# FACTORS AFFECTING LOW SEED : OVULE RATIOS IN A SPRING WOODLAND HERB, *TRILLIUM GRANDIFLORUM* (MELANTHIACEAE)

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Female fertility in flowering plants is commonly observed to be submaximal. The fraction of ovules in a flower that produce seeds is commonly less than 1.00, particularly in outcrossing species. Here we investigate through controlled pollinations factors that may contribute toward low seed : ovule ratios in *Trillium grandiflorum*, a self-incompatible woodland herb from eastern North America. Reduced fruit and seed set in open-pollinated plants in comparison with hand cross-pollinated plants demonstrated that pollen limitation was a contributing factor to low female fertility. However, seed : ovule ratios of hand cross-pollinated flowers averaged only 0.66. Experimental pollinations involving the manipulation of pollen age, the number of pollen donors, the timing of pollination, and the application of self-pollen before cross-pollen each had no effect on fertility. This indicated that aspects of pollen delivery were not responsible for low seed : ovule ratios and that resources might influence variation in seed : ovule ratios. An analysis of the relation between plant size and seed set in hand cross-pollinated plants provided evidence that resources limited fertility when ovules of *T. grandiflorum* were not pollen limited. We discuss these results in the context of the stochastic pollination environment that characterizes the early spring flowering period of *T. grandiflorum*.

*Keywords:* seed : ovule ratios, pollen limitation, resource limitation, *Trillium grandiflorum*, female fertility.

## Introduction

It is commonly observed that female fertility in flowering plants rarely reaches its maximum of one fruit per flower and one seed per ovule in outcrossing species. Diverse genetic and environmental factors can contribute toward low fertility in plant populations (Stephenson 1981; Sutherland and Delph 1984; Wiens 1984; Sutherland 1986; Lee 1988; Charlesworth 1989a, 1989b). Pollen limitation because of insufficient pollinator service has been most commonly investigated, particularly in animal-pollinated plants (reviewed in Burd 1994; Larson and Barrett 2000). Pollen limitation of fertility can be demonstrated experimentally when the addition of supplemental pollen to stigmas increases fruit and/or seed set. In a survey of 258 angiosperm species, Burd (1994) found that fruit and seed set were pollen limited in 60% and 45%, respectively, of the species he examined. However, even when abundant compatible pollen is applied to stigmas in experimental hand-pollinations, not all flowers produce fruit or ovules in a flower mature seeds. If so, the ratio of fruits per flower and/or seeds per ovule (hereafter, S : O ratio) is less than 1. For example, of 29 species that were pollen limited in Burd's survey, none had S : O ratios of 1.0 when manually cross-pollinated (Burd 1994). Clearly, factors other than pollen limitation must also contribute to variation in female fertility in plants.

Resources influence many aspects of plant reproduction, including flower and fruit production and the number of seeds that are matured. Resource limitation of fertility can be demonstrated when the addition of physical resources (e.g., water, nutrients) increases fruit and/or seed set compared with unmanipulated conditions (Lee and Bazzaz 1982; McCall and Primack 1987; Campbell and Halama 1993). However, in perennial species, reproductive success may be influenced by resource conditions in both the current and previous seasons; hence, long-term experiments are often necessary to detect resource limitation. Another means of indirectly detecting resource limitation of fertility is through an examination of the relation between seed production and plant size or status (fig. 1). If resources limit fertility, larger plants with more resources for seed production might be expected to mature a greater proportion of seeds per ovule than would smaller, resource-limited plants. This should result in an accelerating relation between plant size and female fertility (fig. 1a) and a positive relationship between the rate of seed production (i.e., S : O ratio) and plant size (fig. 1b). Together, resources and pollen availability are likely to be the principal joint determinants of variation in plant fertility (Bell 1985; Haig and Westoby 1988; Campbell and Halama 1993; Burd 1994; Goodwillie 2001).

Other less well studied factors can also result in reduced fertility in plant populations, including genetic load and features of the pollination process beyond the total amount of pollen delivered to stigmas. Genetic load influences fertility through the abortion of developing embryos that are homozygous for deleterious alleles (Charlesworth 1989a). Such an effect is most likely to occur through inbreeding in normally outcrossing species. Mechanisms that operate during pollination include the quality of pollen available to fertilize ovules, such as the number and/or relatedness of pollen donors (Marshall and Ellstrand 1986; Waser and Price 1989) and the viability of pollen (Thomson and Thomson 1992). Self-pollen

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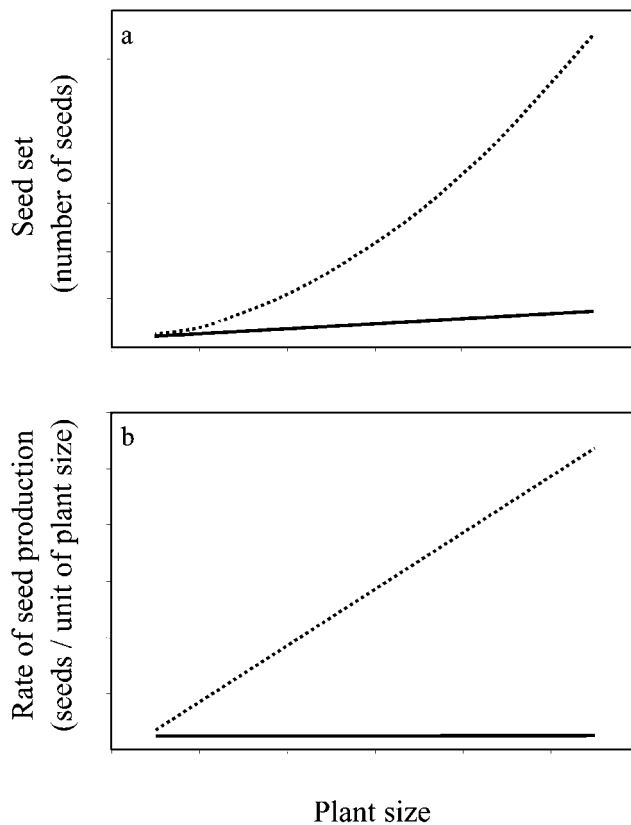
**Fig. 1** Hypothetical relations between seed production and plant size (*a*) and the rate of seed production and plant size (*b*). Note that we use the seed : ovule ratio as a convenient measure of the rate of seed production. When pollen is limiting (solid line), seed set (*a*) is a linear function of plant size. When pollen is abundant (dashed line), there is an overall increase in seed set for plants of all sizes. Small plants that are resource limited set seed at a lower rate than larger plants that are not resource limited. This leads to an accelerating relation between seed set and plant size. A means of detecting such a relation is to take the derivative of seed set with respect to plant size (i.e., the rate of seed production; *b*). When pollen is limiting, the rate of seed production will be the same for plants of all sizes. When pollen is abundant, the accelerating relation between seed production and size will result in a positive linear relation between the rate of seed production and plant size.

