

FAILURE OF REPRODUCTIVE ASSURANCE IN THE CHASMOGAMOUS FLOWERS OF *POLYGALA LEWTONII* (POLYGALACEAE), AN ENDANGERED SANDHILL HERB¹

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Hypothetically, a species with both cleistogamous (CL) flowers and delayed selfing chasmogamous (CH) flowers should display high levels of reproductive assurance because, over time, obligate selfing by CL flowers should reduce inbreeding depression and delayed selfing in CH flowers should compensate for the absence of outcross pollen. We used pollinator-exclusion experiments to investigate reproductive assurance in the CH flowers of *Polygala lewtonii*, an herb with a mixed mating system. We followed CH flowers from bud-break to flower/fruit abscission to quantify fruit initiation and maturation and rates of floral development. We also evaluated the efficacy of the selfing mechanism, conducted pollinator watches to assess the likelihood of pollinator limitation, and performed regression analysis to determine the effect of flower position on fruit production. Pollinator exclusion significantly reduced fruit initiation and maturation. Investigation of floral development demonstrated that the selfing mechanism is largely dysfunctional in CH flowers, indicating the failure of reproductive assurance. Low observed rates of insect visitation appear to contradict high rates of CH fruit production in open-pollinated plants, particularly given the rarity of delayed selfing. In both treatments, flower position significantly affected fruit initiation, suggesting a role for resource limitation in both pollinator-excluded and open-pollinated flowers.

Key words: autogamy; delayed selfing; pollinator exclusion; *Polygala lewtonii*; Polygalaceae; reproductive assurance; selfing.

At least a third of all flowering plants maintain mixed mating systems that permit both self- and cross-fertilization (Lande and Schemske, 1985; Schemske and Lande, 1985; Barrett and Eckert, 1990; Barrett et al., 1996; Richards, 1997; Vogler and Kalisz, 2001). Each mode of sexual reproduction entails advantages and disadvantages that have been the subject of investigation ever since Darwin (1876, 1877). While many plant species have developed traits that prevent or limit self-fertilization (dioecy, self-incompatibility, herkogamy, dichogamy, heterostyly), many others have developed traits that permit, promote, or ensure it (hermaphroditism, self-compatibility, cleistogamy, autogamy). Obligate outcrossing species may suffer partial or complete sexual reproductive failure due to scarcity or absence of mates or pollinators. Selfing may provide reproductive assurance (Darwin, 1876; Lloyd, 1980, 1992; Holsinger, 1996) by guaranteeing sexual reproduction in the absence of outcrossing. In addition, by avoiding the “cost of meiosis,” selfing has an “automatic selection advantage” (Jain, 1976) because selfed offspring contain two copies of maternal genes compared to only one for outcrossed offspring. The main disadvantage of selfing is inbreeding depression, due primarily to the expression of deleterious recessive alleles (Lloyd, 1980; Barrett and Harder, 1996; Holsinger, 1996; Johnston, 1998).

Species with mixed mating systems may have chasmogamous (CH = open-pollinated) flowers only or a combination

of CH and cleistogamous (CL = obligately selfing) flowers (Cruden and Lyon, 1989; Richards, 1997). At least 287 species in 56 angiosperm families have both CH and CL flowers (Lord, 1981). In mixed mating system species that lack CL flowers, CH flowers generally have morphological or phenological adaptations to promote or at least allow selfing (Lloyd, 1979, 1992; Richards, 1997). Selfing can occur within (autogamy) or between (geitonogamy) CH flowers on a plant, and autogamy may be either autonomous (no insect required) or facilitated (insect required) (Lloyd, 1979, 1992). In addition, autonomous crosses may precede flower opening, co-occur with insect-vectored cross-pollinations, or occur as a fail-safe mechanism to ensure fertilization in the absence of insect pollination (“delayed selfing” sensu Lloyd, 1992).

Although the ability to spontaneously self-fertilize when mates or pollinators are in short supply may provide reproductive assurance (Darwin, 1877; Lloyd, 1980; Holsinger, 1996), its value is undercut if pollen or ovules that would otherwise have been involved in outcrosses are preempted (referred to respectively as pollen and seed discounting; Lloyd, 1992; Holsinger, 1996; Herlihy and Eckert, 2002), or if selfed progeny are significantly inferior to outcross progeny due to inbreeding depression (Charlesworth and Charlesworth, 1987; Holsinger, 1991, 1996; Uyenoyama et al., 1993; Barrett and Harder, 1996; Herlihy and Eckert, 2002). Thus, geitonogamy, selfing between flowers on the same plant, provides no reproductive assurance because it involves both pollen and seed discounting (de Jong et al., 1993). Facilitated autogamy, insect-mediated selfing within a flower, usually does not provide reproductive assurance for the same reason (Schoen and Lloyd, 1992; Eckert, 2000). In contrast, delayed spontaneous selfing (autonomous autogamy) generally provides a large measure of reproductive assurance because it occurs only after flowers have had the opportunity for outcrossing (Lloyd and Schoen, 1992; Barrett and Harder, 1996; Johnston, 1998).

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Most studies of reproductive assurance have relied on species that produce only CH flowers, in which selfing is facultative and perhaps rare (Motten, 1982; Dole, 1992; Klips and Snow, 1997; Pannell and Barrett, 1998; Eckert and Schaefer, 1998; Mallick, 2001; Fausto et al., 2001; Kalisz and Vogler, 2003). In such species, inbreeding depression may be a frequent problem, thus undercutting the advantages of selfing (Herlihy and Eckert, 2002). In contrast, in species with both CL and CH flowers, inbreeding depression may be attenuated due to purging of deleterious recessives (Schoen and Lloyd, 1984; Charlesworth and Charlesworth, 1987; Barrett and Charlesworth, 1991; Barrett and Harder, 1996; Husband and Schemske, 1996; Byers and Waller, 1999; Keller and Waller, 2002). In fact, recent studies of plants with CH/CL flowers have generally found low levels of inbreeding depression (Schmitt and Ehrhardt, 1990; Culley, 2000, 2002). However, recent studies have also shown that even species that routinely self can maintain high levels of inbreeding depression (Lande et al., 1994; Eckert and Barrett, 1994; Dudash et al., 1997; Byers and Waller, 1999).

