998). Yet, there has been little functional analysis of dichogamy in natural populations (e.g., Jonsson, Rosquist, and Widén, 1991; Preston, 1991; Dinnétz, 1997) and no experimental manipulation of the degree of dichogamy to determine the impact on self-fertilization.

The extent to which dichogamy reduces self-fertilization depends on (1) the timing of stigma receptivity in relation to anther dehiscence, and (2) the rate at which outcross pollen is deposited on stigmas during female phase and removed from anthers during male phase (Preston, 1991). In protandrous species, autogamous selfing may occur if viable pollen remains in anthers when receptive stigmas are presented. For example, Brunet and Eckert (1998) detected substantial (50%) selfing in strongly protandrous *Aquilegia caerulea* despite an average of 2.9 d separating the dehiscence of the last anther and the beginning of stigma receptivity. Likewise, in protogynous species, autogamous selfing may occur if stigmas are receptive and unfertilized ovules are still available when anthers shed self pollen. Further opportunities for self-fertilization arise in protandrous species if stigmas are presented and can receive self pollen before they become receptive. Consequently, the functioning of dichogamy depends on the rates of pollen de-

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position and removal, which, in turn, depend on pollinator visitation rates and foraging behavior.

In this study, we investigated the functional significance of protogyny in reducing self-fertilization in *Aquilegia canadensis* L. (Ranunculaceae), a spring flowering, short-lived perennial herb native to eastern central North America. This species usually occurs in small populations (<200 flowering plants) located on rocky outcrops and in dry woods (Payson, 1918; Munz, 1946). Individual plants bear one to several large, pendant, red flowers with deep nectar spurs that hold dilute nectar. Although floral morphology and coloration suggest adaptation to hummingbird pollination (Grant and Grant, 1968; Macior, 1978; Faegri and van der Pijl, 1979; Grant and Temeles, 1992), solitary bees and especially bumble bees may also be important pollinators (Schneck, 1901; Macior, 1966).

The flowers of *A. canadensis* were first described as protogynous by Schneck (1901) who observed that the styles ''protrude and are ready to receive pollen from other flowers before the anthers in the same flower are ready to shed pollen'' and that ''close-pollination is avoided by the protogynous condition of the flower.'' The occurrence of protogyny in these large, rewarding flowers suggest that this species practices high levels of outcrossing. However, there are 3 observations that suggest that protogyny is either ineffective at reducing selfing and/or that there is extensive overlap between stigma receptivity and anther dehiscence. First, like other species of *Aquilegia* for which data are available, the flowers of *A. canadensis* are highly autofertile; they set almost a full complement of seeds via automatic self-pollination when isolated from pollinators (Macior, 1978; Eckert and Schaefer, 1998; Routley, Mavraganis, and Eckert, 1999; for other species see Miller, 1978, 1985; Miller and Willard, 1983; Brunet and Eckert, 1998). This suggests that protogyny is, to some extent, incomplete; stigmas are still receptive when self-pollen is shed. Second, Eckert and Schaefer (1998) observed very low levels of pollen deposition before anther dehiscence in one population, suggesting that even if flowers are protogynous, the opportunity for cross-pollination before anther dehiscence provided by protogyny is not realized. Finally, allozyme analysis of progeny arrays sampled from ten natural populations from eastern Ontario, Canada, indicated very high levels of self-fertilization (average = 75%; range = 17–100%; Routley, Mavraganis, and Eckert, 1999). Comparing the levels of selfing in these populations to the inbreeding coefficients of mature plants further suggested that inbreeding depression is very strong. Outcrossed offspring are more than ten times as fit as selfed offspring (Routley, Mavraganis, and Eckert, 1999).

Here, we investigate the nature of protogyny in *A. canadensis* and determine the extent to which it is, or could be, effective at reducing self-fertilization. We begin by describing floral development and quantifying variation in pollen receipt and pollen tube growth across floral development in both the greenhouse and field to assess the extent to which cross-fertilization may precede opportunities for self-fertilization. We then quantify temporal variation in floral attractiveness to see whether there are floral cues associated with the onset of stigma receptivity and anther dehiscence that may affect the timing of cross-pollination relative to self-pollination. Our results suggest that contrary to previous observations, flowers of *A. canadensis* are not protogynous; there is almost complete overlap between stigma receptivity and anther dehiscence, and self- and cross-pollination appear to occur simultaneously.

High levels of self-fertilization combined with very strong inbreeding depression suggest that protogyny would be advantageous in populations of *A. canadensis* if it promoted cross-fertilization. That protogyny does not occur in eastern Ontario populations of *A. canadensis* is particularly puzzling because the genus *Aquilegia* contains closely related species that appear to be strongly protogynous (Chase and Raven, 1975; Miller, 1978) as well as species that are strongly protandrous (Miller, 1978, 1985; Miller and Willard, 1983; Brunet and Eckert, 1998). Moreover, the species in this genus seem to have differentiated rapidly and very recently from each other (Hodges and Arnold, 1994), suggesting that the nature and degree of dichogamy are evolutionarily labile and can be rapidly altered by selection. Because the pattern of floral development in *A. canadensis* enables the creation of artificially protogynous flowers, we can investigate whether protogyny might be an effective mechanism for reducing self-fertilization in *A. canadensis.* We used floral manipulations in conjunction with marker-gene analysis to compare the level of self-fertilization in experimentally protogynous flowers vs. nondichogamous control flowers.

MATERIALS AND METHODS

Flower development—To determine the temporal separation of stigma presentation and anther dehiscence, we monitored flower development in May and June of 1995 in a large population from eastern Ontario, Canada (CCR1; population descriptions and locations in Mavraganis, 1998). We randomly chose 39 plants, each with at least three unopened buds, and excluded them from pollinators with a wire mesh cylinder covered with fine bridal veil. Flowers were either cross- or self-pollinated when anthers began shedding pollen (for details, see Routley, Mavraganis, and Eckert, 1999). Every day throughout flower and fruit development we recorded flower color, the degree to which stigmas were exserted, and the position and dehiscence of anthers. Based on these observations, we divided flower and fruit development into 13 morphologically distinct stages that will be described in detail in the results (Table 1).

Variation in pollen receipt and pollen tube growth across floral development in hand-pollinated flowers—We examined variation in stigma receptivity and pollen tube growth across floral development in a greenhouse experiment involving two populations from eastern Ontario. Before the onset of flowering in early May 1997, 19 and 20 plants were randomly collected from populations CCR1 and QSP1, respectively, and grown in a randomized array in a common greenhouse environment. Each plant was housed in a 7.5-cm pot filled with soilless potting medium (Sunshine Mix™ Number 1) and grown to flowering under ambient light and temperatures of 15°–25°C.

