American Journal of Botany 86(9): 1239–1247. 1999.

THE MECHANISM OF DELAYED SELFING IN *COLLINSIA VERNA* (SCROPHULARIACEAE)[1]

SUSAN KALISZ, DONNA VOGLER, BROOK FAILS, MATT FINER, ERICA SHEPARD, TARA HERMAN, AND REBECCA GONZALES

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Collinsia verna, blue-eyed Mary, has floral attributes of an outcrossing species, yet most flowers readily self-pollinate under greenhouse conditions. Here we describe the mechanism of self-pollination in *C. verna* via changes in relative positions of the stigma and anthers and late timing of receptivity, resulting in delayed selfing. Each flower contains four anthers that dehisce sequentially over ~1 wk. Pollen that is not collected by pollinators accumulates in the keel petal and retains high viability (>80% pollen germination) up to the time of corolla abscission. The stigmatic surface does not become receptive until after the third anther dehisces. This overlap in the sexual phases is concurrent with a change in herkogamy during floral development. In most flowers (70%), the stigma has moved to the front of the keel and is positioned near the anthers when the third anther dehisces. Under field conditions, fruiting success of plants within pollinator exclosures was ~75% of the fruiting success in open-pollinated plants (33% fruiting success via autogamy vs. 44% fruiting success, respectively). *Collinsia verna* plants in pollinator exclosures exhibit variation in autogamy rates within natural populations (range 0–80%). In addition, only half of naturally pollinated, receptive flowers examined had pollen tubes growing in their styles. In contrast, shortly after corolla abscission, nearly all flowers examined (96%) had pollen tubes in their styles. Thus we find that in *C. verna*, autogamy occurs late in floral development, which has the potential to provide substantial reproductive assurance, and that individuals vary in their ability to set fruit through this mechanism. We suggest that delayed selfing mechanisms may be overlooked in other species and that variable pollinator availability may play a significant role in the maintenance of mixed mating in species with delayed selfing, such as *C. verna*.

Key words: autogamy; *Collinsia verna*; delayed selfing; floral morphology; herkogamy; plant mating systems; pollinator limitation; reproductive assurance, Scrophulariaceae.

If a plant population relies solely on animal vectors to move pollen among individuals and if pollinators are absent or in low numbers at certain times or years, individuals that can self-pollinate if not previously outcrossed will be at a selective advantage (Lloyd, 1979). This reproductive assurance process has been termed ''delayed'' selfing (Lloyd, 1992; Sakai, 1995) in that self-pollination is delayed until after the opportunity for outcrossing has passed (see also ''facultative xenogamy'' of Cruden and Lyon [1989]). Delayed selfing incurs no cost under conditions of high outcrossing (i.e., no pollen discounting, Holsinger, 1991), but is selectively advantageous when pollinators or pollen is limiting. Since the absence of pollinators decreases both seed production and pollen-mediated mating opportunities, delayed selfing is expected to benefit both female and male function. In this context, self-fertilization can be considered a bet-hedging strategy that can increase either the probability of maintaining an individual's genes within a population or a population's reproductive output in the face of unpredictability of pollinator service, or both.

[1] Manuscript received 17 September 1998; revision accepted 2 February 1999.

The authors thank Samantha Vosick, Phil Brautigam, and Devon Drown for their unquestionable dedication to the project; Tom Harper for his assistance with electron microscopy and photo layout, and Ellen York for valuable greenhouse assistance. This research was supported by a University of Pittsburgh's Central Research Development Fund Grant and a National Science Foundation Grant to S.K. (DEB94-21782); REU supplements to the NSF grant provided support for B.F., E.S., M.F., and R.G.

[2] Author for correspondence (tel. 412-624-4281; FAX 412-624-4759; e-mail: kalisz+@pitt.edu).

In general, two suites of floral morphological traits have been shown to modify the degree of self-pollination within flowers. First, the proximity of male (anthers) and female parts (stigmas) within a flower will determine how readily self pollen can be deposited on the stigma. The distance between the anthers and stigmatic surface (herkogamy) has been shown to be positively correlated with outcrossing rates (e.g., Holtsford and Ellstrand, 1992; Belaoussoff and Shore, 1995; Karron et al., 1997). Second, the separation in time of the expression of male and female phases (dichogamy) functions in the same fashion as spatial distances. The longer the time lag between the male and female phases the lower the expected selfing rate (Bertin and Newman, 1993). Thus delayed selfing may be achieved by either a partial overlap in timing of male vs. female phases or changes in the relative positions of anther and stigma during development. For example, delayed selfing in *Hibiscus laevis* (Klips and Snow, 1997) and *Campanula* species (Faegri and van der Pijl, 1979) is characterized by a progressive downward curling of the stigmatic area in towards the style where anthers or pollen are located. Conversely, in the protogynous *Aquilegia canadensis* (Eckhert and Schaeffer, 1998) the stamens progressively elongate towards the exerted stigma. In *Kalmia latifolia* (Rathcke and Real, 1993) and *Sanguinaria canadensis* (Lyon, 1992), anthers collapse onto the stigma on the final day of floral development, thereby achieving self-pollination. A related phenomenon, whereby the abscissing corolla drags the anthers across the stigma has been described in both *Lupinus nanus* (Juncosa and Webster, 1989) and *Mimulus guttatus* (Dole, 1990) and was termed ''corolla dragging''

by Dole (1990). Others have found an increase in self-pollination late in floral life without changes in morphology. The breakdown of self-incompatibility as the flower ages in both *Lilium longifolium* (Ascher and Peloquin, 1966) and in *Campanula rapunculoides* (Richardson et al., 1990) is attributed to degradation of the proteins that control self-incompatibility (e.g., the S-RNases) and can be viewed as another form of delayed selfing.