Polygala lewtonii Small (Polygalaceae), a federally endangered (USFWS, 1999), short-lived, perennial herb endemic to xeric uplands maintained by frequent fire in central Florida (Menges and Weekley, 2003), has both CL and CH flowers (James, 1957; C. Weekley, unpublished report). *Polygala lewtonii* is one of about three dozen amphicarpic species (Cheplick, 1987) in which CL flowers occur on underground stems. CH flowers, which occur on aboveground terminal racemes, are small and pea-like with stigmas and anthers in close juxtaposition. As in other members of its genus, the CH flowers of *P. lewtonii* possess a bi-lobed stigma with one sterile and one fertile lobe (Venkatesh, 1956; Miller, 1971; Brantjes, 1982; Zomlefer, 1989). Typically in Florida species, pollen is deposited on the sterile lobe and transferred to the fertile lobe as the flower ages if it has not been picked up by visiting insects (Zomlefer, 1989). This mechanism putatively ensures autonomous selfing in the absence of insect pollination. Because autonomous selfing occurs only after flowers have been exposed to insects for several days, the mechanism provides for delayed selfing (*sensu* Lloyd, 1992). We have previously documented high fruit set in open-pollinated CH flowers (C. Weekley, unpublished report; Menges and Weekley, 2003), but we do not know what proportion of open-pollinated fruits are the products of outcrossing, geitonogamy or some form of autogamy.

In this study, we focused on the CH flowers of *P. lewtonii* and the efficacy of pollination with and without insect vectors. We tested the hypothesis that delayed selfing in CH flowers provides reproductive assurance. Our expectation was that fruit set in pollinator-excluded CH flowers would approximate fruit set in CH flowers given access to pollinators. Because the small size and intricate arrangement of floral structures in *P. lewtonii* precluded floral manipulations (e.g., emasculations, hand pollinations) usually employed in mating system studies (Schoen and Lloyd, 1992), we relied on pollinator-exclusion experiments to estimate rates of spontaneous selfing (autonomous autogamy) in *P. lewtonii*. Our study had five objectives: (1) to compare percentage fruit initiation and fruit maturation in open-pollinated vs. pollinator-excluded flowers, (2) to compare rates of floral development in the two treatments, (3) to document the mechanics of self-pollination, (4) to investigate the effect of flower position on fruit initiation and fruit maturation in the contrasting treatments, and (5) to quantify insect visitation rates to open-pollinated flowers.

MATERIALS AND METHODS

Study species—*Polygala lewtonii* is a multi-stemmed herb narrowly endemic to yellow sand, xeric upland habitats on the Mount Dora and Lake Wales Ridges of central peninsular Florida (USFWS, 1999). Adults grow to 20 cm in height with few to many (3–30) erect, decumbent, or prostrate stems. Populations typically occupy open areas in longleaf pine-wiregrass (*Pinus palustris* Mill.-*Aristida stricta* Michx. var. *beyrichiana* Trin. & Rupt.) sandhills historically characterized by frequent (2–5 yr) lightning-ignited fires (Myers, 1990; Menges, 1999). *Polygala lewtonii* plants are killed by fire, but postfire seedling recruitment from a long-lived dormant seedbank often results in order-of-magnitude increases in aboveground population size (Menges and Weekley, 2002). Aboveground populations decline with time-since-fire, but even in long unburned sites small populations are maintained by periodic seedling recruitment, particularly during wet winters (e.g., El Niño years).

Bright pink to purple CH flowers occur on many-flowered terminal racemes and are visited by a variety of insects. White CL flowers occur on few-flowered underground rhizomes. In addition, solitary, pale pink CL flowers may occur in the axils of aboveground leaves, although these are extremely rare. CH flowers are zygomorphic and bisexual with the ovary superior and two-loculed, with one ovule in each locule (Zomlefer, 1989). Two of the five sepals are petal-like in color and size, mimicking the wings of papilionoid legumes, and persist after the corolla, stamens and stigma/style abscise. The corolla is reduced to three petals; the upper petals are fused, and the lower petal is a keel with a fringe of fingerlike projections. The fruit is a dehiscent, two-seeded capsule. Seeds bear stiff hairs and two aril-like outgrowths at the micropyle that attract ants (Zomlefer, 1989; Menges and Weekley, 2002).

Like other members of its genus, *P. lewtonii* has a sexual morphology that apparently ensures delayed selfing (Venkatesh, 1956; Miller, 1971; Brantjes, 1982; Zomlefer, 1989). In Florida species, flowers have a bi-lobed pistil consisting of an apical sterile lobe with a tuft of hairs and a sticky fertile lobe (Fig. 1; Zomlefer, 1989). The androecium comprises eight stamens whose fused filaments form a tube partly enclosing the ovary and the style, with the bi-lobed stigma nested among the anthers. During flower opening, pollen is deposited on the sterile lobe. Insect visitors land on the fringed keel petal to access nectar secreted at the base of the flower, picking up pollen from the sterile lobe in the process. Insect visitors may also deposit either outcross pollen or self-pollen on the fertile lobe, thus effecting cross-pollination, facilitated autogamy (if the pollen is from the same flower), or geitonogamy (if the pollen is from another flower on the same plant). As the flower ages, the two stigmatic lobes are thought to fold together, thereby effecting spontaneous self-pollination (Zomlefer, 1989). The functioning of this mechanism in *P. lewtonii* has not been previously investigated.