Each plant produced at least four flowers, and one flower on each plant was hand-pollinated at stages 3, 5, 6, or 8 (Table 1). For 3 of 39 plants, a flower was not pollinated at stage 3. Hand-pollinations involved cross pollen from a single freshly dehisced anther taken from another plant in the same population. To prevent contamination of stigmas with self-pollen, undehisced anthers were removed from flowers each day as they moved towards the stigmas on unfurling filaments. For each flower, a stigma from one of the n carpels (n = 3–5, median = 5) was collected before pollination to examine variation in the morphology of the stigma surface across floral development. Unfortunately, most of these samples were accidentally destroyed and only fragmentary information could be obtained (see Discussion). The remaining $n - 1$ carpels were pollinated and collected 24 h later. The pollen grains on the stigmas of one or two of these carpels (median = 2) and the pollen tubes in all $n - 1$ styles were visualized and counted as follows.

Stigmas were squashed in fuschin jelly under a glass coverslip, and the pollen grains on and close to each stigma were counted (Kearns and Inouye, 1993, pp. 115–119). If more than one stigma was assayed, the number of pollen grains per stigma (N_p) was averaged to yield one value per flower. Pollen tubes were visualized using fluorescence microscopy following Kearns

TABLE 1. Flower and fruit development in *Aquilegia canadensis*. Spur color changed as flowers developed from yellow-green (YG) through green (G), green-red (GR), and red (R). Means ± 1 SD for duration were calculated with the individual flower as the unit of observation. Morphological definitions of stages 10–13 are given in the text.

Floral stage	Spur color	Stigmas presented	No. anthers dehisced	Duration of stage (d)	*N* flowers/ *N* plants
1	YG	No	0	1.7 ± 1.0	13/9
2	G	No	0	3.4 ± 1.6	61/27
3	GR	Yes	0	2.5 ± 1.4	105/33
4	R	Yes	0	1.5 ± 0.7	115/34
5	R	Yes	0	1.0 ± 0.5	118/34
6	R	Yes	1–10	0.9 ± 0.6	124/34
7	R	Yes	11–29	1.5 ± 0.8	133/34
8	R	Yes	>30	0.9 ± 0.7	133/34
9	R	Yes	All	1.1 ± 0.8	133/34
10	—	—	—	0.8 ± 0.8	133/34
11	—	—	—	2.7 ± 1.4	133/34
12	—	—	—	4.0 ± 3.0	133/34
13	—	—	—	15.1 ± 4.3	133/34

and Inouye (1993, pp. 124–129). The carpels from each flower were fixed in FAA (formalin-acetic acid-alcohol) for 24 h and then stored in 70% ethanol. Individual carpels were then separated using forceps, softened in 5 mol/L NaOH overnight (from 8 to 24 h), rinsed three times with tap water, and stained in 0.1% (w/v) aniline blue solution decolorized overnight in 0.1 mol/L K_3PO_4. Each carpel was mounted on a microscope slide with 10% glycerol and squashed under a glass cover slip. Pollen tubes were viewed under a Leica DMRB™ stereo microscope at 200× using 350-nm excitation and 420-nm barrier filters.

Each style was visually divided into k 1-mm sections ($k \approx 14$)and the number of pollen tubes in each section (x_i) was recorded ($i = 1 - k$). The mean length of pollen tubes (L) in each style was calculated as:

$$L = \frac{\sum_{i=1}^{k} \left\{ (x_i - x_{i+1}) \left(\frac{d_i + d_{i+1}}{2} \right) \right\}}{x_1},$$

where d_i is the distance from the beginning of the i^{th} segment to the stigma ($d_1 = 0$ mm). We also recorded the maximum number of pollen tubes in any segment of the style (x_{max}, usually $x_{max} = x_1$). These two variables (L and x_{max}) were averaged across carpels (usually three) to yield one value for each variable per flower.

We assessed variation in these measures of pollen receipt (N_p) and pollen tube growth (L and x_{max}) among floral developmental stages and between populations using repeated-measures ANOVA with plant as subject, population as a random, between-subject effect and developmental stage as a fixed, within-subject effect (Neter, Wasserman, and Kutner, 1990, pp. 1057–1066). The F test for population used the MS for plant[population] as denominator. All other F tests used the residual MS as denominator.

Number of pollen grains/stigma (N_p) was square root transformed and L and x_{max} were $\log_{10}$ transformed to meet the assumptions of ANOVA. However, even after transformation the residuals from the analysis of N_p were not entirely independent of the predicted values (Pearson $r = +0.18$, $N = 153$, $P = 0.025$). In this case, analysis of the raw data, transformed data, and even ranked data yielded the same qualitative results, suggesting that the analysis presented below is probably robust to violation of ANOVA assumptions. These analyses and others described below were performed using JMP (version 3.2.1; SAS, 1997).

TABLE 2. Analysis of variation in pollen receipt and pollen tube growth among stages of floral development in hand-pollinated flowers from two populations of *Aquilegia canadensis*. The overall ANOVA model was significant for all variables (all P's < 0.05). The residual df = 108 in all analyses. Means are in Fig. 1.

Source of variation	df	SS	*F*	*P*
a) $\sqrt{\text{Pollen grains/stigma}}$, $r^2 = 0.41$				
Population	1	96.8	8.0	0.0075
Plant [Population]	37	448.6	1.0	0.43
Developmental stage	3	183.0	5.2	0.0021
Population × Stage	3	144.0	4.1	0.0083
b) $\log_{10}$[Pollen tubes/style + 1], $r^2 = 0.62$				
Population	1	1.22	3.3	0.076
Plant [Population]	37	13.55	1.4	0.089
Developmental stage	3	30.68	39.3	<0.0001
Population × Stage	3	0.68	0.9	0.46
c) $\log_{10}$[Mean pollen tube length + 1], $r^2 = 0.53$				
Population	1	0.06	1.4	0.24
Plant [Population]	37	1.72	1.2	0.28
Developmental stage	3	3.05	25.3	<0.0001
Population × Stage	3	0.09	0.8	0.51

Variation in pollen deposition and pollen tube growth across floral development in naturally pollinated flowers—To determine the schedule of pollen deposition and pollen tube growth across floral development in naturally pollinated flowers, we compared stigmatic pollen loads and pollen tube growth across seven stages of floral development (stages 3–9, see Table 1) in four eastern Ontario populations (AHB4, AKA1, CCP3, and CCR4) during May 1995. Ten flowers per stage (one per plant) were haphazardly sampled on a single day in each population. We counted the number of pollen grains on each stigma of each flower (as above) and calculated the average number of pollen grains per stigma for each flower. We assayed pollen tube numbers in all styles of each flower (as above) and recorded the maximum number of pollen tubes in each style as well as the number of tubes at the base of each style.

We assessed variation in pollen grain and pollen tube numbers among developmental stages in each population using one-way ANOVA with Tukey-Kramer multiple contrasts. We assessed overall variation among populations and developmental stages and whether differences between stages varied among populations using two-way mixed-model ANOVA with floral stage as a fixed effect and population as a random effect. F tests for both population and floral stage used the interaction MS as denominator. Because early floral stages had very few pollen grains on stigmas and almost no pollen tubes growing in the styles, the data were not normally distributed, there was strong heteroscedasticity in sample variances, and the data could not be successfully transformed to meet assumptions of ANOVA. Log_{10} transformation best improved normality of residuals and somewhat reduced the correlation between residuals and predicted values. Transformation did not qualitatively change the effect of developmental stage, which was very strong and highly significant for all variables. However, $\log_{10}$ transformation did affect the significance of the interaction between population and developmental stage. Hence, anal-

yses based on $\log_{10}$ transformed data are reported below and should be interpreted with caution.