on stigmas can also interfere with outcrossing, resulting in reduced seed set (reviewed in Barrett 2002). Each of these factors has been demonstrated to influence seed set in particular species of herbaceous flowering plants. However, few studies have simultaneously examined how these pollination-related factors may contribute to low S : O ratios in natural populations.

*Trillium grandiflorum* (Melanthiaceae) provides a useful model system to examine ecological factors limiting reproductive success in flowering plants. The species is a bee-pollinated, self-incompatible, spring woodland herb that is widespread in deciduous forests in eastern North America (Case and Case 1997; Sage et al. 2001). *Trillium* species, including *T. grandiflorum*, often exhibit reduced fruit and seed production (Kawano et al. 1986; Lubbers and Lechowicz

1989; Ohara et al. 1990; Lapointe 1998; Smith 1998; Kalisz et al. 1999; Wright and Barrett 1999; Irwin 2000). Spring ephemerals are notorious for unpredictable pollinator service (Schemske et al. 1978; Baker et al. 2000), and low visitation rates to *T. grandiflorum* may account for pollen limitation of fertility in some populations (Smith 1998). However, previous work has demonstrated that even when abundant cross-pollen is manually applied to stigmas of *T. grandiflorum*, the S : O ratio increases but does not reach a maximum of 1 (Smith 1998; Wright and Barrett 1999; Irwin 2000). This raises the question of what other factors might limit female fertility in *T. grandiflorum*. Extensive populations composed of thousands of plants and a large, easily manipulated flower make *T. grandiflorum* useful for experimental studies aimed at investigating the proximate mechanisms influencing S : O ratios in flowering plants.

The goals of this study were to examine factors that may contribute to the low S : O ratios observed in *T. grandiflorum* at a site in southern Ontario, Canada. First, as discussed above, pollen limitation is a common cause of low fertility. Therefore, we assessed the degree of pollen limitation of fruit and seed set by comparisons of open- and hand cross-pollinated flowers. Second, as pollinator visitation rates are unreliable in spring, we examined the relation between the exposure time of flowers to pollinators and fertility. Third, using experimental pollinations, we investigated four hypotheses concerned with the control of seed set in *T. grandiflorum*. We briefly outline each hypothesis and its predictions.

*The number of pollen donors affects S : O ratios.* Multiple paternity increases seed set per fruit in *Raphanus sativus* (Marshall and Ellstrand 1986). If a similar mechanism occurs in *T. grandiflorum*, the S : O ratio should increase as the number of pollen donors increases. Presumably, this effect arises because a higher pollen donor number increases the probability of favorable mating combinations.

*Even when abundant pollen is supplied, reduced viability of pollen leads to low S : O ratios.* Pollen of *Erythronium grandiflorum*, another early spring–flowering herb, only germinates on the day of anther dehiscence (Thomson and Thomson 1992). If pollen of *T. grandiflorum* becomes less potent over time, then the S : O ratio should be high when fresh pollen is used for cross-pollination and should decrease when older pollen is applied.

*Reduced S : O ratios occur if some ovules are not mature when pollination occurs.* Asynchronous ovule development has been demonstrated in *Narcissus triandrus* (Sage et al. 1999). If ovules mature differentially in *T. grandiflorum* and remain receptive for only a few days, pollination on a single day may not fertilize all ovules since some are undeveloped or unreceptive. Consequently, pollination on multiple days should increase seed set in comparison with pollination on a single day only. Alternatively, ovules might mature sequentially and remain receptive until fertilized. Thus, there would be few mature ovules when the flower has just opened, but all ovules are mature by the end of floral life. If so, pollination late in floral life should lead to increased S : O ratios compared with pollinations soon after flower opening.

*Self-pollination interferes with later cross-pollination, thus resulting in reduced fertility.* The inhibitory effects of prior self-pollination on the seed set of hand cross-pollinated flowers

have been demonstrated in several species (Waser and Price 1991; Broyles and Wyatt 1993). Prior self-pollination may reduce seed set if self-pollen physically blocks the stigma or style (Shore and Barrett 1984), causes flower senescence (Reid 1989; Visser 1986), or curtails ovule development (ovule discounting *sensu* Barrett et al. 1996), thus preventing subsequent fertilization by cross-pollen. If so, flowers of *T. grandiflorum* without self-pollen (i.e., because they have been emasculated before anthesis) should have higher S : O ratios following cross-pollination than flowers that are self-pollinated before cross-pollination.

Finally, in light of the results we obtained from these experimental pollination studies, we also examined the potential for resource limitation of S : O ratios by investigating the relation between plant size and seed production using the graphical model presented above.

## Material and Methods

### Study Site

We performed this study at Joker's Hill Research Station near Newmarket, southern Ontario, Canada (44.02973°N, 79.52715°W) in May–July 1999. Here, *Trillium grandiflorum* is an understory herb in mixed deciduous forest dominated by sugar maple and beech. The study site is typical of the species' habitats (Case and Case 1997), and populations at the site contain thousands of *T. grandiflorum* plants. We performed the work in an area of ca. 2 ha.