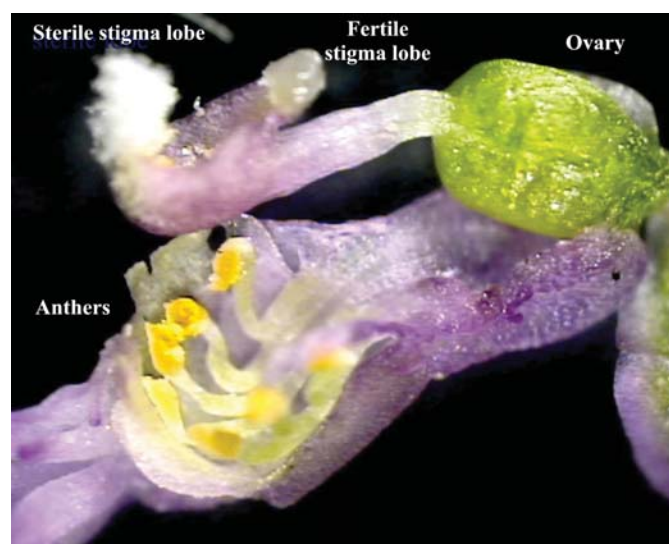


Fig. 1. Microphotograph of *Polygala lewtonii* flower with corolla partially removed to show position of pistil with sterile and fertile stigmatic lobes. The pistil has been lifted free of fused stamens among which it is nested in vivo from tip of anthers to base of ovary is ~ 4 mm.

Study site—We conducted the pollinator-exclusion experiments and the flower visitor observations in spring 2004 at the Lake Wales Ridge National Wildlife Refuge (Carter Creek; latitude 27° 30' N; longitude 81° 22' W) within a population of *Polygala lewtonii* occurring on a sandhill site that had been burned under prescription in 2001. Some of the study plants were postburn seedling recruits, but others may have been older individuals in unburned microsites. Flowers used for photo-documentation of floral development were collected in 2003 from the same site. All lab work was conducted at Archbold Biological Station.

Pollinator-exclusion experiments and floral development observations—In March 2004, we selected 20 pairs of *P. lewtonii* plants at the Carter Creek site. Paired plants were within 0.5 m of each other and thus occupied a shared microhabitat. One member of each pair was enclosed in a Delnet heat-sealed, non-woven pollination bag (Applied Extrusion Technologies, Middleton, Delaware, USA) to exclude pollinators (pollinator-exclusion treatment) and the other was left unbagged (open-pollinated treatment). We chose Delnet pollination bags because they are made of a fine-mesh, polyethylene material with high ratings for air permeability and are designed to minimize alteration of the microenvironment. Bags were supported by bamboo skewers and held in place by sand piled around the base of the bag.

On each plant, we marked three bud-bearing stems with color-coded thread and counted the number of flower buds; altogether, 1645 buds were included in the experimental setup. However, we excluded one pollinator-excluded plant on which only a single flower opened and its paired open-pollinated plant, reducing to 38 the number of paired plants included in the analyses below. An additional 134 buds were either lost to herbivores or failed to develop. Thus, we actually observed 1468 developing flowers over the course of the experiment (794 in the open-pollinated treatment and 674 in the pollinator-excluded treatment). There was no significant difference in the mean number of flowers per plant (paired *t* test: $t = 1.333$, $df = 18$, $P = 0.199$) or per raceme ($t = 1.738$, $df = 56$, $P = 0.088$) in the two treatments. However, plants in the pollinator-excluded treatment had a significantly higher ($Z = -2.797$, $P = 0.005$) percentage of open flowers than plants in the open-pollinated treatment.

In *P. lewtonii*, blooming occurs acropetally (from the base to the tip of the raceme), thereby facilitating tracking of flower and fruit development. On each marked raceme, we numbered enlarging buds sequentially from the base of the raceme to its apex. Conspicuous abscission scars marked the location of abscised flowers or fruits. For 59 days, we followed developing flowers daily from bud enlargement through flower opening to flower or fruit abscission. We recognized five stages of flower development: (1) swollen bud, (2) sepals expanded, (3) flower open, (4) flower closed, and (5) developing fruit. Flowers usually opened between 0900 and 1000 hours and closed between 1600 and 1700 hours on successive days. Flowers may open and close daily for several days, but apparently once a flower is pollinated it ceases to re-open. To evaluate rates of floral development, we compared the number of days that flowers in the two treatments spent in each of the five stages. We used survival analysis to compare rates of fruit abscission and also used the resulting survival curves to distinguish between fruit abortion and fruit maturation.

We defined fruit initiation as the point at which the petals, stamens, style, and stigmas abscised, leaving the two petaloid sepals between which the enlarging ovary/developing fruit could be seen. Fruits were defined as mature if they could be detached from the raceme with a slight touch and were fully enlarged or if abscission occurred more than 22 d after initiation. We arrived at the 22-d threshold by calculating the mean time to abscission of all developing fruits and by looking for breakpoints in the survival curves.

Microphotographic documentation of floral development—In 2003, using the same Carter Creek population as in the pollinator-exclusion experiments, we chose a group of *P. lewtonii* plants to provide flowers for microscopic observation and documentation of floral development. Five plants were bagged with Delnet mesh pollination bags to exclude pollinators, and five others were left unbagged to permit insect pollination. On each plant, 3–5 flowering stems were marked with color-coded thread. We collected 10–20 flowers of known age daily from bagged and unbagged plants. Flowers were placed in plastic petri dishes for transport to the lab and were generally examined within 2–3 h of collection. Altogether we dissected and examined approximately 300 *P. lewtonii* flowers, ranging from 1 to 15 days in age. For comparison of floral functioning, in 2004, we collected flowers from three widespread congeners, *P. nana*, *P. rugellii*, and *P. setacea*, which, in central Florida, occupy mesic to wet flatwoods habitats. Flowers of all four species

were dissected in the lab using an Olympus SZX12 (Olympus America, Melville, New York, USA) dissecting stereomicroscope and photographed using an Optronics 1-CCD digital video camera (Optronics, Goleta, California, USA) and Image-Pro Plus (version 4.1) image analysis software (Media Cybernetics, Silver Spring, Maryland, USA).