Variation in nectar availability and flower size across floral development in naturally pollinated flowers—To assess the potential attractiveness of flowers to pollinators in relation to periods of stigma receptivity and anther dehiscence, we measured nectar availability and components of size for flowers in stages 4, 5, 6, 7, and 8 (see Table 1) in two eastern Ontario populations (CCR1 and QBS1) in May 1997. At least ten flowers per stage (one per plant) were haphazardly sampled in each population. Nectar availability was measured as the volume of nectar in the spur tips (standing crop) by inserting a 1×50-mm strip of chromatography paper (Whatman 3 MM) into each of two randomly chosen nectar spurs. The strips were left in the spurs for 1 min to soak up all the nectar. Paper strips were then removed and the length of nectar migration on the chromatography paper was measured to 0.1 mm using Vernier calipers. Migration lengths of the two spurs for each flower were strongly correlated (Pearson $r = +0.83$, $N = 124$, $P < 0.00001$ for QBS1; and $r = +0.91$, $N = 100$, $P < 0.00001$ for CCR1), hence they were averaged to yield one value per flower.

A standard curve of migration length over known volume of artificial nectar with the same sucrose concentration as nectar taken from flowers in natural populations (25% sucrose; see Macior, 1978) was calculated and used to convert the average migration length for each flower into a nectar volume. The standard curve used the migration lengths of ten replicate strips for each of five known volumes (2, 4, 6, 8, and 10 μL). A regression of length (in millimetres) over volume (in microlitres) forced through the origin yielded the following conversion: volume $= 0.262 \times$ length ($r^2 = 0.99$).

We measured three components of flower size to 0.1 mm using vernier calipers: (1) the maximum length of one randomly chosen spur; (2) the length of one randomly chosen sepal; and (3) sepal spread, measured as the distance between the tips of two randomly chosen opposite sepals.

We assessed variation in nectar availability and components of flower size among developmental stages in each population using one-way ANOVA with Tukey-Kramer multiple contrasts. We assessed overall variation in both flower size and per-flower nectar availability among populations and stages and whether differences among stages varied between populations using two-way ANOVA with developmental stage as a fixed effect and population as a random effect. *F* tests for both main effects used the interaction MS as denominator. Because flowers in early developmental stages (4 and 5) produced very little nectar, average nectar volumes were not normally distributed, and there was strong heteroscedasticity in sample variances. Thus, the data were inverse transformed ($Y' = 1/[Y + 1]$) to meet assumptions of ANOVA. Likewise, sepal length was $\log_{10}$ transformed to meet assumptions of ANOVA. The other two analyses used untransformed data.

Experimental manipulation of protogyny—In May and June 1998, we compared the level of self-fertilization in artificially protogynous flowers with non protogynous control flowers in three large populations (>150 flowering plants) in eastern Ontario (QBC1, QCA1, QWM1). We randomly chose plants with one or more flowers in which no anthers had dehisced (<stage 6) and used one flower per plant. A flower on each plant was assigned to one of four treatments. Protogynous flowers (treatment "P") were created by removing the 19 anthers that would normally dehisce first (out of an average total of 39 anthers per flower). Stamens are inserted in a column of whorls around the style and anthers most distal to the pedicel reflex and dehisce before proximal anthers. Thus the order in which anthers dehisce can be accurately predicted. These protogynous flowers may self-fertilize at a lower level than unmanipulated flowers for two reasons: (1) because artificial protogyny allows cross-fertilization before opportunities for self-fertilization; and (2) because they contain much less self-pollen. Accordingly, the protogynous flowers were compared with control flowers from which 19 randomly chosen anthers were removed before dehiscence (treatment "C"). Compared to C flowers, P flowers experienced about one day of stigma receptivity before anthers started shedding self-pollen. We also performed a third treatment in which the last 19 anthers to dehisce were removed before they started shedding pollen ("L"). In unmanipulated flowers, the 39 anthers dehisce over a 3–4 d period, however, stigmas usually acquire enough pollen to fertilize all ovules in the first day of anther dehiscence (see Results). Consequently, removing the last 19 anthers should have no effect on the level of self-fertilization.

To minimize the effect of other factors that might influence the level of self-fertilization (e.g., display size, position in the flowering sequence, time of flowering), we treated quartets of plants matched for inflorescence number and flower number. One flower on each plant received one of the three treatments (P, C, or L) or was left unmanipulated ("U"). The treated flowers were further matched for their time of flowering and position within the flowering sequence. Floral manipulations were performed between 5 May and 1 June, during which time at least ten plants in each population were flowering on any given day. We treated between 25 and 40 quartets for each population, but attrition due to herbivory, seed predation, and drought greatly reduced sample sizes. The number of fruits collected from P, C, L, and U flowers, respectively, was 20, 18, 13, and 15 in QBC1; 12, 15, 11, and 10 in QCA1; and 18, 9, 15, and 10 in QWM1. We collected most fruits just before the follicles split open and counted the plump, black glossy seeds in each complete fruit. There was an average (±1 SE) of 10.0 ± 0.5 seeds/follicle ($N = 160$), which did not vary significantly among populations or treatments (two-way ANOVA, whole-model $F_{11\,148} = 1.34$, $P = 0.21$). We also collected fruits from as many additional unmanipulated plants ("X") to increase the sample of fruits used to obtain a baseline estimate of self-fertilization for each population (N for U and X combined = 35 in QBC1, 32 in QCA1 and 29 in QWM1).

We assayed ten seeds per fruit (total = 2283) for two polymorphic allozyme loci (PER and IDH) following Routley, Mavraganis, and Eckert (1999). There were three alleles for each locus, although one allele for each was rare and not found in all populations. To obtain multilocus and single-locus estimates of the level of outcrossing (t_m and t_s, respectively) for each treatment (P, C, and L), we used Ritland's maximum-likelihood program MLTR, which estimates mating parameters for different classes of plants that experience a common set of pollen allele frequencies (Ritland, 1986, 1990). Newton-Raphson iteration was used to find maximum-likelihood estimates, and standard errors were calculated as the SD of 1000 bootstrap values generated with the progeny array as the unit of resampling. The multilocus estimate of the proportion of seeds produced through selfing was calculated as $s = 1 - t_m$. We obtained an estimate of the baseline level of self-fertilization for each population in a similar fashion using only the progeny arrays from unmanipulated flowers (U and X). For all analyses, iterations from any set of starting values converged on only one set of maximum-likelihood estimates.