For species that are animal pollinated, variation in pollinator frequency (resulting in episodes of outcross pollen limitation) may be a significant force in the maintenance of mechanisms that induce autogamy late in floral life or late in the season (Cruden and Lyon, 1989). Moreover, under conditions of variable pollinator service, species with delayed selfing could exhibit variable outcrossing rates across flowering seasons. Thus species with delayed selfing traits are likely to be classified as mixed-mating species. Mixed-mating is expressed when an individual plant's seed production in one season is the result of both selfing events and outcrossing events. Recently, Schoen, Morgan, and Bataillon (1996) presented a model of mixed-mating evolution and asserted that reproductive assurance is an under-appreciated selective force in the evolution of selfing rates. As a result of this inattention, the link between the forces that select for reproductive assurance selfing (including delayed selfing) and mixed-mating systems in plants is poorly understood.

Previous investigations with *Collinsia verna* (Scrophulariaceae), blue-eyed Mary, suggest that this winter annual may have evolved delayed selfing as one of a suite of bet-hedging strategies in chronically variable environments. Prior work has shown that populations of *C. verna* experience dramatic annual fluctuations in fecundity and population growth rates (Kalisz, 1991; Kalisz and McPeek, 1992, 1993; Thiede, 1996). Over the 3–6 wk flowering period in April and May, plants experience dramatic variation in biotic and abiotic conditions that affect pollinators (unpublished data). In addition, natural populations of *C. verna* exhibit significant variation in outcrossing rates both within and among years (detailed in Materials and Methods). The range of outcrossing rates in *C. verna* populations ($t = 0.60$–0.93) suggests that progeny are produced by a combination of selfing and outcrossing each year. A similar 30% range of outcrossing rates ($t = 0.32$–0.64) was reported for its congener *C. heterophylla* in four populations in a single year (Mayer, Charlesworth and Meyers, 1996).

Given the variable outcrossing rates and variable environment during the flowering season of *C. verna*, we hypothesize that this species maintains delayed selfing for reproductive assurance. Thus, we were motivated to examine the mechanism of autogamy as a first step towards understanding its role in the evolution of the mating system of this species. Our objectives were to quantify and/or characterize: (1) changes in the relative positions of the stigma and dehiscent anthers and overlap of sexual phases over the floral life span, (2) timing of morphological changes in stigmatic surface and the rate of autogamous pollen deposition over the floral life span, (3) timing of stigmatic receptivity relative to pollen tube growth, (4) pollen longevity in the field, (5) pollinator behavior relative to floral developmental stage, and (6) the variance in autogamy rates in natural populations.

MATERIALS AND METHODS

Study species—*Collinsia verna*, Nutt., blue-eyed Mary (Scrophulariaceae, $2n = 14$) is a winter annual of the eastern United States, which germinates in the autumn. Plants bloom in the early spring coincident with the spring ephemeral flora. Each plant produces up to 40 flowers (Kalisz, 1989, 1991; Kalisz and McPeek, 1992, 1993) arranged in 5–9 whorls around the stem. The zygomorphic flowers have five sepals, five petals (the two upper petals are white, the three lower petals are blue), four anthers, and one ovary containing four ovules. The middle blue petal is folded into a keel, which encloses the male and female parts throughout their development. The four stamens are adnate to the corolla; thus, corolla and stamens abscise as a unit at the end of floral life. Flowers provide pollen and nectar rewards that are collected by a diverse array of native bees (particularly *Bombus*), honey bees, and occasional lepidopterans and dipterans (S. K., personal observations).

Collinsia verna exhibits mixed mating. Outcrossing rates for three MI and one IL populations of *C. verna* over 5 yr indicate that progeny are produced by a combination of selfing and outcrossing each year ($t = 0.60$–0.93; S. Kalisz, unpublished data). Even within a single population, outcrossing rate varied by >20% from one year to the next. Despite the relatively high to intermediate outcrossing rates in *C. verna*, inbreeding depression is consistently low to moderate (i.e., $\delta \leq 0.3$) (Kalisz, 1989; S. Kalisz, unpublished data).

Data presented in this paper were collected either on plants grown in the greenhouse, in natural field populations, or in an experimental garden. Four populations were used in the greenhouse studies of the morphological correlates of delayed selfing, three natural populations were used in the field studies of autogamy rate, and ~1000 plants from each population were planted into an experimental garden to examine pollinator visitation frequency with floral age. *Collinsia verna* plants used in the greenhouse studies were collected as seeds from natural populations, germinated, and grown to flowering in the University of Pittsburgh greenhouse. All populations used in this study were of large size ($N \gg 10^6$ plants). The locations of populations used in the greenhouse and field studies were BT = Braddock Trail Park (Westmoreland Co., Pennsylvania), EF = Enlow Fork (Washington Co., Pennsylvania), TMC = Ten Mile Creek (Washington Co., Pennsylvania), and TU = TU Avenue (Kalamazoo Co., Michigan).

Greenhouse experiment 1: changes in stigma-anther separation (herkogamy) during development—To document changes in the relative positions of anthers releasing pollen and the stigma, single flowers from 25 individuals from the TU population were monitored every 8 h to determine the timing of anther dehiscence over the course of floral lifetime. Each flower was scored for the number of anthers dehisced (zero anthers up to four anthers), the position of the dehisced anthers, and the position of the stigma relative to the anthers (herkogamy). On the basis of these data, flowers were classified into one of six stages. Stages 0–4 reflect the number of anthers dehisced, and stage 5 indicates that the corolla has abscised. Representative photographs were taken of flowers at each stage.

Greenhouse experiment 2: stigma morphology—To determine the extent to which morphological changes in the stigma occur during floral development, 30 flowers at each stage for stages 1–5 for a total of 150 flowers were randomly sampled. Fifty plants from the TU population were used. Styles were dissected from each flower and immediately placed on a glass microscope slide with a drop of polyvinyl-lacto-phenol fixative. Styles were viewed under 400× magnification of a light microscope, and measurements were made of the stigmatic width and style width using an ocular micrometer.

