

AN ABSTRACT OF THE THESIS OF

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Title: The Influence of Pollinators on the Maintenance of Mixed Mating in a
Population of the Blue Columbine, *Aquilegia coerulea* (Ranunculaceae)

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Pollination ecology may play an important role in the maintenance of selfing in populations of self-compatible hermaphroditic plants where both selfing and outcrossing occur (mixed mating). Behavior and abundance of pollinators can influence the two major modes of selfing; autogamy (selfing within a flower) and geitonogamy (selfing between flowers on the same plant). Autogamy may be selected for as a method of reproductive assurance during times of reduced or inefficient pollinator service. In contrast, geitonogamy is a negative and non-adaptive consequence of pollinators visiting multiple flowers per plant. Pollinator behavior and abundance, which can both influence levels of selfing, varies among pollinator types. Evidence suggests that of the two major pollinators of the blue columbine, *Aquilegia coerulea*, hawkmoths may decrease levels of selfing compared to bumblebees. The goals of this study are to 1) quantify the contribution of autogamy and geitonogamy to the overall selfing rate, 2) determine whether hawkmoths decrease levels of selfing compared to bumblebees and other floral visitors, and 3) examine how pollinator behavior influences levels of selfing in a Colorado population of *A. coerulea*.

First, we estimated levels of selfing from groups of emasculated and control flowers using allozyme data to measure the genetic contribution of geitonogamy and autogamy to the population selfing rate. Second, contribution of autogamy to seed set was quantified as the difference in seed set between control and emasculated flowers. Third, we compared the realized levels of autogamy measured from allozyme data to

the potential for autogamy, measured as seed set in absence of pollinators. Fourth, we compared the realized levels of geitonogamy measured from allozyme data to the potential for geitonogamy, calculated from the flowering phenology. Fifth, we conducted pollinator observations to document the number of different pollinators, their behavior, and their variation in abundance over three years of study. Finally, we determined if there was a relationship between yearly abundance of each pollinator type and yearly selfing rate.

The allozyme data showed that geitonogamy was the primary contributor to the intermediate levels of selfing found in this population, suggesting that selfing is due to the negative consequence of pollinators visiting multiple flowers per plant and has no adaptive explanation. The realized level of geitonogamy was greater than the potential for geitonogamy, and resulted from pollinator preferences for a floral gender. In contrast, the realized level of autogamy was negligible, which suggested that reproductive assurance was not being selected for in this population. However, seed set from control flowers were significantly greater than that of emasculated flowers, indicating that autogamy contributed to an increase in seed set. The discrepancy between genetic and seed set data could be explained by bumblebee preference for control relative to emasculated flowers. Although the realized level of autogamy was minimal, plants in the population retained a high potential for autogamy, which may be selected for as a mechanism of reproductive assurance during years of low pollinator abundance. Pollinators in this population of *A. coerulea* included bumblebees, hawkmoths, solitary bees, wasps and flies. The abundance of all pollinators varied significantly among years except for solitary bees and wasps. In addition, we documented a significant variation in selfing rate (0.23-0.59) over the three years of study. A relationship was noted between years with increased hawkmoth abundance and a decrease in selfing rate. No relationships were observed between yearly selfing rate and abundance of the other pollinator types. The behavior most likely responsible for the decrease in selfing is that, unlike other pollinators, hawkmoths prefer female-phase flowers, which reduces levels of geitonogamy. Thus, the abundance and behavior of distinct pollinator types can differentially influence levels of selfing.

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The Influence of Pollinators on the Maintenance of Mixed Mating in a
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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Heather R. Sweet, Author

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For my parents, Bob and Donna Sweet
And brother, Wylie Sweet

The Influence of Pollinators on the Maintenance of Mixed Mating in a Population of the Blue Columbine, *Aquilegia coerulea* (Ranunculaceae)

Chapter 1

INTRODUCTION

Approximately 80% of flowering plants are hermaphroditic and thus potentially capable of reproducing via self- (selfing) or cross-fertilization (outcrossing). The evolutionary consequences of selfing and outcrossing have been the focus of many studies since Darwin's seminal work in 1876. Numerous investigations have found that offspring produced via selfing have significantly reduced fitness compared to those produced through outcrossing (Darwin 1876; Lloyd 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Holsinger 1991; Husband and Schemske 1995, 1996; Johnston and Schoen 1995; Barrett and Harder 1996). Inbreeding depression, the relative reduction in fitness of progeny due to selfing versus outcrossing, is usually caused by the expression of deleterious homozygous recessive alleles (Barrett and Charlesworth 1991; Dudash and Carr 1998; Willis 1999). However, individuals that self benefit from a 50% genetic transmission advantage over outcrossers because selfers transmit their entire genome to their progeny while outcrossers can only contribute half of their genome to their offspring (Fisher 1941; Jain 1976; Holsinger 1988, 1992). Furthermore, selfing may provide an additional advantage by ensuring seed set in plants when potential mates and/or pollinators are rare or absent (Jain 1976; Wyatt 1988; Parker et al. 1995).