To determine whether experimental protogyny reduced the level of self-fertilization, we contrasted estimates of s between treatments within populations with a priori, nonorthogonal, pairwise comparisons (P vs. C, and C vs. L), and a per-comparison α of 2.5%. Because we expected that protogyny should reduce selfing, the P vs. C comparison was one-tailed (e.g., H_O: $s_P = s_C$; H_A: $s_P < s_C$). Because we expected that the removal of late anthers should have no effect on selfing, the C vs. L comparison was two-tailed (e.g., H_O: $s_C = s_L$; H_A: $s_C \neq s_L$). The one-tailed contrast was considered significant if <2.5% of the differences ($s_P - s_C$) between randomly paired bootstrap estimates were >0 (Eckert and Barrett, 1994). The two-tailed contrast was considered significant if <1.25% of the differences ($s_C - s_L$), were >0 or <0. Maximum-likelihood estimates of s and individual bootstrap estimates were averaged across populations to yield the average level of self-fertilization for the three emasculation treatments (P, C, and L). The standard error of the average was calculated as the SD of the 1000 average bootstrap values (see Eckert and Barrett, 1994, for details). These average estimates were then contrasted with the same procedure used for the single population estimates.

RESULTS

Floral development—We divided flower and fruit development into 13 relatively distinct morphological stages (Table 1). Stage-1 flowers were small yellow-green buds. The development of clearly defined nectar spurs and a hint of a reddish color marked the onset of stage 2. At stage 3, nectar spurs

were well defined, buds were a deeper red, and styles elongated to present stigmas outside closed buds. At stage 4, the sepals began to open and expose the lamellae (openings of the nectar spurs). The stamens were curled back inside the corolla and surrounded the carpels. At stage 5, the sepals had spread farther and some stamens had straightened out to bring anthers towards the stigmas, though none had dehisced. Collectively, stages 3–5 are called ''stigma presentation.''

Stage 6 was marked by the beginning of anther dehiscence. Fewer than ten stamens were fully unfurled to place dehiscing anthers close to (0–10 mm) the stigmas. Sepals were usually fully open at this stage. Stage-6 flowers were deep red with bright yellow lamellae. Stage-7 flowers were much like stage-6 flowers except that more anthers (11–29) had dehisced. At stage 8, almost all (>30) anthers had dehisced. At stage 9, all anthers had dehisced and the stigmas had started to curl. Collectively, stages 6–9 are called ''anther dehiscence.'' Flowers spent more time in stigma presentation (mean ± 1 SE = 5.2 ± 1.9 d) than anther dehiscence (3.2 ± 0.9 d, paired $t = 3.7$, $P = 0.0004$).

At stage 10, nectar spurs, sepals and stamens abscised, thereby exposing the carpels. At stage 11, the unfused, thin, green carpels (follicles) had begun to swell. At stage 12, follicles were noticeably swollen. At stage 13, follicles were a darker green and were separating from one another. Stage 13 ended when mature follicles split to release seeds. On average, fruit development (stages 10–13) averaged 22.7 ± 3.1 d.

Variation in pollen receipt and pollen tube growth across floral development in hand-pollinated flowers—The results of the greenhouse pollination experiment suggest that although stigmas are presented well outside flowers several days before anther dehiscence, they are not very receptive until anthers start to dehisce (Fig. 1). The number of pollen grains deposited on stigmas via hand-pollination varied somewhat with developmental stage, although the pattern of variation differed among the two populations (Table 2). In CCR1, pollen receipt increased more or less monotonically from an average (±1 SE) of 60.3 ± 11.1 grains on stage-3 stigmas to 159.4 ± 23.7 grains on stage-8 stigmas (Fig. 1). In QSP1, pollen loads were highest on stage-3 stigmas (166.1 ± 23.3) with only a moderate increase from stage 5 (99.6 ± 17.3) to stage 8 (145.9 ± 19.3). For both populations, the number of pollen grains deposited on stigmas at all stages was much greater than the average number of ovules per carpel (average = 25 ovules; Routley, Mavraganis, and Eckert, 1999).

Variation in pollen germination and tube growth across floral development was much more striking than the pattern of variation in pollen receipt. ANOVA revealed a strong increase in pollen tube growth from stage 3 to stage 8 for both measures of pollen tube development (Table 2). In contrast, there was no significant variation in tube growth between populations, and the pattern of variation across developmental stages did not differ significantly between populations. Twenty-four hours after hand-pollination, there were very few pollen tubes in the styles of flowers pollinated before anther dehiscence (mean ± 1 SE of stages 3 and 5 = 4.6 ± 1.3 tubes) compared to those pollinated after anther dehiscence (stages 6 and 8 = 33.2 ± 4.8 tubes). Similarly, the mean length of pollen tubes was much lower in flowers pollinated before (0.78 ± 0.14 mm) vs. after (2.04 ± 0.13 mm) anther dehiscence.

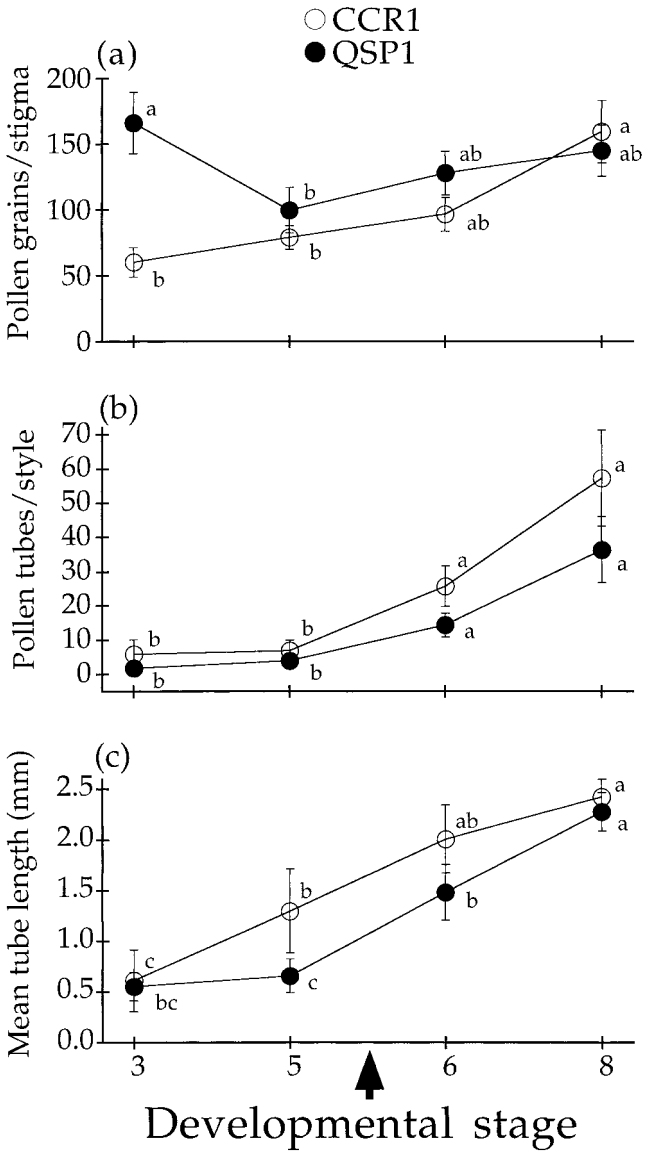

Fig. 1. Variation in pollen receipt and pollen tube growth among stages of floral development in hand-pollinated flowers from two populations of *Aquilegia canadensis* grown under greenhouse conditions. Points are means (±1 SE) based on 20 flowers per stage per population. Letters next to points show the results of multiple contrasts among developmental stages performed for each population separately; those not sharing a letter are significantly different. Arrows beneath the *x*-axis mark the onset of anther dehiscence. Analysis of these data is in Table 2.