### Fertility Studies

*Pollen limitation.* To investigate the extent of pollen limitation in *T. grandiflorum*, we randomly assigned plants to one of two treatments: open-pollinated (OP) and hand cross-pollinated (CP; hereafter referred to as cross-pollinated). Pollinations were carried out on May 5, 10, and 15 at two sites separated by ca. 500 m. However, as the results from the timing of pollination experiment demonstrate (see "Results"), there is no apparent effect of pollination date on the levels of fruit or seed set. Thus, in the "Results" section, we pooled data across days within treatments. Unless otherwise noted, cross-pollination refers to flowers that we pollinated with one anther from each of three donor plants (i.e., three anthers in total). Donor plants were at least 1 m but not more than 10 m distant from recipient plants. Irwin (2001) demonstrated no effect of "outcrossing distance" on variation in seed set in *T. grandiflorum*. All plants in these experiments were marked with a metal tag and a 1-m-tall wire with a flag to enable relocation of plants for fruit collection. Bridal veil was used to bag flowers before and after pollination. Only single-flowered plants were used, since this was the predominant condition in populations at both sites. Fruits were placed in 70% ethanol in early July when they were close to maturity. We counted the number of seeds, shrunken seeds, and apparently unfertilized ovules ("Discussion") under a dissecting microscope. The total number of ovules was calculated as the sum of seeds, aborted seeds, and unfertilized ovules. In this study, we define the S : O ratio as the number of mature seeds divided by the total ovule number per flower.

To analyze the extent of pollen limitation of fruit set in *T.*

*grandiflorum*, we used logistic regression, with site, pollination treatment, and the interaction of site and treatment as effects. Site and its interaction were treated as random effects. As the interaction was not significant, we removed it from the final model. We used ANCOVA to explore variation in S : O ratios. For this analysis, seed production per flower was the response variable, and we included site, treatment, ovule number, and their interactions as effects. Site and its interactions were treated as random effects. However, none of the interactions in this analysis was significant, and therefore they were removed from the pollen limitation model. We performed these and all subsequent analyses with JMP (SAS Institute 2000).

*Manipulations of flower exposure to pollinators.* Flowers of *T. grandiflorum* are long-lived, remaining open with non-senescent tepals for 17–20 d (Ashman and Schoen 1994; Sage et al. 2001), depending on temperature and whether flowers have been pollinated. Presumably, extended floral longevity ensures pollination during a time when pollinator activity is unpredictable. To test this hypothesis, we manipulated the period during which *T. grandiflorum* flowers were exposed to pollinators. We tagged ca. 170 plants with flowers that opened on the same day. Two days later (May 7), and every 2 d thereafter for 12 d, we emasculated and bagged a random subset of 15 plants, thus preventing any subsequent pollination. However, to meet the $\chi^2$-test requirement of adequate sample sizes per cell, we pooled treatments of 2 d and 4 d, 6 d and 8 d, and 10 d and 12 d. Twenty-five plants were left unmanipulated and unbagged to serve as an open-pollinated control. In addition, we cross-pollinated 20 plants with one anther from each of three donors to serve as a cross-pollinated control. We used a $\chi^2$-test to analyze fruit set among treatments. We could not analyze seed production for this experiment because low fruit set per treatment gave insufficient data for an ANCOVA of seed set.

*Effect of number of pollen donors.* To test the hypothesis that the number of pollen donors may affect the S : O ratio, we manipulated the number of pollen donors per pollination. We bagged and emasculated flowers on 100 plants before anthesis. Plants were then randomly assigned to one of four pollination treatments: one, two, three, or six pollen donors. For each treatment, flowers were pollinated with a total of six anthers divided among the donors: six anthers from one donor, three anthers from each of two donors, two anthers from each of three donors, and one anther from each of six donors. All pollinations for this experiment were performed on May 14. We used a $\chi^2$-test to analyze fruit set per treatment and ANCOVA to analyze S : O ratios. For the ANCOVA, seed production per flower was the response variable, pollination treatment was the main effect, and ovule number was the covariate. This ANCOVA model was used for all subsequent experiments.

*Effect of pollen age.* To test the hypothesis that pollen viability may decrease with age and affect S : O ratios, we pollinated flowers with young and old pollen. We bagged and emasculated flowers on 120 plants before anthesis. To control for pollen age under field conditions, we used donor pollen that came only from flowers that had opened on May 6. Starting May 7 and every 2 d thereafter for 10 d, we cross-pollinated a random subset of 20 plants.

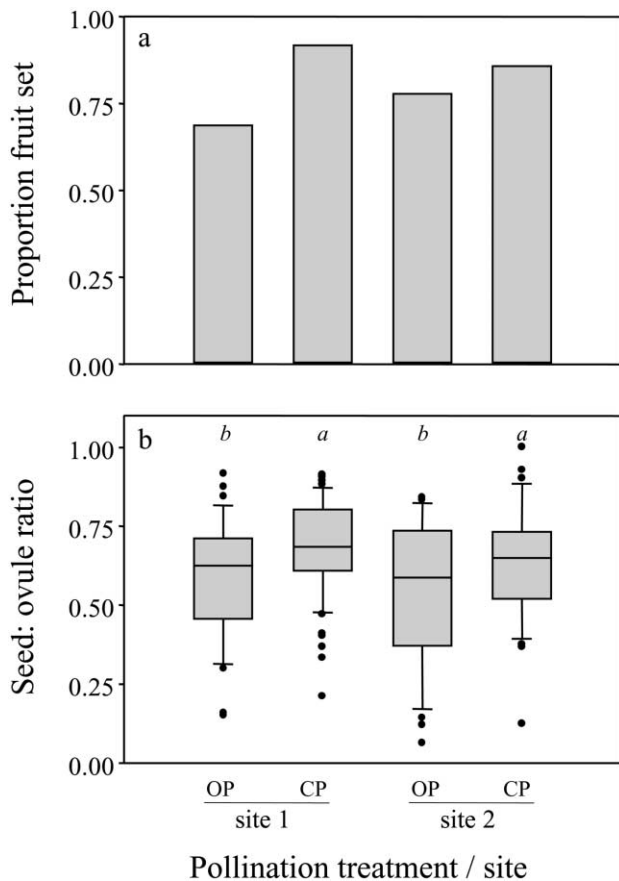*Effect of the timing of pollination.* If ovule development