Effect of flower position on fruit initiation and maturation—In our monitoring scheme, flower number was an index of flower position along the raceme. Thus, in following flower development, we were also able to evaluate the effect of flower position on fruit initiation and maturation.

Insect visitation to open-pollinated plants—During the spring 2004 flowering season, we conducted 25 30-min pollinator watches in four patches of *P. lewtonii* at Carter Creek, each patch with at least seven flowering plants. We focused each watch on a single focal plant within the patch and recorded data on the number of insect visitors, the number of visits to the patch and to the focal plant, and the number of flowers visited. At the beginning of each watch, we recorded the temperature and noted whether the sky was clear, partly cloudy, or overcast.

Statistical methods—For analysis of data on fruit initiation, fruit maturation, and rate of floral development, mean values for each plant in the two treatments constituted the response variable. These data were generally nonnormal and could not be transformed to normality. Therefore, we used the nonparametric Wilcoxon signed rank test to evaluate the significance of differences between the two treatments. Survival analysis of rates of fruit abscission was based on survival curves and on the Wilcoxon (Gehan) statistic. We used regression analysis to evaluate the effect of flower position on fruit initiation and maturation. All statistical tests were performed in SPSS (version 11.5; SPSS, 2002).

RESULTS

Fruit initiation and maturation of pollinator-excluded vs. open-pollinated flowers—Fruit initiation, fruit maturation as a percentage of flowers available, and fruit maturation as a percentage of fruits initiated were all significantly lower in pollinator-excluded flowers than in open-pollinated flowers (Fig. 2). Overall, the 794 open-pollinated flowers initiated 656 fruits (82.6%), while the 674 pollinator-excluded flowers initiated only 69 fruits (10.2%). On a per-plant basis, percentage fruit initiation was significantly greater for open-pollinated than for pollinator-excluded plants ($Z = -3.823$, $P < 0.0001$). In the open-pollinated treatment, the mean percentage initiation (\pm SD) was $81.5 \pm 8.8\%$ and ranged from 66.7 to 100%. In the pollinator-exclusion treatment, the mean percentage initiation was $12.2 \pm 14.4\%$; three pollinator-excluded plants initiated no fruits and for the 16 that did so, percentage initiation ranged from 2.6 to 50.0%.

Overall, 58.8% of open-pollinated flowers produced fruits (467/794), while only 2.8% of pollinator-excluded flowers (19/674) did so. On a per-plant basis, the percentage fruit maturation as a proportion of total flowers available was significantly greater in the open-pollinated than in the pollinator-excluded plants ($Z = -3.724$, $P < 0.0001$) with a mean of $55.6 \pm 18.4\%$ for the open-pollinated plants, but only $2.7 \pm 3.7\%$ for the pollinator-excluded plants. For open-pollinated plants, the percentage maturation as a proportion of flowers ranged from 0 (one plant) to 82.8%; corresponding values for pollinator-excluded plants ranged from 0 (for 11 plants) to 16.0%.

Open-pollinated and pollinator-excluded flowers also differed significantly in the percentage of fruit initiations resulting in mature fruits. Globally, 71.2% of open-pollinated flowers that initiated fruits successfully matured them (467/656), while only 27.5% of pollinator-excluded flowers (19/69) did so. On

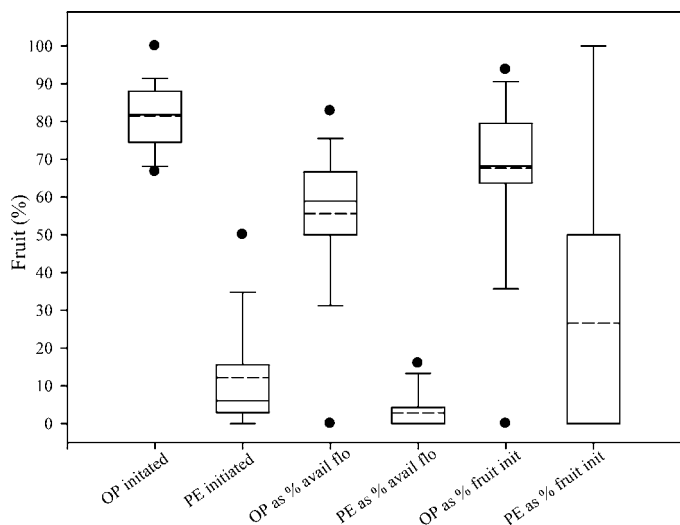


Fig. 2. Boxplots of percentage of fruits initiated and matured for the open-pollinated (OP) and pollinator-excluded (PE) treatments (“avail flo” = available flowers; “fruit init” = fruits initiated). On each boxplot, lower and upper solid horizontal bars represent the 25th and 75th percentiles, respectively, the solid middle bar represents the median, and the dashed bar represents the mean. The lower and upper “whiskers” show the 10th and 90th percentiles, respectively, and solid dots represent outliers. When percentage fruit initiated has a median of 0 (as with the fourth and sixth boxplots), the lower horizontal line is the median, below which there are no lower values.

a per-plant basis, open-pollinated plants matured $67.7 \pm 21.0\%$ of fruits initiated, while pollinator-excluded plants matured only $26.6 \pm 36.6\%$, and again the difference was significant ($Z = -3.332, P = 0.001$). With one exception, all open-pollinated plants produced mature fruits, with the percentage yield for the other 18 plants ranging from 33.3 to 93.8%. Ten pollinator-excluded plants produced no mature fruits, with percentage yield in the other nine pollinator-excluded plants ranging from 7.7 to 100%.