Variation in pollen deposition and pollen tube number across floral development in naturally pollinated flowers—In all four populations, the number of pollen grains on naturally pollinated stigmas increased greatly across developmental stages (Fig. 2; $P < 0.0001$ for all one-way ANOVAs). Overall, there was significant variation in pollen load among developmental stages but not populations (Table 3). On average (±1 SE), only 7.9 ± 1.9 pollen grains were deposited on each stigma by just before anther dehiscence compared to 125.1 ± 21.6 grains just after the onset of anther dehiscence and 239.4 ± 19.1 grains by the end of anther dehiscence. Given that there was an average of 25 ovules in each carpel, it seems that sufficient pollen to fertilize all ovules was received by stigmas

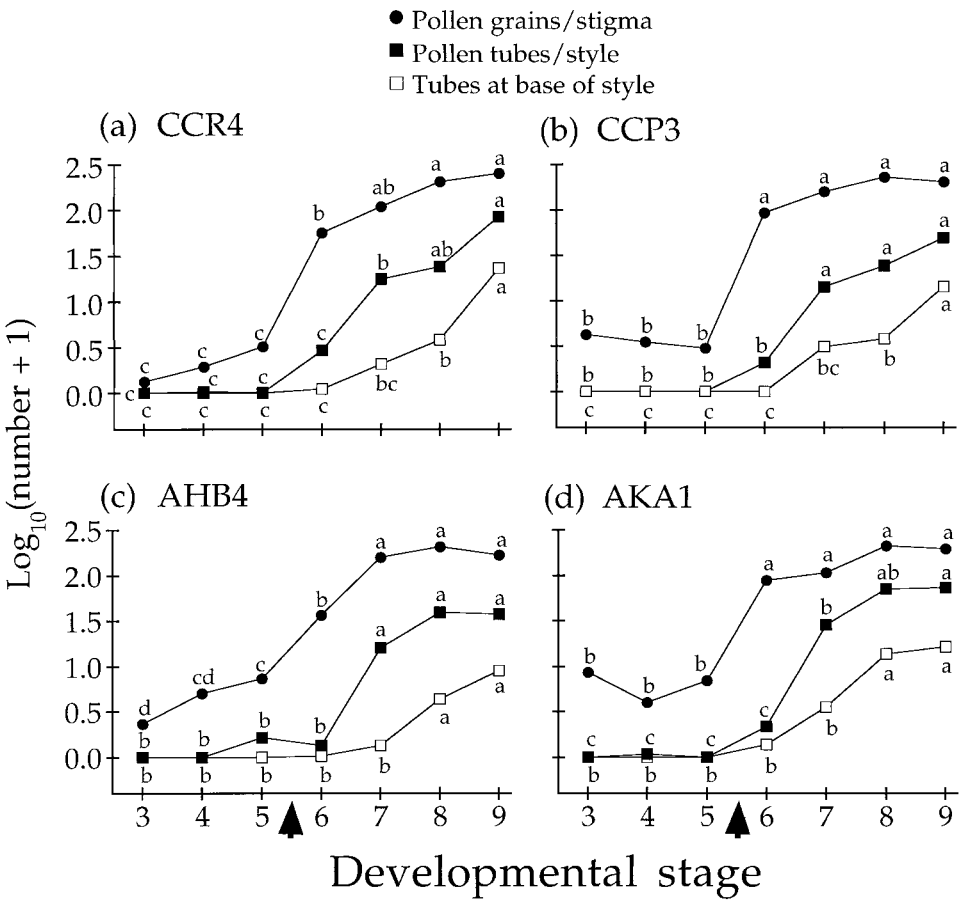

Fig. 2. Variation in pollen deposition and pollen tube growth among stages of floral development in naturally pollinated flowers from four populations of *Aquilegia canadensis.* Points are means based on ten flowers per stage per population. Error bars have been ommitted to improve clarity. ● = the number of pollen grains per stigma, ■ = the maximum number of pollen tubes in each style, and □ = the number of pollen tubes reaching the base of each style. Letters next to points show the results of multiple contrasts among developmental stages; those not sharing a letter are significantly different. Arrows beneath the *x*-axes mark the onset of anther dehiscence. Analysis of these data is in Table 3.

within the first day of anther dehiscence. The relation between developmental stage and pollen load varied significantly among populations. However, this interaction effect was small ($F = 2.1$) compared to the large effect of developmental stage ($F = 91.3$; Table 3). The largest increase in pollen load occurred between stages 5 and 6 in all populations (increase: range = 5–55-fold, average = 21-fold).

Variation in the number of pollen tubes in the style closely reflected the pattern of pollen deposition (Fig. 2, $P < 0.0001$ for all one-way ANOVAs). Again, there was significant variation in pollen tube numbers among developmental stages but not populations (Table 3). However for this variable, the increase in tube numbers across floral development did not vary among populations. By the end of stigma presentation, styles contained an average of only 0.39 ± 0.24 pollen tubes compared to 69.5 ± 5.5 by the end of anther dehiscence. The increase in pollen tube numbers between stages generally peaked between stages 6 and 7 and strongly declined between later stages.

Variation in pollen deposition and tube growth across floral development was also reflected in the number of pollen tubes reaching the base of the style (Fig. 2; $P < 0.0001$ for all one-way ANOVAs). Again, there was significant variation in pollen tube numbers among developmental stages but not among populations; and the interaction between these main effects was not significant (Table 3). No pollen tubes had reached the base of the style in any of the flowers collected before anther dehiscence. The number of pollen tubes at the base continued to increase throughout the period of anther dehiscence and showed little sign of leveling off by stage 9. By the end of anther dehiscence 20.6 ± 1.9 pollen tubes had reached the base of the style. Although pollen grains accumulate very quickly on stigmas, fertilization of all ovules is probably not complete until 4 d after the beginning of anther dehiscence, when flowers have just begun to fall apart.

Variation in nectar availability and components of flower size across floral development in naturally pollinated flowers—In both populations, there was a large increase in nectar availability as flowers developed (Fig. 3; $P < 0.001$ for both one-way ANOVAs). Overall, nectar volume varied significantly among developmental stages and between populations (Table 4). On average (±1 SE), each spur contained only 0.53 ± 0.12 μL of nectar before anther dehiscence, compared to 3.00 ± 0.17 μL after the onset of anther dehiscence. Average nectar volume was 1.9-fold higher at QBS1 (2.5 ± 0.3 μL) than at CCR1 (1.4 ± 0.3 μL). The relationship between developmental stage and nectar volume also varied among