**Fig. 2** Pollen limitation of fruit set (*a*) and seed set (*b*) of *Trillium grandiflorum* at Joker's Hill, Ontario, in 1999. The two treatments, open-pollinated (*OP*) and hand cross-pollinated (*CP*), were performed at two sites. OP fruit set was significantly lower than CP fruit set ($P = 0.006$; see table 1). Lowercase letters above box plots of seed : ovule ratios indicate significantly different contrasts using Tukey's test ($P < 0.05$; see table 2 for analysis). Sample sizes for each treatment: fruit set, $n = 70, 73, 41, 36$; seed set, $n = 48, 66, 32, 32$.

and receptivity are asynchronous in *Trillium*, a single pollination may not result in the fertilization of all ovules. Pollination on multiple days, however, should result in the fertilization of a greater fraction of ovules. To test the hypothesis that the timing of pollination influences fertility, we compared seed set from multiple and single pollinations. We bagged and emasculated flowers on 100 plants before anthesis. Plants were randomly assigned to one of four pollination treatments. There were three treatments in which flowers were pollinated once: flowers in the early treatment were pollinated on May 9, the middle treatment was pollinated on May 12, and the late treatment was pollinated on May 15. We pollinated flowers in the fourth treatment on three different days: May 9, 12, and 15. To control for the amount of pollen and the number of donors, we pollinated plants in the early, middle, and late treatments with one anther from each of three donors, while we used one anther from one donor on each day and used a different donor each day to pollinate plants in the multiple pollination treat-

ment. Thus, all flowers in this experiment were pollinated with three anthers from three different donors.

*Effect of prior self-pollination on outcrossed seed set.* To test the hypothesis that prior self-pollination may inhibit seed set of outcrossed flowers, we compared fertility of cross-pollinated flowers with and without prior self-pollination. We bagged 34 *T. grandiflorum* flowers before anthesis. We pollinated 34 plants with self-pollen and then emasculated the flowers. Seventeen plants were cross-pollinated using three donors 1 d later, while another 17 plants were cross-pollinated with three donors 2 d later. For the control group, we emasculated and bagged an additional 20 plants before anthesis and cross-pollinated these plants with pollen from three donors.

*Resource limitation of seed : ovule ratios.* We examined the relation between plant size and seed production to assess if resources might have been a factor limiting fertility in *T. grandiflorum*. We compared high- and low-fertility plants under conditions of both pollen limitation (i.e., open-pollinated) and pollen abundance (i.e., hand cross-pollinated). We ranked open-pollinated *T. grandiflorum* by S : O ratio ($n = 68$) as well as cross-pollinated *T. grandiflorum* from the pollen limitation study ($n = 92$). For each group, we designated the lowest twenty-fifth percentile as the low S : O group and the highest twenty-fifth percentile as the high S : O group. Total ovule number is highly correlated to overall plant size in *T. grandiflorum* at Joker's Hill and elsewhere (Wright and Barrett 1999; Irwin 2000). Therefore, ovule number is a convenient proxy for plant size. We analyzed variation in ovule number between S : O ranks for both open- and hand cross-pollinated flowers in a factorial ANOVA, with pollination treatment, S : O rank, and their interaction as effects. We square root–transformed ovule number to meet parametric assumptions of homoscedasticity and normal distributions within treatments and used Tukey's HSD test to perform pairwise contrasts of ovule number among treatments.

## Results

### Factors Affecting Fertility in Trillium grandiflorum

*Pollen limitation of fruit and seed set.* Comparison of open- and cross-pollinated fruit set (fig. 2*a*) and S : O ratios (fig. 2*b*) indicated that *Trillium grandiflorum* was pollen limited in 1999 at Joker's Hill (logistic regression of fruit set, table 1; ANCOVA of seed set, table 2). On average, open-pollinated flowers exhibited a 47% reduction in seeds per flower (i.e., fruit per flower × seeds per fruit) in comparison with cross-

**Table 1**

**Logistic Regression Analysis of Pollen Limitation of Fruit Set of *Trillium grandiflorum* at Two Sites at Joker's Hill, Ontario, in 1999**

| Source | df | $\chi^2$ | P |
|---|---|---|---|
| Site | 2 | 0.2 | 0.69 |
| Treatment | 1 | 11.2 | 0.0006 |

Note. Whole model: $r^2 = 0.06$; $P = 0.003$. Site is a random effect, and the nonsignificant interaction term has been removed from the model. For details, refer to text.