Rate of floral development in pollinator-excluded vs. open-pollinated flowers—There were no differences between treatments in the number of days that a flower spent in stages 1 (bud enlarged) or 2 (sepals expanded). While stages 3 (flower open) and 4 (flower closed) were significantly more rapid in the open-pollinated treatment, stage 5 (developing fruit) was significantly more protracted in the open-pollinated flowers (Fig. 3). Pollinator-excluded flowers opened on twice as many days as open-pollinated flowers (4.4 ± 0.7 vs. 2.2 ± 0.4 days), and the difference was significant ($Z = -3.823, P < 0.0001$). Pollinator-excluded flowers spent about 2.5 times as long in the closed flower stage as open-pollinated flowers (13.7 ± 4.9 vs. 5.7 ± 1.2 d; $Z = -3.823, P < 0.0001$), probably reflecting a longer time to petal abscission in unpollinated flowers. However, fruits from open-pollinated plants abscised significantly later than their pollinator-excluded counterparts (22.2 ± 4.7 vs. 10.4 ± 10.1 d; $Z = -3.219, P = 0.001$).

Survival analysis also determined that fruit abscission was significantly more rapid in the pollinator-excluded than in the open-pollinated plants (Fig. 4; Wilcoxon (Gehan) statistic = 65.415, $df = 1, P < 0.0001$). On a per plant basis, median time to abscission was 26.8 d for open-pollinated fruits, but only 7.6 d for pollinator-excluded fruits. By day 5 post-initiation,

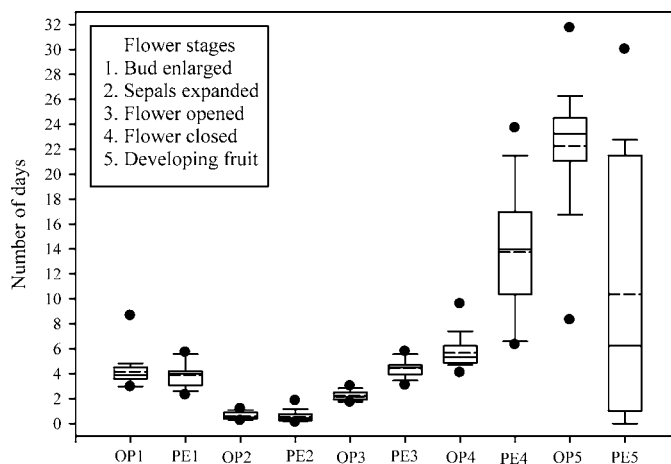


Fig. 3. Boxplots of number of days that flowers within each treatment spent in each of five stages of flower development. On each boxplot, lower and upper solid horizontal bars represent the 25th and 75th percentiles, respectively; the solid middle bar represents the median and the dashed bar represents the mean. The lower and upper “whiskers” show the 10th and 90th percentiles, respectively, and solid dots represent outliers. OP = open-pollinated, PE = pollinator-excluded. Five stages of flower development are explained in the legend.

approximately 50% of the pollinator-excluded fruits had abscised, while less than 10% of open-pollinated fruits had done so. After day 22, abscission in the open-pollinated treatment accelerated rapidly and there was also a small but obvious jump in abscission in the pollinator-excluded plants.

Microphotographs of floral development—In microscopic examination of approximately 300 *Polygala lewtonii* flowers observed over a 15-d period, we found no evidence of the closing together of the two stigmatic lobes to effect self-pollination. By day 6, some open-pollinated flowers had developing fruits (Fig. 5A), but no pollinator-excluded flowers did. However, in some pollinator-excluded flowers

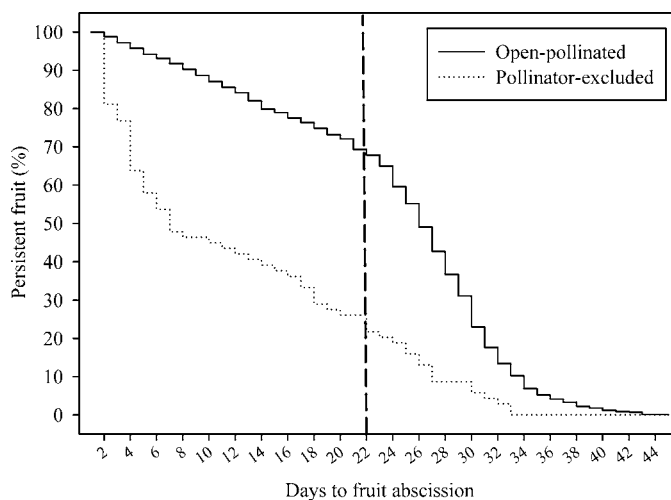


Fig. 4. Survival curves showing time to abscission of developing fruits in the open-pollinated and pollinator-excluded treatments. The vertical reference line at day 22 shows approximate breakpoint for fruit maturation.