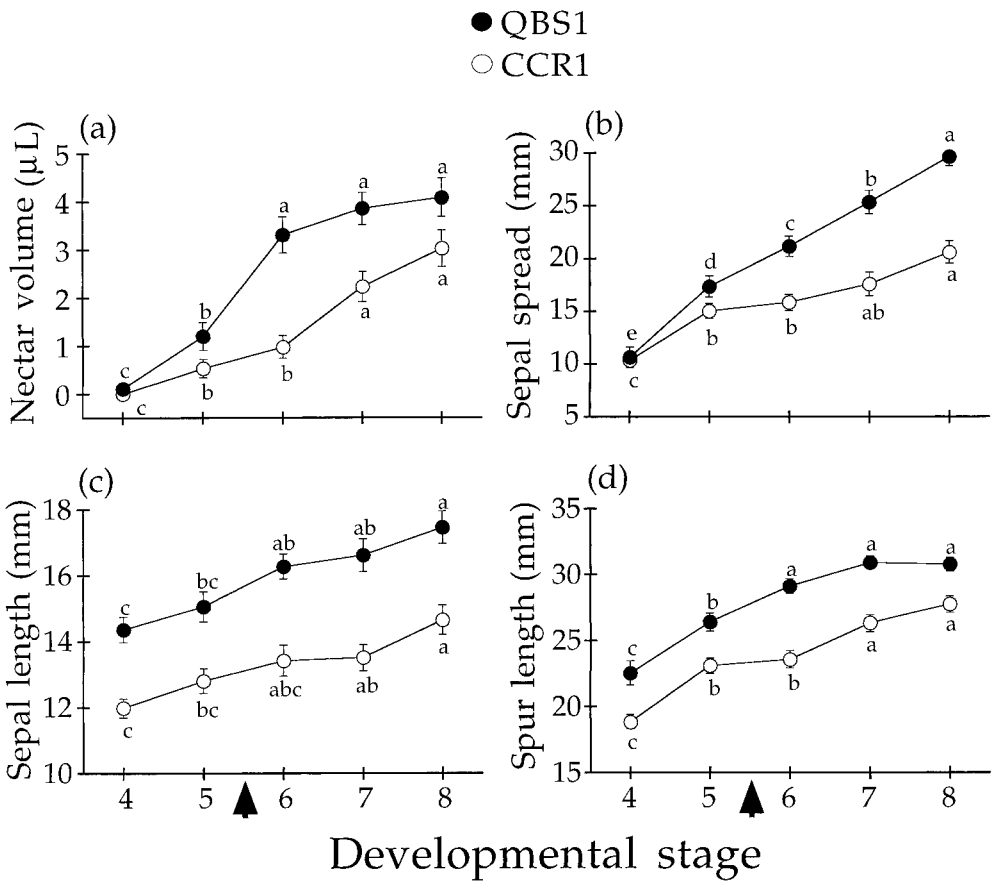

Fig. 3. Variation in nectar availability and components of flower size among stages of floral development in naturally pollinated flowers from two populations of *Aquilegia canadensis.* Points are means (±1 SE) based on at least ten flowers per stage per population. Letters next to points show the results of multiple contrasts among developmental stages performed for each population separately; those not sharing a letter are significantly different. Arrows beneath the *x*-axes mark the onset of anther dehiscence. Analysis of these data is in Table 4.

populations. At QBS1 the largest increase in nectar volume between stages occurred between just before and just after anther dehiscence (2.8-fold increase between stages 5 and 6). At CCR1, the largest increase in nectar volume occurred between stages 6 and 7 (2.3-fold increase).

TABLE 3. Analysis of variation in pollen grains/stigma, pollen tubes/style, and pollen tubes at the base of the style among stages of floral developmental in naturally pollinated flowers from four populations of *Aquilegia canadensis.* The overall ANOVA model was significant for all variables (all *P*'s < 0.0001). The residual df = 257 in all analyses. Means are in Fig. 2.

Source of variation	df	SS	*F*	*P*
a) $\log_{10}$[Pollen grains/stigma + 1], $r^2 = 0.83$				
Population	3	1.8	1.9	0.17
Developmental stage	6	176.1	91.3	<0.0001
Population × Stage	18	5.8	2.1	0.0057
b) $\log_{10}$[Pollen tubes/style + 1], $r^2 = 0.81$				
Population	3	0.6	1.4	0.28
Developmental stage	6	146.9	166.0	<0.0001
Population × Stage	18	2.6	1.0	0.41
c) $\log_{10}$[Tubes at base of style + 1], $r^2 = 0.64$				
Population	3	0.94	2.0	0.14
Developmental stage	6	51.11	55.6	<0.0001
Population × Stage	18	2.76	1.3	0.20

Developmental variation in flower size reflected the pattern of nectar availability, except that the components of flower size increased more regularly across floral development (Fig. 3). All three measures of flower size showed roughly the same pattern. There was significant variation in all three components of flower size among developmental stages and between populations (Table 4). The most striking temporal variation occurred for sepal spread, which more than doubled between stages 4 and 8, though the increase was more pronounced in QBS1 than in CCR1 (Table 4). Although flower size increased steadily throughout floral development, the onset of anther dehiscence or nectar availability was not associated with a particularly large increase in any component of flower size.

The effect of experimental protogyny on the level of self-fertilization—The three populations chosen for this experiment were predominantly self-fertilizing in 1998. The average $s \pm 1$ SE of unmanipulated (U and X) flowers was 0.771 ± 0.094. None of the differences in *s* between populations were significant (0.822 ± 0.063, 0.703 ± 0.084, and 0.788 ± 0.100 for QBC1, QCA1, and QWM1, respectively; *P* = proportion of bootstrap differences above or below zero >0.16 for all comparisons). These population-level estimates of *s* were well within the range of those obtained for ten other eastern Ontario populations in 1998 (B. Ozimec, C. R. Herlihy, and C. G.

TABLE 4. Analysis of variation in per-flower nectar availability (μL) and components of flower size (mm) among stages of floral development in naturally pollinated flowers from two populations of *Aquilegia canadensis.* The overall ANOVA model was significant for all variables (all *P*'s < 0.0001). The residual df = 213 in all analyses. Means are in Fig. 3.

Source of variation	df	SS	*F*	*P*
a) 1/[Nectar volume + 1], $r^2 = 0.57$				
Population	1	1.11	7.5	0.052
Developmental stage	4	13.87	23.2	0.0050
Population × Stage	4	0.60	2.6	0.0348
b) Sepal spread, $r^2 = 0.63$				
Population	1	1399	10.7	0.031
Developmental stage	4	4993	9.5	0.025
Population × Stage	4	525	6.7	<0.0001
c) $\log_{10}$[Sepal length], $r^2 = 0.41$				
Population	1	0.345	439.9	<0.0001
Developmental stage	4	0.182	58.2	0.0008
Population × Stage	4	0.003	0.2	0.93
d) Spur length, $r^2 = 0.63$				
Population	1	955	81.9	0.0008
Developmental stage	4	1810	38.8	0.0019
Population × Stage	4	47	1.5	0.21

Eckert, Queen's University, unpublished data; see also Routley, Mavraganis, and Eckert, 1999).

Contrary to expectations, the removal of early-dehiscing anthers did not reduce the level of self-fertilization in any of the three populations (Fig. 4). In QBC1 and QWM1, protogynous flowers actually self-fertilized a little more than control flowers. In QCA1, the maximum-likelihood estimate for P flowers was slightly lower than the estimate for C flowers, but the difference was far from significant. None of the minor differences in selfing between C and L flowers neared significance. The level of selfing averaged across populations was 0.821 ± 0.043 for P flowers, 0.713 ± 0.070 for C flowers, and 0.783 ± 0.063 for L flowers. There was no difference in selfing between control (C) and unmanipulated (U and X) flowers in any of the three populations (Fig. 4; all *P*'s > 0.14).