**Table 2**

**Analyses of Covariation (ANCOVA) in Seed Set per Fruit of *Trillium grandiflorum*
at Joker's Hill, Ontario, in 1999**

| Source | df | SS | F | P |
|---|---|---|---|---|
| Number of pollen donors ($r^2 = 0.71$, $P < 0.001$): | | | | |
|     Treatment | 3 | 0.6 | 0.7 | 0.54 |
|     Ovule number | 1 | 45.0 | 155.2 | <0.0001 |
|     Treatment × ovule number | 3 | 1.8 | 2.0 | 0.12 |
|     Error | 67 | 19.4 | | |
| Age of pollen ($r^2 = 0.68$, $P < 0.0001$): | | | | |
|     Treatment | 5 | 1.57 | 0.8 | 0.52 |
|     Ovule number | 1 | 50.1 | 135.1 | <0.0001 |
|     Treatment × ovule number | 5 | 1.5 | 0.8 | 0.54 |
|     Error | 85 | 6.2 | | |
| Timing of pollination ($r^2 = 0.58$, $P < 0.0001$): | | | | |
|     Treatment | 3 | 1.9 | 2.0 | 0.12 |
|     Ovule number | 1 | 27.9 | 86.1 | <0.0001 |
|     Treatment × ovule number | 3 | 1.0 | 1.0 | 0.39 |
|     Error | 67 | 21.7 | | |
| Timing of cross-pollination after self-pollination ($r^2 = 0.67$, $P < 0.0001$): | | | | |
|     Treatment | 2 | 1.8 | 2.2 | 0.13 |
|     Ovule number | 1 | 12.5 | 30.5 | <0.0001 |
|     Treatment × ovule number | 2 | 1.5 | 1.8 | 0.19 |
|     Error | 32 | 13.1 | | |
| Pollen limitation ($r^2 = 0.73$, $P < 0.0001$):[a] | | | | |
|     Site (random) | 2 | 1.2 | 3.4 | 0.06 |
|     Treatment | 1 | 4.7 | 13.2 | 0.01 |
|     Ovule number | 1 | 151.2 | 420.1 | <0.0001 |
|     Treatment × ovule number | 1 | 0.3 | 0.9 | 0.35 |
|     Error | 160 | 57.5 | | |

Note. Ovule number was a covariate in all analyses. For details, refer to text.

[a] Site is a random effect, and nonsignificant interactions except "Treatment × ovule number" have been removed from the model.

pollinated flowers; this indicates that pollen delivery limited seed set at both sites. Hand cross-pollination significantly increased the S : O ratio from 0.56 in open-pollinated flowers to 0.66 in cross-pollinated flowers.

*Exposure to pollinators.* Flowers with experimentally shortened exposure to pollinators were 33%–50% less likely to produce fruit than were flowers with longer exposure periods (fig. 3; $\chi^2 = 29.0$, df = 4, $P < 0.0001$). None of the open-pollinated treatments, regardless of exposure time, reached the 100% fruit set observed in cross-pollinated flowers.

*Effect of number of pollen donors.* Seed set from a single pollen donor was not significantly different from seed set when two, three, or six pollen donors were used in cross-pollinations (fig. 4a; ANCOVA, table 2). Fruit set was greater than 95% for all treatments and did not differ significantly among treatments ($\chi^2 = 4.0$, df = 3, $P = 0.26$).

*Effect of pollen age.* There was no significant difference in fruit set (>95% for all treatments; $\chi^2 = 0.0$, df = 5, $P = 1.00$) or seed set (fig. 4b; ANCOVA, table 2) from pollinations that used pollen that ranged in age from 2 to 12 d.

*Effect of timing of pollination.* There was no significant difference in fruit set (>95% for all treatments; $\chi^2 = 2.8$, df = 3, $P = 0.43$) or seed set (fig. 4c; ANCOVA, table 2) between flowers that were pollinated once compared with those that were pollinated on three separate occasions. Furthermore,
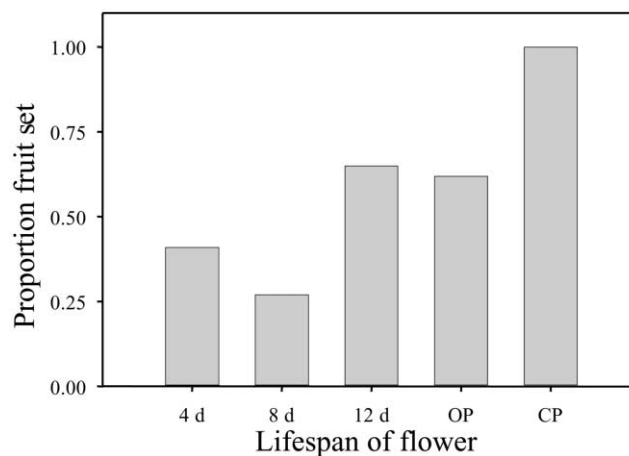


**Fig. 3** Effect of experimental manipulation of floral life span on fruit set of *Trillium grandiflorum* at Joker's Hill, Ontario; *4 d*, *8 d*, and *12 d* indicate the number of days between anthesis and bagging; *OP* = open-pollinated; *CP* = hand cross-pollinated and bagged. Fruit set was significantly different among treatments ($\chi^2$-test, $P < 0.001$). Sample sizes for each treatment: $n = 22, 22, 26, 21, 16$.
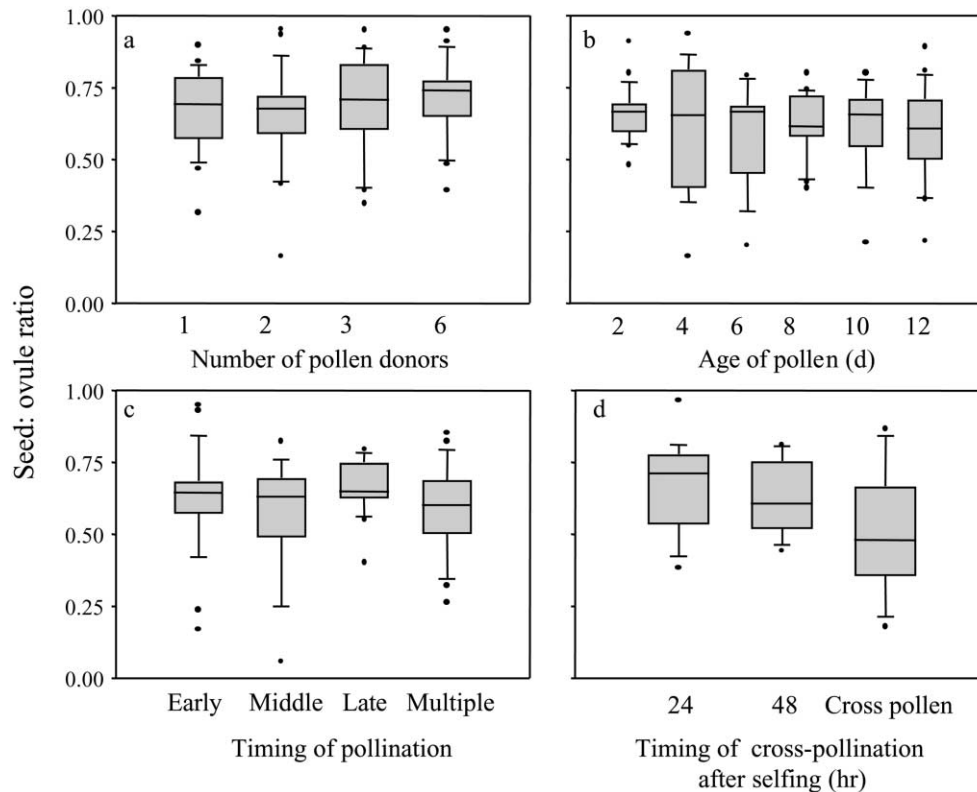
**Fig. 4**  Comparison of seed set from four different pollination treatments of *Trillium grandiflorum* at Joker's Hill, Ontario. *a*, Number of pollen donors. *b*, Age of pollen. *c*, Timing of pollination. *d*, Prior self-pollination. Within each experiment, there was no difference in seed set among treatments (ANCOVA, *P*'s > 0.12; see table 2 for analyses). Sample sizes for each treatment: number of pollen donors, $n = 22, 21, 16, 17$; age of pollen, $n = 22, 14, 15, 17, 12, 19$; timing of pollination, $n = 23, 19, 19, 20$; timing of cross-pollination after self-pollination, $n = 17, 15, 21$.