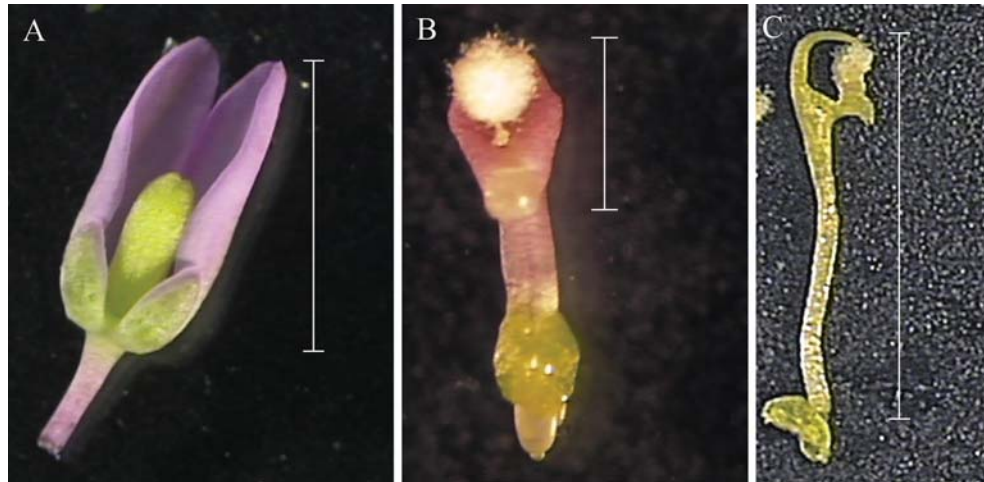


Fig. 5. Comparative floral development in two species of *Polygala*. (A) Developing fruit in an open-pollinated flower of *Polygala lewtonii*. Scale bar = 6 mm. (B) Pistil from 7-d-old pollinator-excluded flower of *P. lewtonii*, showing no sign of closure between the sterile and fertile stigmatic lobes; note pollen on sterile lobe and in trough between the lobes. Scale bar = 3.75 mm; distance between stigmatic lobes is approximately 0.5 mm. (C) Flower of *P. rugellii*, several days old, with pollen-bearing stigmatic lobe folded to make contact with fertile lobe. Scale bar = 4 mm.

pollen was detected on the fertile lobe even though the lobes were still well separated (Fig. 5B), and pollen was frequently seen in the trough between the lobes in flowers of both treatments. In contrast, we readily documented the pollen-bearing sterile lobe folding back to deposit pollen on the fertile lobe in the congeners *P. rugellii* (Fig. 5C), *P. nana*, and *P. sectacea*.

Effect of flower position on fruit initiation and maturation—There was a significant negative correlation between flower position and percentage fruits initiated (Fig. 6A) for both the open-pollinated ($r^2 = 0.688$, $P < 0.0001$) and the pollinator-excluded ($r^2 = 0.317$, $P = 0.006$) treatment. In both treatments, a higher percentage of fruits was initiated near the base of the raceme than near the apex. There was also a significant negative relationship between flower position and percentage fruit maturation (Fig. 6B) for the open-pollinated treatment ($r^2 = 0.273$, $P = 0.004$), indicating that many initiated fruits failed to develop in this treatment. However, in the pollinator-excluded treatment, the relationship was weak and nonsignificant ($r^2 = 0.007$, $P = 0.70$).

Insect visitation at open-pollinated flowers—We observed insect visitors in only 12 of the 25 watches, with a total of 16 visitors representing three orders and at least seven species (Table 1). Sulfur butterflies were the most frequent visitors, but bee-flies visited the most flowers. Twenty-one watches took place between 0900 and 1200 hours, with two watches before 0900 and two shortly after 1200 hours. We observed no visitors before 0900 hours and 12 of 16 between 0900 and 1200 hours. Thirteen of 16 visitors appeared on partly cloudy days (0.867 visitors per watch), three on clear days (0.375 visitors per watch), and none on overcast days. Visitors seemed most active during watches when the temperature was between 21 and 30°C; however, only four watches occurred during periods when it was either cooler or warmer. During the 12.5 h of observations, insects visited a total of 60 flowers on plants within the four study patches, for a visitation rate of 0.3 flowers per insect per hour.

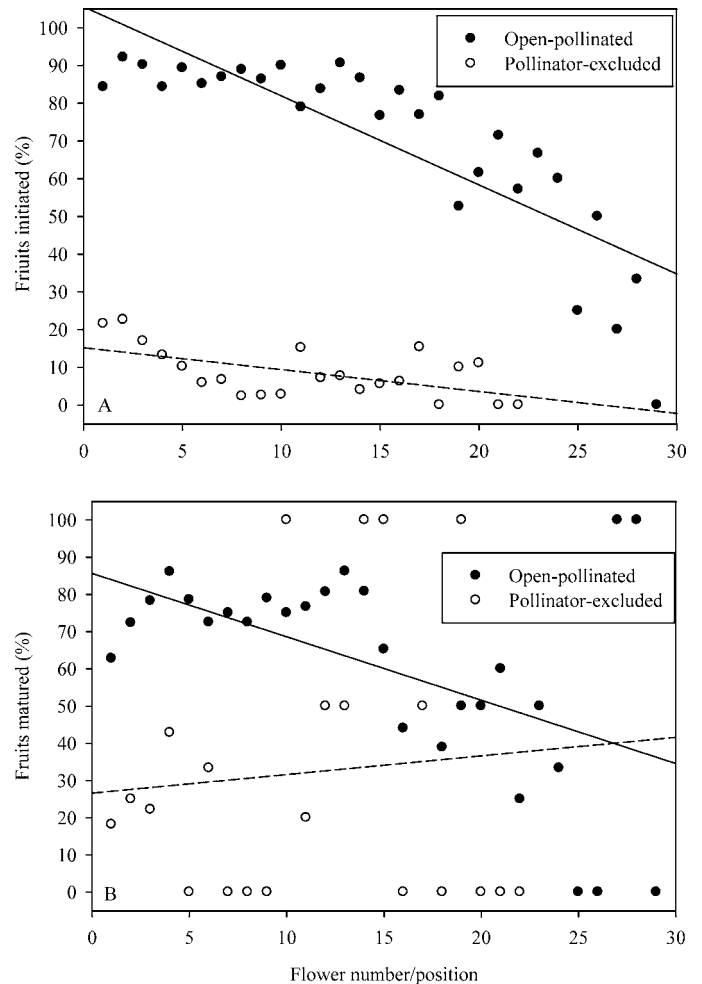


Fig. 6. Effects of flower position on fruit production. Fruit initiation (A) and fruit maturation as percentage of fruits initiated (B) of open-pollinated and pollinator-excluded flowers of *Polygala lewtonii* as a function of position of flower on the raceme. Flowers are numbered along the raceme from the base to the apex.