To examine fine-scale morphological changes in the stigma, the styles from five randomly chosen flowers (one of each of stages 1–4) were excised and mounted for scanning electron microscopy (SEM). Samples were gold sputter-coated with a Polaron Instruments SEM Coating Unit

(model E5100) for 2 min at 15 mA. Micrographs were taken on a JOEL JSM-35C electron microscope using Polaroid Type 55 4 × 5 inch film exposed at F8.

Greenhouse experiment 3: self-pollination and autogamous pollen loads—To determine both the timing of self-pollination during floral development and the number of pollen grains in autogamous pollen loads, stigmas of 20 flowers per stage from stages 1–4 were examined for a total of 80 flowers. Flowers were taken from greenhouse-grown plants derived from the EF population. Stigmas were readily excised without contacting the anthers by spreading the two lower petals apart and affixing both petals to the sticky portion of a Post-It Note (TM). This secured the keel petal in an open position and spread the four anthers and the style apart, allowing determination of floral stage. Flowers (still attached to the Post-It Note) were placed under a dissecting microscope, and the style was removed with a pair of fine scissors. The upper portion of the style was fresh mounted in a 1:1 solution of glycerol : 1% acetocarmine stain. Stigmas were examined under a microscope for the presence/absence of pollen grains. When pollen grains were present, they were counted under 400× magnification.

Experimental garden: test of independence of floral stage and pollinator visitation—To determine whether pollinators expressed a preference for particular floral stages, pollinators were observed for 0.5 h on each of four different sunny days in May of 1997 in an experimental garden. This garden contained 3000 *C. verna* individuals planted at natural densities. As a pollinator visited and left a flower, the visited flower would be checked and its stage (0–4) recorded (pollinators do not visit flower without corolla, i.e., stage 5). A *G* test was performed on the data to determine whether pollinators preferentially visited any stages (H_0 = all stages visited with equal frequency).

Field experiment 1: stigmatic receptivity—The timing of stigmatic receptivity was examined in the BT, EF, and TMC field populations in two ways. First, our indirect test of receptivity was an assay that detects the presence of stigmatic peroxidases. When receptive styles are placed in a 3% solution of hydrogen peroxide, vigorous bubbling occurs on the stigmatic surface (Kearns and Inouye, 1993, p. 68). Stigmas that are not receptive do not produce bubbles. In the field, gynoecia were dissected from flowers of all six stages described in greenhouse experiment 1. Individual styles were immediately sandwiched between two cover slips with a drop of hydrogen peroxide (with the ovary outside the cover slip). The stigmatic area was examined under a dissecting microscope in the field. Stigmas were scored as positive for peroxidase activity only if we observed vigorous bubbling across the entire surface of the stigma. [Note: We determined that small amounts of pollen deliberately placed on the stigmatic surface of young and presumably unreceptive stigmas produced very weak and localized bubbling (also noted by Kearns and Inouye, 1993). Thus, in the field, styles with weak bubbling following the application of the hydrogen peroxide were presumed to be false positives and were scored as unreceptive.] At each of the three Pennsylvania populations, 75 styles were tested for peroxidase activity for each of the six floral developmental stages (N = 1350).

Second, our direct test of stigmatic receptivity was to score for the presence of pollen tubes in the styles. A subset of the styles that were field tested for peroxidase activity were preserved in 70% EtOH and transported back to the laboratory (total N = 234 styles: 42, 62, and 130 styles from BT, EF, and TMC populations, respectively). At a later date, the styles were digested in 1 mol/L NaOH for 1 h and subsequently stained with aniline blue (Martin, 1959) and mounted on glass slides. The stained pollen tubes were resolved using fluorescence microscopy. All 234 styles were scored for the presence/absence of pollen tubes.

The styles (described above) with at least one pollen tube were used to determine the number of pollen grains received under field conditions. From that subset, we randomly selected 30 stage-4 slide preparations and 30 stage-5 slide preparations. Stigmatic tissue was spread by gently pressing on the coverslip. All pollen grains adhering to the stigma were counted at 400× magnification.

Field experiment 2: pollen longevity—To determine the duration of pollen viability during a flower's lifetime, ten flowers were collected directly from flowering plants (fresh pollen), and another ten from recently abscised flowers on the forest floor (old pollen) at the BT site on 12 and 22 May 1997. Pollen was shaken out of the keels onto petri dishes filled with a modified Brewbaker and Kwack (1963) medium (10% sucrose, 100 mg/L boric acid, and 150 mg/L of Ca_2HPO_4 in a 1.5% agar base). After 4 h at room temperature, the number of germinated grains out of a sample of 300 grains was determined by examining a transect across the diameter of the petri dish under a microscope.

Field experiment 3: autogamy rates and fruiting success in natural populations—To determine the extent to which plants differ phenotypically in their ability to self-pollinate via autogamy, we placed 0.3 m^2 pollinator exclusion boxes constructed from fiberglass window screening and polyvinylchloride pipe at 40 locations in each of the three populations (BT, EF, and TMC). These exclosures were set over naturally occurring groups of 10–20 plants prior to flowering (early April). A second set of 40 boxes with top screens only (controls) was installed as pairs to each of the exclosures. All control and exclosure screens were removed at the end of flowering period, and fruits were allowed to develop. Prior to seed dispersal (early June), we collected all plants from both exclosures and controls and counted total number of flowers and fruits. For plants within the exclosures, the ratio of fruits/flowers for each plant represents reproductive assurance under a ''worst-case'' scenario of no pollinator activity. We term this ratio in the exclosures ''autogamy rate.'' Autogamy rate per plant is a ratio, and ratios are inaccurate estimators when the numbers in the ratios are small. To correct for this, we restricted our autogamy rate sample to those plants that produced ≥5 flowering whorls. This ensured that the autogamy rate was based on ≥10 flowers/plant. In addition, we counted flowers and fruits only in the four lowest flowering whorls. This ensured that the fruits were developed earliest in the season, when the plants were least likely to be resource limited (Haig and Westoby, 1988). For the control plants, the fruits/flowers ratio per plant represents the reproductive output under open-pollination conditions of each site in 1998. We term this ratio in the controls ''fruiting success.'' As was done with the exclosure plants, we calculated the fruiting success for all plants with at least five flowering whorls, again restricting our flower and fruit tallies to the lowest four flowering whorls.