there were no significant differences in seed set among single pollinations performed on different days.

*Effect of prior self-pollination.*    There was no significant difference in fruit set (>95% for all treatments; $\chi^2 = 3.0$, df = 2, $P = 0.21$) or seed set (fig. 4*d*; ANCOVA, table 2) between flowers that were self-pollinated and then cross-pollinated compared with flowers that were cross-pollinated only.

*Resource limitation of S : O ratios.*    The analysis of seed production and plant size indicated that resources limit S : O ratios when pollen supply does not constrain female fertility. When *T. grandiflorum* plants were ranked by S : O ratio, open-pollinated fruits with high S : O ratios did not differ significantly in total ovule number from fruits with low S : O ratios (fig. 5; ANOVA, table 3). Note that *T. grandiflorum* at Joker's Hill was pollen limited in 1999 (fig. 2*b*). Thus, when pollen is limiting, resources do not appear to limit seed production; there is no relation between the S : O ratio and plant size (i.e., solid line in fig. 1*b*). However, when cross-pollinated fruits are ranked by S : O ratio, fruits with high S : O ratios had significantly more ovules than those with low S : O ratios (fig. 5; table 3). Therefore, it appears that when pollen is in excess, larger plants benefit from greater seed production than do smaller plants (i.e., dashed line in fig. 1*b*).

## Discussion

Submaximal fertility is commonly observed in flowering plants, especially among animal-pollinated, outcrossing perennials (Sutherland and Delph 1984; Wiens 1984; Sutherland 1986; Charlesworth 1989*a*, 1989*b*; Burd 1994; Larson and Barrett 2000). *Trillium grandiflorum* is no exception, and previous studies have reported low seed : ovule ratios and/or evidence for pollen limitation of seed set at other sites (Lubbers and Lechowicz 1989; Smith 1998; Kalisz et al. 1999; Irwin 2000). The goal of this study was to examine some of the potential causes of submaximal fertility in *T. grandiflorum* using experimental approaches. We found that fruit and seed set were pollen limited in 1999, probably as a result of infrequent pollinator visitation. However, as reported earlier in *Trillium* spp., even when flowers were provided with abundant cross-pollen, the mean S : O ratio was substantially less than 1, implying that factors other than low pollinator service limit fertility. To investigate this possibility, we manipulated aspects of the timing of pollination and the quantity and quality of pollen that flowers received. However, in no case were we able to raise the average S : O ratio above 0.65; this implicates resources as a factor limiting seed set. An analysis of the relation between plant size and seed production provided support for
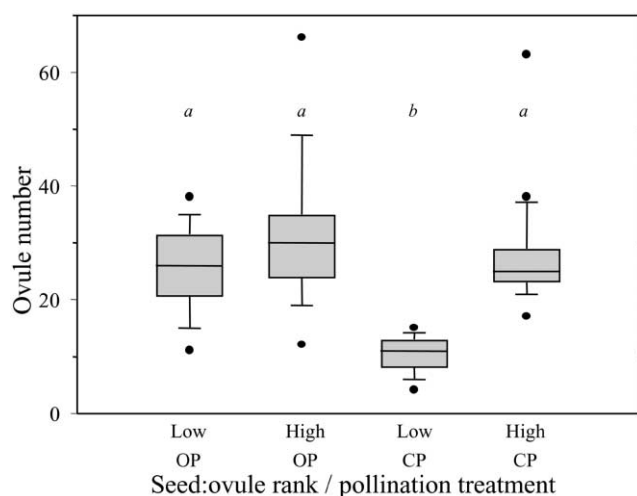
**Fig. 5** Differences in plant size (measured by ovule number) between high and low fertility (measured by S : O ratio) plants of *Trillium grandiflorum* with different pollination treatments; *OP* = open-pollinated; *CP* = hand cross-pollinated. For open-pollinated plants, the lower twenty-fifth percentile of the S : O ratios ranged from 0.03 to 0.40 (mean = 0.18, $n$ = 15), and the upper twenty-fifth percentile ranged from 0.76 to 1.00 (mean = 0.84, $n$ = 23); for cross-pollinated plants, the lower twenty-fifth percentile ranged from 0.21 to 0.55 (mean = 0.45, $n$ = 23), and the upper twenty-fifth percentile ranged from 0.81 to 1.00 (mean = 0.90, $n$ = 23). Lowercase letters above box plots indicate significantly different contrasts (Tukey's HSD; see table 3 for ANOVA).

this, but only in the instance when pollen does not limit reproduction. We discuss these findings in light of the stochastic pollination environment experienced by early spring–flowering woodland herbs, such as *T. grandiflorum*.