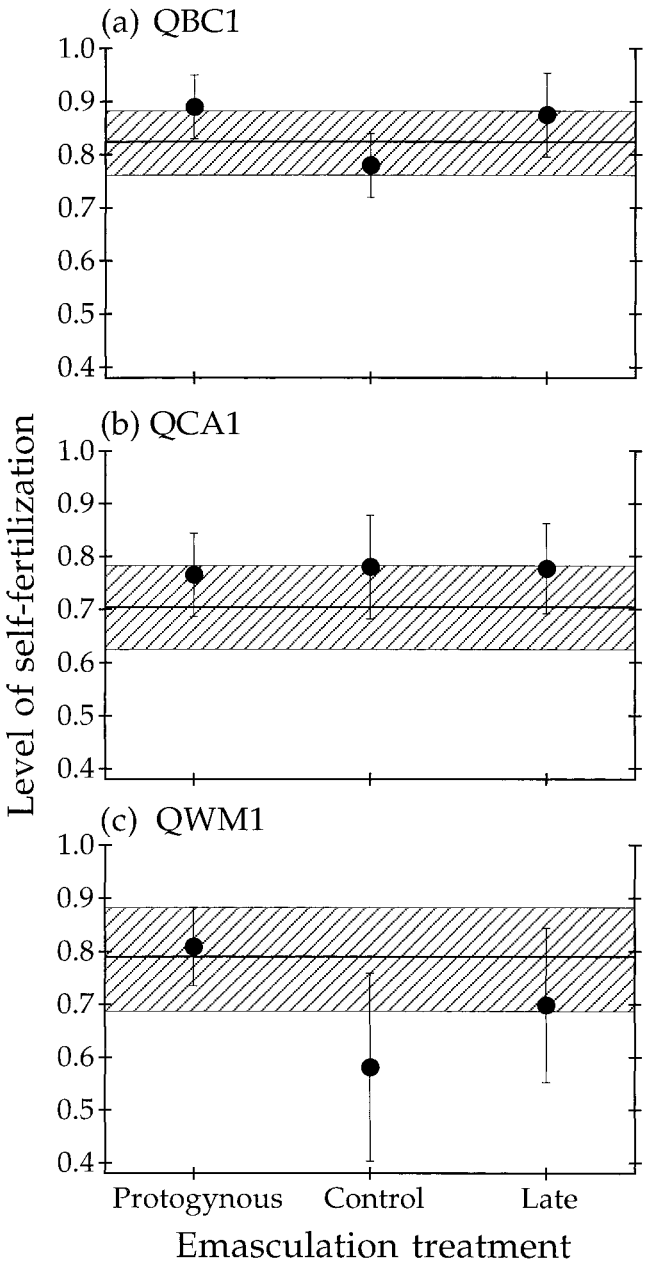

Fig. 4. The effect of experimental protogyny on the level of self-fertilization in three populations of *Aquilegia canadensis.* Points are maximum-likelihood estimates (±1 SE) of the proportions of seeds produced through self-fertilization for each emasculation treatment. Protogynous flowers had the first 19 anthers removed before dehiscence; late flowers had the last 19 anthers removed; control flowers had a random 19 anthers removed. The horizontal line is the average level of selfing in the population based on progeny arrays from unmanipulated flowers. The hatched box shows ±1 SE around the population mean. Sample sizes are in the Materials and Methods.

DISCUSSION

Morphological vs. functional dichogamy—Although dichogamy is one of the most common floral mechanisms in angiosperms (Bertin and Newman, 1993), the extent to which dichogamy separates sexual functions in time and the effect of such temporal separation on mating patterns have rarely been investigated. The extent of dichogamy is usually inferred from rough morphological cues such as the relative timing of style elongation, stigmatic swelling, and anther dehiscence (Lloyd and Schoen, 1992; Bertin and Newman, 1993). However, these cues may often be misleading (e.g., Preston, 1991). Such is the case in *A. canadensis.*

Schneck (1901) described *A. canadensis* as protogynous because styles elongate to present stigmas well outside the corolla several days before the filaments unfurl and anthers dehisce. The sepals and nectar spurs are almost fully developed during the period of stigma presentation before anther dehiscence. However, results from our greenhouse experiment suggest that stigmas are relatively unreceptive to pollen germination and tube growth until after anthers start to dehisce (Fig. 1b–d). In some species, including other species of *Aquilegia*, the onset of stigma receptivity is associated with swelling of the stigmatic papillae or some other obvious morphological change to the stigma (Bertin and Newman, 1993; Brunet and Eckert, 1998). In *A. canadensis,* stigmas sometimes appear increasingly swollen as floral development progresses, but the change is subtle and inconsistent (see Materials and Methods; C. G. Eckert and C. R. Herlihy, Queen's University, personal observations).

Even though stigmas become receptive at the same time as anthers start shedding self-pollen, floral development in *A. canadensis* may afford some opportunity for cross-fertilization to occur before self-fertilization. Styles elongate to bring stigmas to their ultimate position with respect to the rest of the flower well before anther dehiscence, and stigmas can receive substantial quantities of cross-pollen (at least via hand-pollination) before they become receptive. If pollen deposited on stigmas before the onset of receptivity remains viable, prior cross-pollination may increase the probability of cross-fertilization. However, our data on the timing of pollen deposition in four natural populations (Fig. 2) suggest that the potential for prior cross-pollination is not realized (see also Eckert and Schaefer, 1998). Very little pollen is deposited on stigmas before anther dehiscence.

That stigmas receive little cross-pollen before anther dehiscence may simply be because flowers are not rewarding and/or attractive to pollinators at this time. In one of the populations we examined (QBS1), nectar availability increased substantially after anther dehiscence (Fig. 3a). In the other population examined (CCR1) nectar reward steadily increased throughout floral development but there was no major increase in nectar coincident with anther dehiscence. Because nectar availability (i.e., standing crop) represents the sum of nectar production minus nectar removal by pollinators, it may not reliably indicate the rate of nectar production. However, in our populations of *A. canadensis*, low pollen loads on stigmas before anther dehiscence suggest very low rates of pollinator visitation. Accordingly, low nectar levels early in floral development are very likely a consequence of low nectar production.

Developmental variation in nectar production has been observed between floral sex phases in several dichogamous taxa, and in most cases variation in nectar production is associated with significant variation in pollinator visitation (Bell et. al., 1984; Devlin and Stephenson, 1985; Klinkhamer and de Jong, 1990; Gonzalez et al., 1995; Aizen and Basilio, 1998; but see McDade, 1986). In *A. canadensis*, the steady increase in components of flower size, especially sepal spread, throughout floral development (Fig. 3b–d) could be used by pollinators to roughly discriminate between rewarding and unrewarding flowers. Our field observations also indicate that some pollinator taxa may also visit *A. canadensis* to collect pollen (see also Macior, 1966). This too may result in flowers being visited infrequently before anthers start shedding pollen (Gonzalez et al., 1995; Inoue, Maki, and Masuda, 1995).