RESULTS

Greenhouse experiment 1: changes in stigma–anther separation during development—In all flowers observed in the greenhouse, the four anthers move toward the front of the flower and dehisce sequentially. Thus, floral development can be categorized into six relatively discrete morphological stages (Fig. 1). *Stage 0.* The flower is in bud with no anthers shedding pollen, the style and anther filaments are not elongated (Fig. 1A). *Stage 1.* As the flower opens fully (Fig. 1B), the front pair of anthers elongates toward the front of the flower and one anther sheds pollen. *Stage 2.* The second anther dehisces while the stigma and the second pair of anthers remains in the rear (Fig. 1C). *Stage 3.* The two rear anthers move to the front, and one sheds pollen (Fig. 1D), thus three out of the four anthers have dehisced. Of the 20 flowers examined at this stage, nine contained stigmas that had elongated to the same position as the anthers, i.e., nearly half of the stage 3 flowers lacked a stigma–anther sepa-

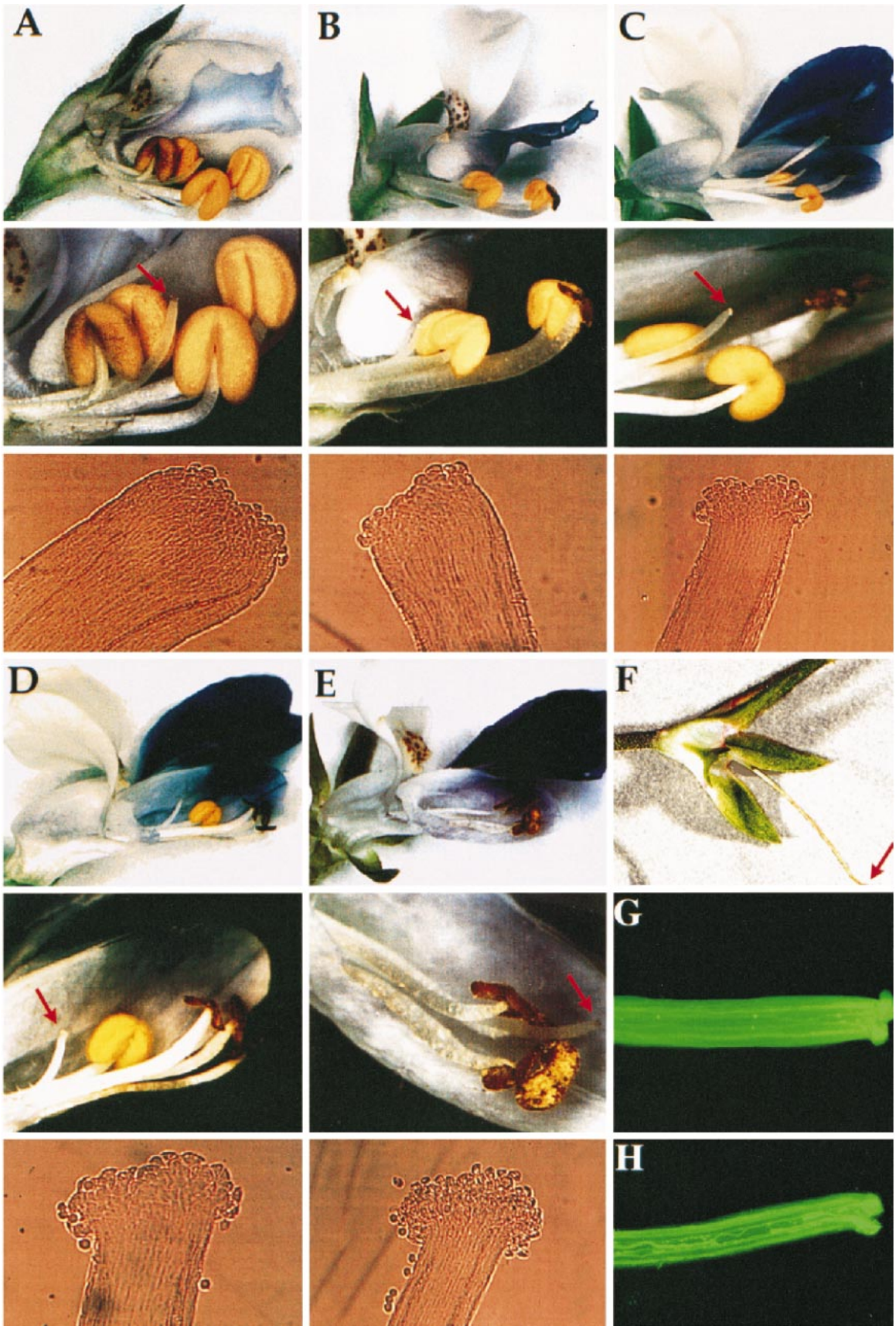

Fig. 1. Six stages of floral development in *Collinsia verna*. (A–E) Whole flower (top panel), anthers and style (middle panel) and stigma (lower panel) for stages 0–4, respectively. Refer to text for description of stages. The keel petal has been partially removed, and the stigma position is indicated by a red arrow. (F) Whole flower at corolla drop, stage 5. (G) Pollen tubes in a stage-4 style. (H) Pollen tubes in a stage-5 style.