TABLE 1. Summary of insect visitors to patches of *Polygala lewtonii* during 25 30-min pollinator watches. Shown are the order and taxon of insect (when known), the number of individuals, the number of visits to a patch and to a focal plant, and the number of individual flowers visited.

Order	Taxon	No. insects	No. visits/patch	No. visits/focal plant	No. flowers visited
Diptera	<i>Geron</i> sp.	2	3	0	18
	Fly	2	4	2	10
	Gnat	2	2	0	4
	"Mosquito fly"	2	6	1	9
Hymenoptera	Halictidae	2	2	0	2
Lepidoptera	Pieridae	5	6	1	14
	Small moth	1	1	0	3
TOTALS		16	24	4	60

DISCUSSION

Fruit initiation and maturation in open-pollinated and pollinator-excluded flowers—Denying the chasmogamous flowers of *Polygala lewtonii* access to insects results in significantly lower fruit initiation, fruit maturation as a percentage of flowers available for pollination, and fruit maturation as a percentage of fruits initiated. Drastically reduced fruit initiation by pollinator-excluded flowers (10.2 vs. 82.6% for open-pollinated flowers) implicates dysfunction or inefficiency of the mechanism that putatively ensures delayed selfing in the absence of insect pollinators. Lower fruit production in the pollinator-excluded treatment (27.5 vs. 71.2%) suggests inbreeding depression in autonomously selfed flowers, although other factors may be involved. Thus, reproductive assurance (Lloyd, 1992; Holsinger, 1996; Kalisz and Vogler, 2003; Kalisz et al., 2004), the ability of a self-compatible species to compensate for pollen- or pollinator-limitation by spontaneously selfing, is severely limited in the chasmogamous flowers of *P. lewtonii* and fruit production is minimal in the absence of insect visitors.

The failure of approximately 16% of pollinator-excluded *P. lewtonii* plants (3 of 19) to initiate fruits indicates a total dysfunction of the selfing mechanism in the bagged flowers on these plants, but fruit initiation as high as 50% in 13 other plants (range 2.6–50.0%) suggests that a modicum of functionality may be maintained in some individuals. The degree of dysfunction may vary by genotype, plant age, or some other life history parameter. The poor reproductive performance of bagged flowers raises the concern that these results are an artifact of the bagging treatment itself. Depending on the materials used, pollination bags may alter the temperature, relative humidity, or solar radiation experienced by bagged flowers (Kearns and Inouye, 1993). We chose Delnet polyethylene bags because their high air permeability is designed to minimize alteration of the microenvironment. In our own field tests, we found that the temperature inside bags differed by less than one degree over a 24-h period from temperatures outside bags and that even short-term differences of up to 5°C fell within the range of temperatures normally experienced by *P. lewtonii* during its flowering season. Similarly, the ranges of relative humidity and solar radiation inside the bags are unlikely to differ significantly from the ambient conditions under which *P. lewtonii* normally flowers. In addition, we found no evidence of the closing together of stigmas in either bagged or unbagged

flowers. We conclude, therefore, that the reproductive failure observed in *P. lewtonii* in this study is not an artifact of the bagging treatment.

In contrast to the high rate of reproductive failure in the pollinator-excluded treatment, in open-pollinated plants a minimum of 66.7% of flowers initiated fruits and in 11 of 19 plants (57.9%) fruit initiation was greater than 80%. Fruit initiation in the open-pollinated treatment may have been due to facilitated (insect-mediated) selfing, geitonogamy (within-plant pollen transfers), or outcrossing, as well as spontaneous selfing via the closing together of the stigmatic lobes. Although pollinator-exclusion experiments provide an estimate of spontaneous selfing (autonomous autogamy) rates, they unfortunately do not allow for partitioning the modes of fruit initiation in open-pollinated flowers. Sire-exclusion analysis using molecular markers such as microsatellites would provide an assessment of the degree of selfing within a population with a mixed mating system, but cannot distinguish between geitonogamy and various modes of autogamy (Schoen and Lloyd, 1992). Both geitonogamy and insect-mediated autogamy are likely in *P. lewtonii*. Neither mode of self-fertilization contributes to reproductive assurance since both co-opt pollen and ovules otherwise available for outcrossing (Schoen and Lloyd, 1992; de Jong et al., 1993; Eckert, 2000).

Fruit maturation as a percentage of fruits initiated in the open-pollinated treatment was 2.6 times greater than in the pollinator-excluded treatment. Only one open-pollinated plant failed to produce mature fruits, and 16 of 18 plants had fruit yields greater than 60%. In contrast, seven of the 16 pollinator-excluded plants (43.7%) that initiated fruits failed to mature them. The high rate of fruit abortion in the pollinator-excluded treatment is undoubtedly due primarily to inbreeding depression. This is a surprising result because of the expectation that in a mixed mating system species, routine selfing by CL flowers would purge most deleterious lethal alleles (Schoen and Lloyd, 1984; Charlesworth and Charlesworth, 1987; Barrett and Charlesworth, 1991; Barrett and Harder, 1996; Husband and Schemske, 1996; Byers and Waller, 1999; Keller and Waller, 2002). Although some studies have found that outcrossed progeny from mixed mating system (CH/CL) species have a fitness advantage over selfed progeny (Schemske, 1978; Waller, 1984; Mitchell-Olds and Waller, 1985), many other studies have shown that selfed progeny are as least as fit as outcrossed progeny over a range of life history stages (Wilken, 1982; Cheplick and Quinn, 1982, 1983; Clay 1983, Clay and Antonovics, 1985; Antlfinger 1986; Gara and Muenchow, 1990; Le Corff, 1996; Berg and Redbo-Torstensson, 1999, 2000). However, few studies have investigated early acting inbreeding depression by quantifying fruit maturation rates as a percentage of fruit initiations (Husband and Schemske, 1996).