## Pollen Limitation of Fertility in *Trillium grandiflorum*

Our comparison of cross- and open-pollinated flowers from two sites at Joker's Hill in southern Ontario (fig. 2) confirms other studies that have found pollen limitation of fertility in *T. grandiflorum* (Lubbers and Lechowicz 1989; Smith 1998; Wright and Barrett 1999; Irwin 2000). Unreliable pollinator activity in early spring is the most likely cause of pollen limitation. Indeed, *T. grandiflorum* and other spring wildflowers commonly experience low pollinator visitation rates (Schemske et al. 1978; Barrett and Helenurm 1987; Baker et al. 2000; Irwin 2000). The extent of pollen limitation can vary in both space and time (Baker et al. 2000), and, indeed, there is evidence for this in *T. grandiflorum*. For example, Lubbers and Lechowicz (1989) found year-to-year variation in pollen limitation in *T. grandiflorum* in Quebec, and Smith (1998) found spatial variation in pollen limitation in a survey of 18 southern Ontario populations. Therefore, studies of numerous sites conducted over several years are needed before general conclusions can be reached regarding the extent of pollen limitation, especially for long-lived perennials such as *T. grandiflorum*.

## Low S : O Ratios in *Trillium grandiflorum*

We investigated under field conditions whether pollen load quality (pollen donor number or pollen age), the timing of pollination, and interference by self-pollen affected patterns of female fertility in populations of *T. grandiflorum* at Joker's Hill. However, despite the generally large sample sizes that we used in these studies, we failed to find experimental evidence that any of these factors significantly influenced S : O ratios. We consider each experiment in turn and attempt to explain some of the reasons why the factors we investigated may be of less importance in *T. grandiflorum* than in other species investigated.

First, if pollen donor number influenced fertility in *T. grandiflorum*, there should have been an increase in seed set with the number of pollen donors in our experimental crosses. Nonetheless, pollen from one donor resulted in seed set equal to pollen from multiple donors (fig. 4*a*). Why might *T. grandiflorum* outcrossed seed set be insensitive to the number of donors represented in outcrossed pollen loads? It is possible that this result may be associated with the low pollinator service that populations of *T. grandiflorum* commonly receive (Broyles et al. 1997; Smith 1998; Wright and Barrett 1999; Irwin 2000; S. R. Griffin and S. C. H. Barrett, unpublished data). Using genetic markers, Broyles et al. (1997) and Kalisz et al. (1999) estimated that the number of male sires per fruit of *T. grandiflorum* averaged one, a result consistent with the observation that because pollinator densities are very low, flowers typically receive only a single pollination visit. If flowers typically receive pollen from only a single donor, it is perhaps not surprising that the fraction of ovules fertilized by the single donor treatment was similar to that found using multiple donors. Strong donor number effects on seed set may be more commonly associated with species that usually experience high pollinator visitation rates and the multiple paternity of fruits.

Second, fresh pollen should have increased S : O ratios in comparison with older pollen if pollen age were an important determinant of fertility in *T. grandiflorum*. However, 12-d-old pollen showed no decline in potency under field conditions compared with 2-d-old pollen (fig. 4*b*). Long pollen longevity in *T. grandiflorum* may be functionally associated with the reproductive biology of the species. Flowers of *T. grandiflorum* are long-lived (Ashman and Schoen 1994; Sage et al. 2001), and populations at Joker's Hill bloom synchronously with the

**Table 3**

**Analysis of Variation (ANOVA) in Plant Size (Measured by Ovule Number) between Open- and Hand Cross-Pollinated *Trillium grandiflorum* with High and Low Fertility at Joker's Hill, Ontario, in 1999**

| Source | df | SS | F | P |
|---|---|---|---|---|
| Pollination treatment | 1 | 30.1 | 48.1 | <0.0001 |
| S : O rank | 1 | 19.4 | 30.4 | <0.0001 |
| Pollination treatment × S : O rank | 1 | 10.8 | 17.2 | <0.0001 |
| Error | 72 | 45.0 | | |

Note. Within each pollination treatment, plants were ranked by seed : ovule (S : O) ratio, and the top and bottom quartiles were designated as high and low fertility, respectively. Whole model: $r^2$ = 0.61; $P < 0.0001$.

vast majority of flowers opening within 3–5 d of each other (see fig. 1 in Sage et al. 2001). Under these conditions, the pollen pool within a population of *T. grandiflorum* may be of relatively uniform age, and long pollen viability would clearly be adaptive where pollinators are scarce, as they frequently are for *T. grandiflorum*. This is because if pollinators visited populations only toward the middle or end of the synchronous blooming period, reproductive success would be severely curtailed if pollen viabilities lasted only a few days.

Third, if some ovules of *T. grandiflorum* were unavailable for fertilization when pollination occurs because they were either immature or unreceptive, then serial pollinations during the life span of the flower might have been expected to result in greater seed set compared with single pollinations. However, one pollination event appears to be sufficient for female reproductive success in *T. grandiflorum*. Serial pollinations did not increase seed set over single pollinations in flowers of any age; this suggests that ovule receptivity is both synchronous and of extended receptivity (fig. 4*c*). Reduced fruit set in flowers with experimentally shortened exposure to pollinators (fig. 3) and the limited number of pollinators observed at Joker's Hill throughout the blooming period (S. R. Griffin and S. C. H. Barrett, unpublished data) indicate that pollinator visitation to *T. grandiflorum* is extremely infrequent. When pollinator visitation rates are low and highly stochastic, theoretical models predict that floral strategies will evolve to ensure maximum female and male reproductive success from one visit (Harder and Wilson 1994; Burd 1995). Synchronous ovule development and extended ovule receptivity and pollen viability may represent such strategies in *T. grandiflorum*.