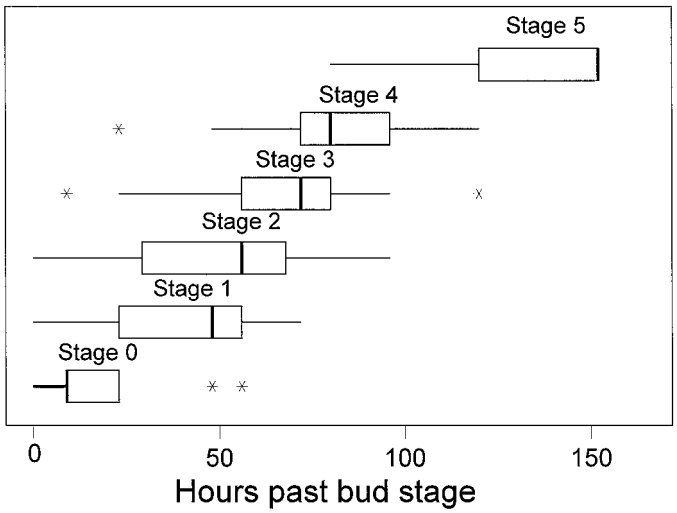

Fig. 2. Box plots of the duration of each of the six floral developmental stages in 25 *C. verna* plants monitored every 8 h from bud to corolla drop. Median values are indicated by a thick vertical line. Plants were grown in a greenhouse from seeds of the TU population.

ration. *Stage 4.* The last anther sheds its pollen. At this stage, 90% of the stigmas examined had elongated past the previously dehisced anthers, placing the stigma at the front of the flower (Fig. 1E). *Stage 5.* The corolla abscises (Fig. 1F). Because the anther filaments are adnate to the corolla, the anthers (and any remaining pollen) abscise as a unit with the corolla. Although the six stages always occur in the sequence described above, the duration of each stage varied among flowers observed in the greenhouse. Flowers were observed to open but persist at Stage 0 (i.e., with no anthers dehisced) from 9 to 23 h (Fig. 2).

The first anther dehisces (stage 1) at a median time of 47 h, the second anther at 56 h (Fig. 2). Substantial variation in the timing of anther dehiscence is indicated by the considerable overlap between stage 1 (23–56 h, interquartile range) and stage 2 (29–63 h, interquartile range). Stages 3 and 4 occurred on the third day at 71 and 83 h, respectively (median values, Fig. 2). The corolla abscised (stage 5) at a median time of 152 h (range: 142–156 h). In the greenhouse, the flowers were open for ~143 h and had pollen available for approximately the last 104 h (Fig. 2).

Greenhouse experiment 2: stigmatic flaring and herkogamy—During stages 0 and 1, the stigmatic surface appears to be tightly compressed [bright-field photographs, lower panel (Fig. 1A, B) and SEM (Fig. 3A, B)]. The stigma (position noted by red arrow in Fig. 1) remains in the throat of the corolla. Stigmatic width increases slightly between stages 0 and 1 (193 ± 12 and 204 ± 11 μm, respectively) with a notable increase at stage 2 (272 ± 10 μm). During stage 3, the individual papillae swell and begin to separate (Fig. 1D, lower panel, and Fig. 3D) contributing to a flaring of the stigmatic surface. During the remainder of floral development (stages 3–5) the increase in stigmatic width is more gradual (291, 322, and 362 ± 11 μm, for stages 3–5, respectively, see bright-field photographs; Fig. 1D, E). We observed pollen on the stigmatic surface of these greenhouse-grown plants at the later developmental stages (Fig. 3D, E), but we rarely observed pollen on younger styles (Fig. 3A–D).

Of the 20 flowers being monitored for floral development in the previous experiment, only two were observed with styles elongated and their stigmas at the same position as the first dehiscing anther at stage 1 (Fig. 4). By stage 4, 18 out of 20 flowers had stigmas near the dehiscing anthers (Fig. 4).

Greenhouse experiment 3: pollen loads via autogamy—In a separate experiment where styles were harvested at developmental stages 1 through 4 and examined for pollen, only 10 and 25% of the stage 1 and 2 flowers, respectively, were autogamously pollinated. The average pollen load on these pollinated stigmas was 2 ± 1.4 grains for stage 1 and 2.6 ± 3.1 grains for stage 2 (Table 1). By stage 3, when the majority of the stigmas in the previous experiment were observed to be in the pollen zone (70%; Fig. 2), less than half of the styles harvested at that same stage were observed to have any pollen (45%; Table 1). Most of the pollen loads on stage-3-pollinated stigmas were <10 pollen grains (median pollen load at stage 3 was six grains; Table 1), but one pollination resulted in 68 grains being deposited, the highest autogamously pollinated load observed. By stage 4, 85% of the flowers had stigmas that received some self-pollen (Table 1). The average load of autogamous pollen on these stage-4 flowers was 23.5 ± 14.6 pollen grains. Autogamous pollen loads on stage-4 flowers ranged from zero (in three of the 20 stigmas examined) to 60 grains (Table 1).

Experimental garden: test of independence of floral stage and pollinator visitation—Observations of 1032 visits to *C. verna* flowers in the experimental garden indicate that bees did not discriminate against flowers based on the number of anthers dehisced ($G = 2.3$, 3 df, $P = 0.746$). Both honey bees and bumble bees were observed to collect pollen, nectar, or both from flowers in these four stages. Pollinators were also observed to probe for nectar in the infrequent open flowers with no anthers dehisced.

Field experiment 1: stigmatic receptivity—Stigmatic peroxidase tests indicate that receptivity occurs at, or subsequent to, stage 3 (Fig. 5, dark bars). Of the styles tested at stage 3, 14% were positive for peroxidase activity, and by stage 4, 74% were scored as positive. While pollen tubes were found only in styles with receptive stigmas in the field, not all receptive pistils were pollinated. At stage 4, of the 57 stigmas that tested positive for peroxidase, 55% (31/57) had pollen tubes. At stage 5 (corolla drop), over 95% of the styles collected in the field had receptive stigmas, and of these, 96% (73/76) had pollen tubes (Figs. 5, 1G and H).