Recent studies of other mixed mating system species have found evidence for (e.g., Motten, 1982; Piper et al., 1986; Dole, 1992; Klips and Snow, 1997; Fausto et al., 2001; Culley, 2002; Elle and Carney, 2003; Kalisz and Vogler, 2003; Kalisz et al., 2004) and against (e.g., Eckert and Schaefer, 1998; Herlihy and Eckert, 2002) the reproductive assurance hypothesis. However, to the best of our knowledge, only one previous researcher (Culley, 2002) has investigated reproductive assurance in the CH flowers of a species with both CH and CL flowers. Culley (2002) found that both the mixed mating (CH/CL) system and delayed selfing in the CH flowers of

Viola pubescens contributed to the reproductive output of the species across heterogeneous environments in which both pollinator service and resources are potentially limiting.

In the case of *Polygala lewtonii*, no assessment of the contribution of CL flowers to its reproductive output has been undertaken. However, our results demonstrate that little reproductive assurance is provided by the CH flowers due to the inefficiency of the delayed selfing mechanism and high rates of fruit abortion due to inbreeding depression and perhaps other causes.

Rates of floral development—The two most important differences in floral development between pollinator-excluded and open-pollinated flowers were the number of days that flowers in the two treatments were open and the number of days from fruit initiation to fruit abscission. Pollinator-excluded flowers opened twice as often as open-pollinated flowers (approximately 4 vs. approximately 2 days), suggesting that open-pollinated flowers cease to open once they have been pollinated by visiting insects and that unpollinated flowers have a mean life span of approximately 4 days. The median time to abscission of fruits in the pollinator-excluded treatment was only about a third of that for open-pollinated plants (7.63 vs. 26.85 days), indicating the much higher rates of abortion in obligately-selfed fruits than in open-pollinated fruits. We interpret the higher abortion rates of pollinator-excluded flowers as evidence of early acting inbreeding depression, although resource limitation may also play a role (discussed later).

Mechanics of delayed selfing—Dissections of *P. lewtonii* flowers of known age, documented by microphotographs, provided no evidence that the two lobes of the bi-lobed stigma close together to effect spontaneous selfing. In contrast, using the same procedures for collecting and examining flowers, we readily documented the closing together of the stigmatic lobes in three other *Polygala* species. Thus, the mechanism putatively ensuring delayed autonomous selfing in *P. lewtonii* appears to be largely dysfunctional. However, approximately 10% of pollinator-excluded flowers did initiate fruits, suggesting that the mechanism is not totally dysfunctional. While the selfing mechanism has been documented in several species of *Polygala* (Venkatesh, 1956; Miller, 1971; Brantjes, 1982; Zomlefer, 1989), we are not aware of any studies which quantify its efficacy or which investigate the level of reproductive assurance provided by delayed selfing in any of the approximately 600 *Polygala* species known worldwide.

Flower position and resource limitation—Resource limitation is a potential source of flower and fruit abortion (Stephenson, 1981) in *Polygala lewtonii*. In both the pollinator-excluded and the open-pollinated treatments, fruit initiation was significantly higher at the base of the flowering raceme than at its apex. In open-pollinated plants this could reflect pollinator foraging behavior, but because the pattern is the same for pollinator-excluded plants, resource limitation may also play a role. Fruit maturation was also significantly higher at the base of the flowering raceme than at its apex for open-pollinated plants, again suggesting resource limitation. The weak correlation between flower position and fruit maturation in the pollinator-excluded treatment reflects the over-all low percentage of fruit initiation in flowers denied

access to pollinators rather than a meaningful difference in patterns of fruit maturation.

In addition, differential resource allocation is also a possible explanation for differences in rates of fruit maturation between pollinator-excluded and open-pollinated *P. lewtonii* plants. The protracted floral phenology of pollinator-excluded flowers compared to open-pollinated flowers may contribute to lower rates of fruit maturation because of the higher energetic costs of repetitively opening flowers and perhaps replenishing nectar rewards. However, it seems unlikely that the cost of maintaining unfertilized flowers would be greater than the cost of fruit development.

Pollinator limitation—It is difficult to reconcile our observations of low insect visitation rates in *P. lewtonii* with the relatively high rates of fruit initiation and maturation. Within patches of plants containing dozens of flowering racemes, the observed rate of insect visitation (number of flowers visited per unit time) was only 0.3 flowers per hour, which seems inadequate to pollinate the hundreds of flowers opening over a span of several days. However, given high levels of fruit abortion in pollinator-excluded plants (suggesting that self-pollinations often result in abortion) and high levels of fruit maturation in open-pollinated plants, it seems that many plants must be adequately serviced; in this study, over 80% of open-pollinated flowers initiated fruits and two-thirds of initiated fruits developed to maturity.

Conclusion—Due to the inadequacy of the delayed selfing mechanism and inbreeding depression in the relatively small number of fruits initiated in the pollinator-excluded CH flowers of *P. lewtonii*, these flowers provide only minimal reproductive assurance. However, underground CL flowers, not investigated here, may provide an additional component of reproductive assurance even in the face of substantial inbreeding depression. Assessment of the role of cleistogamy in the overall reproductive output of *Polygala lewtonii* awaits the development of a protocol for monitoring the development of underground CL flowers and fruits.

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