Last, it had been demonstrated that prior self-pollination can interfere with subsequent cross-pollination in some species but not others (reviewed in Barrett 2002). We found no evidence for the inhibitory effects of prior self-pollination on outcrossed seed set in *T. grandiflorum* (fig. 4*d*). Unfortunately, little is known about the specific mechanisms of pollen interference or why some species are more susceptible than others to such effects. *Trillium grandiflorum* has a relatively strong self-incompatibility system in which self-pollen is largely inhibited on the stigma surface (Sage et al. 2001). Stigmatic self-incompatibility may preclude opportunities for physical or biochemical interference in the style or ovary. Many of the clearest examples of the inhibitory effects of prior self-pollination occur in species with late-acting self-incompatibility systems (Waser and Price 1991; Broyles and Wyatt 1993; Sage et al. 1999; Barrett 2002). The absence of such effects in *T. grandiflorum* is therefore probably not fortuitous. The large open flowers of this species do not display strong herkogamy or dichogamy, and natural levels of autonomous and insect-mediated self-pollination are high because of prolific pollen production (S. R. Griffin and S. C. H. Barrett, unpublished data). With this type of floral biology, there may be strong selection for postpollination physiological mechanisms that limit the deleterious effects of self-interference.

Together, these data from experimental crosses are best explained in light of the floral biology of *T. grandiflorum*. Floral traits in this species have likely evolved to maximize reproductive success under a highly stochastic pollinator environment in which many flowers receive only a single pollinator visit. Nonetheless, when ample cross-pollen is applied, all ovules do not produce seeds. Visual inspection of our data revealed that the distribution of S : O ratios across the different pollination treatments were remarkably similar (fig. 4). In all instances, there were some individuals with high S : O ratios approaching 1.0 and some individuals with low S : O ratios of 0.25 and below. Nevertheless, each treatment averaged ca. 60% of the ovules producing mature seed, with a grand mean S : O ratio of $0.62 \pm 0.01$ ($n = 418$). Thus, other mechanisms must be limiting fertility in *T. grandiflorum*.

## The Status of Ovules in Trillium grandiflorum

Two alternative explanations for the submaximal S : O ratios in *T. grandiflorum* that we cannot address with our data relate to the true status of unfertilized ovules that we counted in fruits. First, it is possible that not all ovules produced within a flower of *T. grandiflorum* are viable and therefore capable of being cross-fertilized. A variety of genetic and developmental factors could result in defective ovules leading to sterility. Such effects would be hard to detect accurately in the type of large-scale visual assessments conducted in this study. Another possibility is that the cryptic early abortion of ovules resulted in some ovules being counted as unfertilized when in fact they had been fertilized (Nakamura and Stanton 1987). The S : O ratios could be much closer to 1 if a sizable fraction of ovules produced by *T. grandiflorum* flowers either are inviable or were aborted very early in development. Structural and functional assays of ovules and early embryo development (Stelly et al. 1984; Scribailo and Barrett 1991; Sage et al. 1999) could be used to determine whether these two possibilities are important in *T. grandiflorum* and other species with low S : O ratios.

## Resource Limitation of Seed Set in Trillium grandiflorum

Resource limitation of female fertility seems likely to contribute to the low S : O ratios often found in outcrossing species. Haig and Westoby (1988) predicted a trade-off between pollen and resource limitation of seed production in plants. Indeed, Campbell and Halama (1993) found that resources and pollen limited whole-plant fertility in distinct ways in *Ipomopsis aggregata*. The addition of a large amount of pollen increased the number of seeds per fruit, whereas resource addition increased the number of flowers on a plant that produced fruit. In many *Trillium* spp., there is little room for resource limitation of fruit set since the vast majority of plants are solitary flowered. When ample pollen is applied to flowers of *T. grandiflorum*, fruit set is generally close to 100%. Hence, resources may primarily limit flowering capacity over the lifetime of a plant and the number of seeds produced per fruit within each season. Earlier work on *T. grandiflorum* in Quebec by Lubbers and Lechowicz (1989) provided evidence that resources can indeed limit seed production. They demonstrated that leaf defoliation, which presumably limits resource supply, reduced seed set but not fruit set.

Our analysis of the relation between seed production and plant size in *T. grandiflorum* provides indirect evidence that resources limit S : O ratios when pollen is not limiting. Cross-pollinated plants with high S : O ratios were significantly larger than cross-pollinated plants with low S : O ratios (fig. 5). Thus, among cross-pollinated plants, there is a relation between S : O ratios and plant size (fig. 1); this implies that resource

status directly influences fertility. Among open-pollinated flowers that were pollen limited in 1999 (fig. 2), plants with high or low fertility were not significantly different in size. Thus, when pollen is in short supply in *T. grandiflorum*, resources do not appear to limit S : O ratios, and there is no relation between the S : O ratio and plant size (fig. 1*b*). A similar result was recently reported in a population of *T. grandiflorum* in Vermont in which Irwin (2000) found no relation between S : O ratio and plant size for open-pollinated flowers in a year in which seed set was pollen limited. An alternative explanation to account for this pattern could be that smaller plants may be genetically inferior to larger plants and, as a result, produce more inviable ovules or early-aborting embryos. For the population we examined at Joker's Hill, we consider this explanation unlikely since much of the variation in plant size is age related (Wright and Barrett 1999).

Future studies on the factors governing female fertility in *Trillium* should include both manipulation of resource levels and the quantity and quality of pollen delivered to flowers. Because *T. grandiflorum* is a long-lived perennial (Wright and Barrett 1999), these studies would need to be conducted over extended periods of several to many years. In addition, detailed information on ovule biology, including information on viability, receptivity periods, and the extent of cryptic early abortion, may be required to fully understand the causes of submaximal fertility in *T. grandiflorum* and other species with low S : O ratios.

## Acknowledgments

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