Preliminary data assessing the number of pollen grains/stigma at each stage using the same field-collected stigmatic esterase activity (SEA)-tested flowers indicate a step function in the number of grains. Pollen was infrequently found on stages 0, 1, 2, or 3. Pollen loads on stage-4 flowers were low ($X = 6.7$ grains; range = 2–16

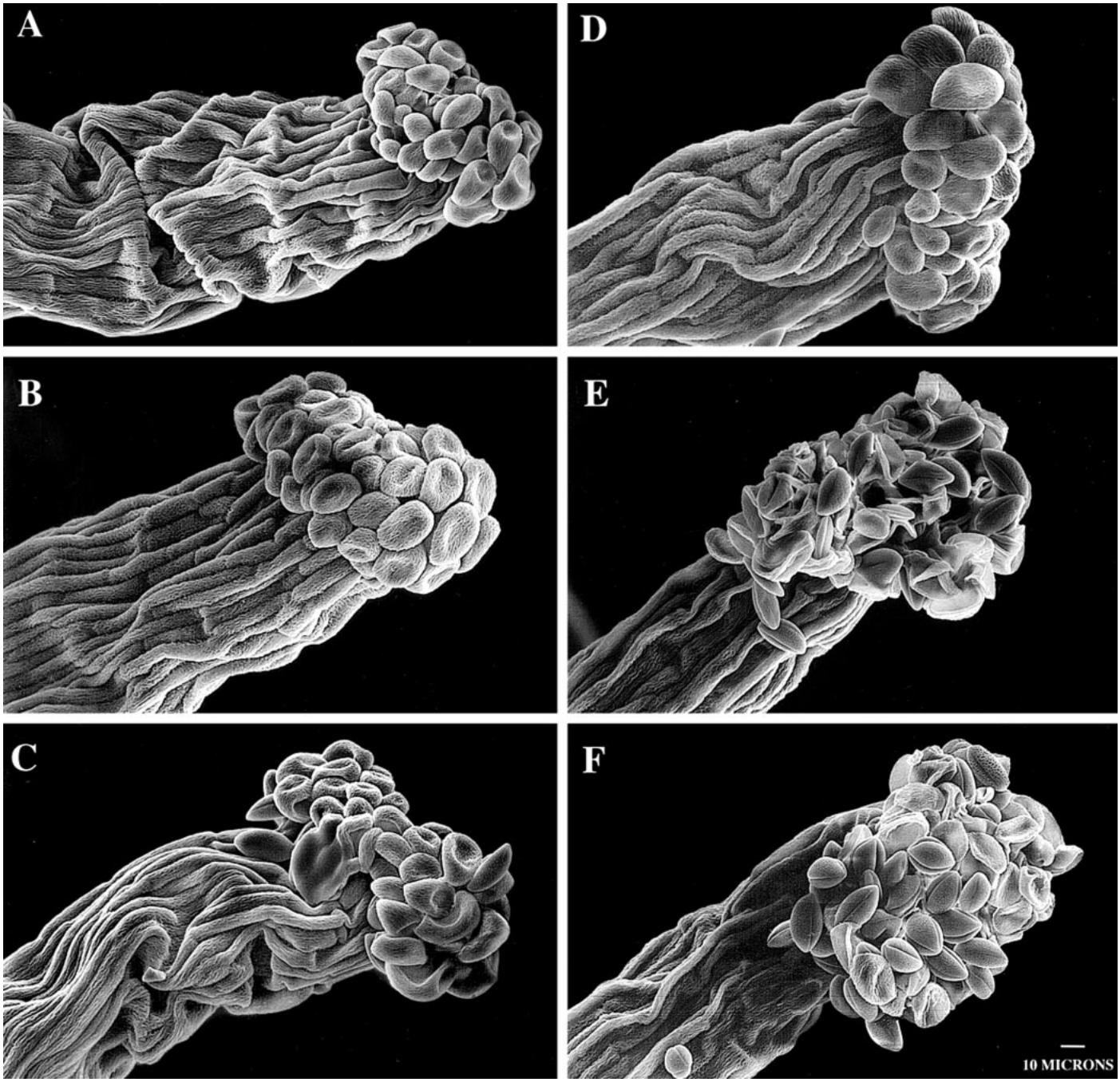

Fig. 3. Scanning electron micrographs of the stigmatic surface of *C. verna.* (A–F) Representative stigmas from flowers at each developmental stage (A = stage 0 through F = stage 5).

grains, $N = 30$), while loads on stage-5 flowers (corolla drop) had more than twice the number of pollen grains ($X = 14.7$; range = 2–34, $N = 30$). These counts, however, are likely to be underestimates because the peroxidase tests and additional staining for pollen tubes probably dislodged some pollen grains from the stigmatic surface. Styles of field-collected (i.e., open-pollinated) plants at stages 4 and 5 were frequently observed to have both pollen and pollen tubes that were easily visualized by fluorescence microscopy. Photographs of fluorescent pollen tubes from a stage-4 and a stage-5 flower are shown in Fig. 1G and H (two pollen tubes vs. 16 pollen tubes), respectively.

Field experiment 2: pollen longevity—Tests of old pollen collected from the keels of flowers that have dropped to the forest floor show germination on an agar-based media equal to fresh pollen collected directly from anthers. Pollen germination trials revealed that both old and fresh pollen had high germination rates (83 vs. 84%, respectively).

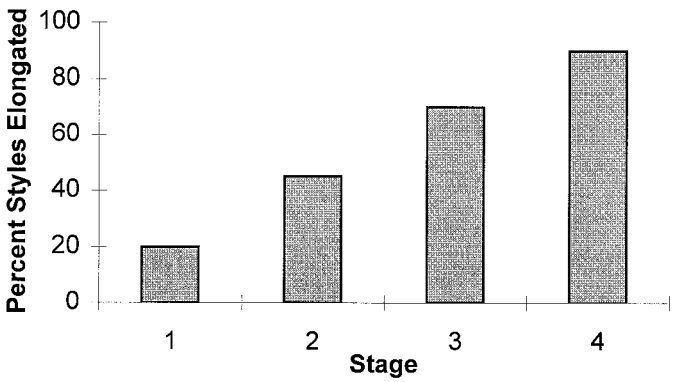

Fig. 4. Percentage of *C. verna* flowers in which the stigma is in the same position as the dehiscing anther(s). $N = 20$ flowers per stage. Plants grown in greenhouse from seeds of the TU population.