Another explanation for low pollen deposition before anther dehiscence is that cross-pollination is simply infrequent throughout floral development regardless of rewards or flower size. In other words, the large increase in pollen deposition following anther dehiscence observed in natural populations was due almost entirely to self-pollination. This is especially likely because *A. canadensis* has a well-developed capacity for automatic self-pollination (Macior, 1978; Eckert and Schaefer, 1998; Routley, Mavraganis, and Eckert, 1999). However, we do not have data on the relative deposition of cross- vs. self-pollen required to directly examine this possibility. However, results from a concurrent emasculation experiment in QBS1 indicated that wholly emasculated flowers receive enough allogamous pollen to achieve the same level of seed set as intact flowers (Eckert and Schaefer, 1998). This suggests that, at least in this population, there is a major increase in the rate of cross-pollination after anther dehiscence.

Would protogyny reduce self-fertilization if it occurred?—The simultaneous onset of stigma receptivity and anther dehiscence combined with close proximity of dehiscing anthers and receptive stigmas would seem to account for the very high levels of self-fertilization observed in eastern Ontario populations of *A. canadensis.* Self-fertilization estimated from progeny arrays in ten populations varied widely among populations (17–100%) and averaged 75% (Routley, Mavraganis, and Eckert, 1999). Comparison of the inbreeding coefficients of adult plants with those expected at inbreeding equilibrium (Ritland, 1990) further indicated that inbreeding depression is probably very strong ($\delta = 1 -$ [fitness of selfed progeny/fitness of outcrossed progeny] = 0.89; Routley, Mavraganis, and Eckert, 1999). Recent experimental results also suggest that although automatic self-fertilization may be favored to some extent as a mechanism of reproductive assurance, it results in severe seed discounting and a substantial net reduction in fitness (C. R. Herlihy and C. G. Eckert, Queen's University, unpublished data).

Given that high levels of selfing amount to a major fitness cost, it would seem that any mechanism that reduces the level of selfing would be strongly selected in *A. canadensis.* This led us to ask whether protogyny could potentially be an effective anti-selfing mechanism in this species. This ''what if'' approach seemed relevant for two reasons. First, many researchers view protogyny as a mechanism that might have evolved to reduce self-fertilization because it provides opportunities for cross-pollination before any possibility of self-pollination (Lloyd and Webb, 1986; Bertin and Newman, 1993). Second, the extent and nature of dichogamy appear to be evolutionarily labile in *Aquilegia* (see Introduction). For instance, *A. elegantula* resembles *A. canadensis* in terms of both floral morphology and pollination ecology but is strongly protogynous (Miller, 1978).

We created protogynous flowers in three populations by removing the first 19 anthers that would have dehisced and compared them to nonprotogynous control flowers from which 19 randomly chosen anthers had been removed. Contrary to our expectations, protogynous flowers did not self-fertilize at a lower level than control flowers in any of the populations, even though our experimental manipulation provided ~1 d during which cross-pollen could arrive on stigmas before any opportunity for self-pollination. We also did not detect any difference in self-fertilization between control flowers and unmanipulated flowers. In what follows, we consider three explanations for this unexpected result.

Protogyny, as we have attempted to simulate it, is primarily effective at reducing self-pollination occurring between anthers and stigmas in the same flower (autogamy). If most self-fertilization involved between-flower self-pollination (geitonogamy) protogyny would have little effect on the mating system. Geitonogamy may be a common form of self-fertilization in plants (de Jong, Waser, and Klinkhamer, 1993; Harder and Barrett, 1996; Snow et al., 1996), and the few studies that have quantified the contribution of geitonogamy to self-fertilization in natural populations have all revealed substantial geitonogamous selfing (Schoen and Lloyd, 1992; Leclerc-Potvin and Ritland, 1993; Eckert, 2000). Preliminary data from an emasculation experiment conducted in nine populations of *A. canadensis* within several kilometres of the ones involved in this study suggest that geitonogamy causes about one-third of the overall level of selfing (C. R. Herlihy and C. G. Eckert, Queen's University, unpublished data). This suggests that most

selfing occurs via autogamy, thus the expectation of lower selfing in protogynous flowers compared to control flowers still holds.

The incomplete dichogamy created by our floral manipulations does not necessarily reduce the overall level of self-pollination; it simply gives cross-pollen a head start in competition for fertilizations. Consequently, protogyny will have little effect on the mating system if the rate at which cross-pollen arrives on stigmas is very low. We currently have no data on the rate of cross-pollination in natural populations. The extremely high level of self-fertilization observed in eastern Ontario populations of *A. canadensis* suggests that cross-pollination is infrequent. However, Eckert and Schaefer (1998) showed that, in QBS1, emasculated flowers eventually receive enough cross-pollen for full seed set. Detailed investigation of the relative timing of self- and cross-pollination will be required to further evaluate this explanation.

Finally, protogyny will be relatively ineffective at reducing selfing if pollinators tend to avoid flowers in female phase (Klinkhamer and de Jong, 1990; Inoue, Maki, and Masuda, 1995). This is particularly likely to occur if pollinators collect pollen (Gonzalez et al., 1995) or flowers produce less nectar during the female than male phase (Bell et al., 1994; Aizen and Basilio, 1998). Pollinators visit flowers of *A. canadensis* infrequently in our study populations. However, the observations we have made to date indicate that flowers are primarily visited by a few bumble bee (*Bombus*) species that either collect pollen, probe for nectar, or rob nectar by biting holes in the spur tips. Bees collecting pollen may avoid our protogynous flowers during their enforced female phase. Likewise, nectar-collecting bees may avoid female-phase flowers if they associate conspicuous, dehiscing anthers with the presence of nectar in the spurs (see Fig. 3a; but see Bell at al., 1994). Currently, we do not have the data required to rigorously address this explanation, although we have observed both pollen- and nectar-collecting bees visiting emasculated flowers. It is worth noting that none of our potential explanations for the ineffectiveness of experimental protogyny at reducing selfing involves methodological artifacts. We also expect that naturally occurring protogynous mutants would fail to enjoy the increased progeny quality associated with reduced self-fertilization. Perhaps this is why protogyny has not evolved in eastern Ontario populations of *A. canadensis*.

Although the functioning of dichogamy depends on how pollinators interact with flowers, much of what we know about this widespread floral mechanism is based on relatively crude observations of the timing of stigma presentation vs. anther dehiscence (Lloyd and Webb, 1986; Bertin and Newman, 1993). Few studies have combined these morphological observations with quantitative assessment of important components of stigma receptivity such as pollen adhesion, germination and pollen tube growth (e.g., Preston, 1991; Navarro, 1997). Fewer still have examined whether temporal separation of anther dehiscence and stigma receptivity reduces the potential for self-fertilization or other forms of pollen-pistil interference in natural populations (e.g., Jonsson, Rosquist, and Widén, 1991; Dinnétz, 1997). Taken together, our results emphasize the importance of field observations and experiments in evaluating the functional significance of dichogamy and other floral traits in natural populations of flowering plants.

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