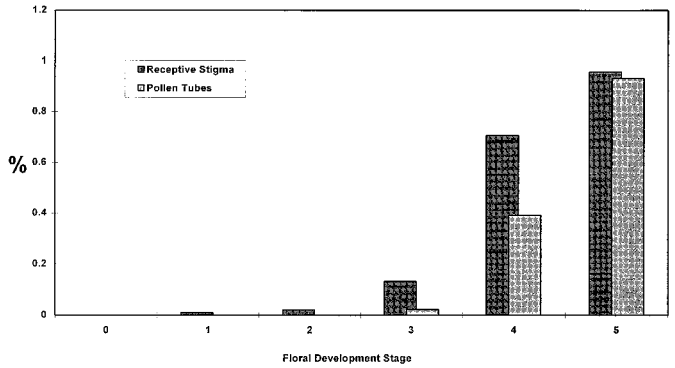

Fig. 5. Timing of stigmatic receptivity and pollen tube growth in field-collected flowers. Receptivity was evaluated by peroxidase activity in 80 flowers in each of the six developmental stages. Pollen tube presence was examined in 234 styles that exhibited peroxidase activity.

Field experiment 3: autogamy rates and fruiting success in natural populations—Vandalism at one site and storm damage at another reduced the number of plants we were able to evaluate for fruit set at two of the three populations (BT and TMC, respectively; Fig. 6). Average fruit set from autogamy by plants in the exclosures was consistently and significantly less than the average fruit set from control plants, which were open-pollinated in the same three populations (exclosures vs. control: BT = 34 vs. 40%, $t = 1.8$, 57 df, $P = 0.04$; EF = 33 vs. 49%, $t = 6.1$, 193 df, $P < 0.0001$; and TMC = 32 vs. 43%, $t = 1.9$, 38 df, $P = 0.03$; Fig. 6). Autogamy rates of individual plants ranged from zero to >80% in these populations, while fruiting success under these field conditions ranged from zero to >90%.

DISCUSSION

Our results indicate that *Collinsia verna* consistently exhibits herkogamy early in floral development. Anthers are matured and pollen is shed from one anther at a time during the average 60 h of floral development quantified in the greenhouse (Figs. 1, 2). In the infrequent early-stage flowers (stages 1 and 2) in which the stigma is adjacent to open anthers, self-pollen loads are low (Table 1) and less than the number of available ovules (four in this species). Thus, the spatial separation of anthers and stigma appears to be sufficient to prevent self-pollination in the first few days after the flower opens. However, reduction in the stigma–anther separation distances late in floral development means that delayed selfing is possible due to the temporal and spatial overlap of receptive stigmas with the late maturing anthers. At the stage prior to corolla abscission, the style has elongated in most (90%) flowers with four anthers dehisced (stage 4), and likewise most (85%) of the flowers at this stage have received some self-pollen in a pollinator-free greenhouse (Fig. 2, Table 1). Even under natural conditions, nearly all flowers (96%, Fig. 5) have been pollinated by the time the corolla drops. If self-pollen were the only pollen available to a flower, the amount deposited autogamously (23 grains, Table 1) on stigmas of late-stage flowers

TABLE 1. Autogamous pollination in *Colinsia verna*. Plants were derived from the EF population and grown undisturbed in a greenhouse. Floral stages refer to the number of anthers dehisced (floral stage 1 = one anther dehisced).

Floral stage	Sample size	Percent of stigmas with pollen	Autogamous pollen load/pollinated stigma	
			Median (range)	Mean (SD)
1	20	10%	2 (1–3)	2.0 (1.4)
2	20	25%	1 (1–8)	2.6 (3.1)
3	20	45%	6 (1–68)	14.1 (20.9)
4	20	85%	20 (2–60)	23.5 (14.6)

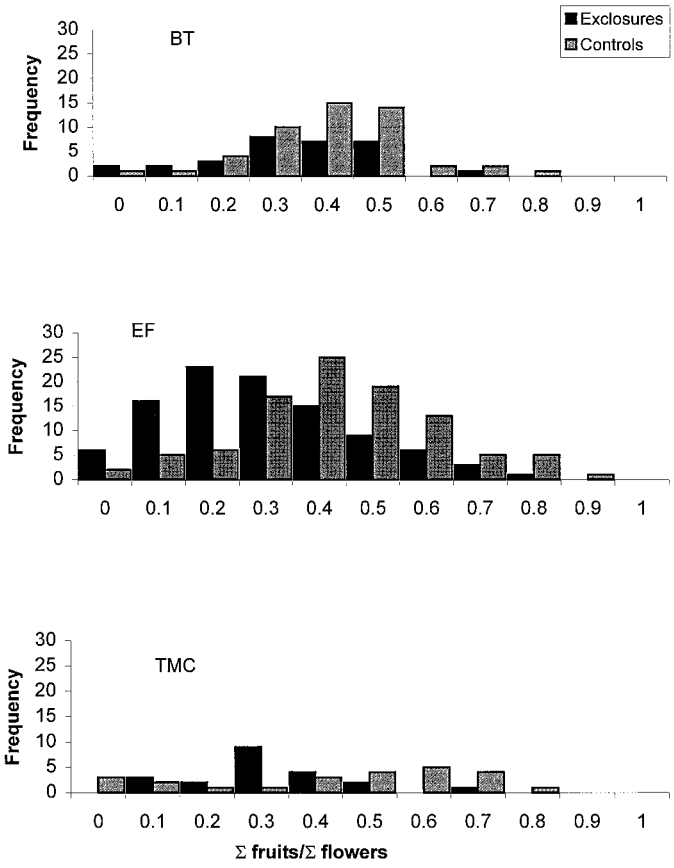

Fig. 6. Frequency of fruit set in plants grown in pollinator exclosure cages (dark bars) and frequency of fruiting success in open-pollinated plants (white bars) in three populations of *C. verna*.

would undoubtedly be sufficient to fertilize all four ovules. Moreover, our study shows that pollen retained in the keel of the flower has a germination rate above 80%, the same as pollen collected from newly dehisced anthers. Thus, accumulated pollen from earlier dehisced anthers may enhance the success of delayed selfing when pollinators are absent.

Our study also reveals that stigmatic receptivity occurs late in a flower's lifetime, generally after the third anther has dehisced. In addition, receptivity is not coincident with the physical flaring of the stigmatic surface (which occurs at the preceding stage). Thus, delayed selfing in *C. verna* is characterized by both diminished herkogamy (Figs. 1, 2; Table 1) and dichogamy (Fig. 5). Our previous observation that most *C. verna* flowers readily set seed autogamously under pollinator-free greenhouse conditions (unpublished data) yet under natural conditions the outcrossing rates are moderate to high (ranging from 60 to 90%, S. Kalisz, unpublished data) is clearly interpretable under the current model of delayed selfing in this species.

Delayed selfing can be selected for if a population experiences unpredictable pollinator service within or among flowering seasons. Two lines of evidence support the idea that our field populations of *C. verna* are pollinator limited and thus will self-pollinate via delayed selfing. First, in our field studies in three natural populations, we found that only about half of the receptive stigmas from stage-4 flowers (i.e., flowers with all four anthers dehisced) had pollen tubes in the style. This result suggests that half of the flowers sampled had not yet been visited by a pollinator, and autogamous pollination was not successful even at this late stage in floral life. However, most (96%) of the stigmas and styles examined shortly after corolla abscission were found to have growing pollen tubes in the styles. Since we know that pollinators do not preferentially visit late-stage flowers, this difference in pollinated styles between stages 4 and 5 suggests that during the year of study (1997), delayed selfing may have been common. We do not know what portion of those pollen tubes observed were from self, rather than from outcross, pollen. Future investigations will determine outcrossing rates in these populations.

The second line of evidence for pollinator limitation and delayed selfing comes from the autogamy rates scored as the number of fruits per flower in plants in the pollinator exclusion treatments in 1998. Autogamy rates ranged from 0 to 80% (average 33%) in the exclosures, whereas the range of successful fruit set under open-pollination was 0–90% (average 44%) (Fig. 6). These data further reveal that for most *C. verna* plants in the pollinator exclosures, autogamy provides substantial and consistent reproductive assurance: average fruit production of plants in the pollinator exclosures was 75% (i.e., 33/44) of the fruit production in open-pollinated plants. While the average autogamy rate in each population was significantly lower than the average fruit set in each population, autogamy rates were nearly constant across the three populations (33, 32, and 34% for BT, EF, and TMC, respectively). The high level of reproductive assurance selfing within the exclosures is consistent with (1) our observation of high levels of pollination late in floral development (Fig. 5) and (2) the fact that styles of most flowers elongate into the zone of dehiscing anthers by the time that the last anther has shed its pollen (Fig. 4). It is notable that in the field exclosures, a few plants (<10%) failed to set any fruit (Fig. 6). Our observations of greenhouse-grown plants revealed that a similarly low percentage of the plants (15%) had styles that failed to elongate into the cluster of dehiscing anthers even after the fourth anther has shed its pollen (Fig. 4). For such individuals with persistent herkogamy, the lack of pollinator activity is likely to result in zero reproductive output. In years in which pollinators fail to visit the population or are present in low abundance, those plants with low autogamy ability could be lost from the population, resulting in an increase in the mean autogamy rate, as was shown by Bixby and Levin (1996) in experimental populations of *Phlox*. Thus, periods in which pollinator activity limits reproductive output may provide a strong selective force maintaining autogamy or, particularly, delayed selfing in these populations, provided the delayed selfing has a genetic basis. While several studies show variation in autogamy rates among cultivars of domesticated plants (reviewed in Rick, 1988), and in wild species (e.g., *Mimulus*—LeClerk-Potvin and Ritland, 1994; *Phlox*—Bixby and Levin, 1996), investigations of interpopulational variation in the *traits* associated with autogamy and delayed selfing are few (e.g., *Mimulus*—Dole, 1992; *Hibiscus*—Klips and Snow, 1997). In general, the genetic variation for delayed selfing traits within and among populations is poorly understood.

The majority of mating system evolution models focus on the role in inbreeding depression (see reviews by Lande and Schemske, 1985; Charlesworth and Charlesworth, 1987, 1990; Uyenoyama, Holsinger and Waller, 1993) and/or population structure (Holsinger, 1991; Ronfort and Couvet, 1995) in determining the equilibrium levels of selfing. However, a model by Schoen and Brown (1991) indicates that if environmental variation exists during the flowering season that could preclude pollinator visitation, reproductive assurance is more important in driving the evolution of mating system than is inbreeding depression and mixed mating will be maintained. The moderate levels of inbreeding depression observed in *C. verna* ($\delta < 0.3$) and the extreme environmental variation experienced by this small winter annual make reproductive assurance models an attractive approach to understand the mating system of this species. Investigations are underway to examine the roles of delayed selfing and environmental heterogeneity in pollinator activity in the evolution of mixed mating in *C. verna*.

Given the fitness advantages of delayed selfing (Lloyd and Schoen, 1992), and its potential role in maintaining mixed mating, it is surprising that fewer than a dozen species have been characterized as having this form of reproductive assurance (see Introduction). We suggest that mechanisms facilitating delayed selfing may be overlooked in many plants. This form of reproductive assurance may be manifested only when pollinators are limiting and will be seen in plants that otherwise appear to be adapted for outcrossing (i.e., large floral display, nectar reward, high pollen/ovule ratio). Although pollen limitation is reportedly common (reviewed in Burd, 1994), it may not occur in all years or at all sites for any given species (e.g., Eckhert and Schaeffer, 1998). Moreover, the

traits associated with delayed selfing may change over the floral life, thus investigations of delayed selfing must be considered within a developmental context, as well as within an ecological context. Information concerning the specific mechanism(s) responsible for self-pollination may provide a crucial insight into understanding how self-fertilization and mixed mating evolve in many natural populations.

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