In 1985 Lande and Schemske developed a theoretical mating system model incorporating gene transmission advantage and inbreeding depression, and where inbreeding depression evolved with the level of selfing. The model predicted that populations evolved to one of two stable endpoints: complete selfing or complete outcrossing. Because selfing plants have a two-fold gene transmission advantage over outcrossing plants, a plant population is expected to become predominantly selfing in

populations where inbreeding depression is smaller than 0.5, while populations with inbreeding depression greater than 0.5 will evolve to complete outcrossing. To date, theoretical models that consider inbreeding depression and the transmission advantage of selfing predict intermediate levels of selfing under fairly restricted conditions. Such conditions include inbreeding depression caused by overdominance (Campbell 1986; Holsinger 1988; Charlesworth and Charlesworth 1990; Uyenoyama and Waller 1991), an increase in inbreeding depression with increased selfing (Damgaard et al. 1992; Latta and Ritland 1993, 1994), fluctuating inbreeding depression (Cheptou and Mathias 2001), or differential dispersal ability of selfed versus outcrossed seed (Holsinger 1986). Given that inbreeding depression is primarily caused by deleterious mutations and not overdominance (Dudash and Carr 1998; Willis 1999), populations maintaining high levels (>20%) of both outcrossing and selfing are not expected to be evolutionarily stable in most plant populations (Lande and Schemske 1985; Charlesworth and Charlesworth 1987; Uyenoyama et al. 1993). However, an estimated 42% of seed plant populations do maintain mixed mating systems (Goodwillie et al. 2005). The discrepancy between theory and empirical data suggest that selective factors in addition to inbreeding depression and gene transmission advantage influence the evolution of self-fertilization in natural plant populations.

The evolution of selfing and the maintenance of mixed mating systems may be strongly influenced by aspects of pollination ecology (Lloyd 1992; Lloyd and Schoen 1992; Holsinger 1996; Vogler and Kalisz 2001). When seed plant populations are separated by their mode of pollination, plants pollinated by abiotic factors (wind and water) fit the theoretically expected bimodal distribution of selfing and outcrossing, while animal-pollinated plants have substantial levels of both selfing and outcrossing (mixed mating) (Schemske and Lande 1985; Aide 1986; Barrett et al. 1996; Vogler and Kalisz 2001). A more recent study, including 345 species in 78 plant families found that plants pollinated by animals are twice as likely to maintain a mixed mating system compared to plants that are pollinated by wind and water (Goodwillie et al. 2005). The

widespread occurrence of mixed mating in populations of animal-pollinated plants suggests that aspects of pollination ecology influence the maintenance of mixed mating in plant populations (Holsinger 1986; Harder and Wilson 1998; Morgan and Wilson 2005; Porcher and Lande 2005).

The majority of angiosperms are pollinated by animals, with the majority being insects. Flowers provide pollinators with vital food resources, usually in the form of nectar or pollen. In return, pollinators transfer pollen among flowers, which many angiosperms partially or completely rely on to produce seed (Proctor et al. 1996). The integration of pollination ecology into the evolution of selfing has highlighted some of the benefits and costs of selfing. While selfing can provide reproductive assurance when pollinators are scarce, pollen or seeds used for selfing may not be available for outcrossing (pollen and seed discounting respectively) (Lloyd 1979, 1980; Holsinger 1991, 1996). Pollination ecology also influences how and when self-fertilization occurs. Selfing can occur via two different modes; autogamy where selfing occurs within a single flower and geitonogamy where selfing occurs between flowers on the same plant. Although the two modes of selfing are genetically equivalent, they have very different fitness consequences. Autogamous selfing, if not facilitated by pollinators, may provide seed set during times of low pollinator abundance, inefficient pollen transfer and/or when potential mates are scarce (reproductive assurance). Therefore, autogamy may be advantageous and be selected for in a plant population when too few outcross pollen are available for fertilization (pollen limitation) (Baker 1965; Lloyd 1979, 1992; Kalisz and Vogler 2003; Kalisz et al. 2004). In contrast, geitonogamy requires the presence of pollinators, thus produces no reproductive advantage. Moreover, geitonogamy reduces the amount of pollen (pollen discounting) and ovules (ovule discounting) that are available for outcrossing. Thus geitonogamy is usually disadvantageous and whenever inbreeding depression occurs, geitonogamy should be selected against. Consequently, geitonogamy may simply be a negative, but unavoidable consequence of cross-pollination when individual plants open more than

one flower simultaneously (Lloyd 1992; Jarne and Charlesworth 1993; Harder and Barrett 1995; Harder and Wilson 1998; Fishman 2000).

Theoretical models that combine genetic and ecological factors have also highlighted the role of pollination ecology, in particular pollen discounting and pollen limitation, in the maintenance of mixed mating system (Holsinger 1991; Johnston 1998; Porcher and Lande 2005). In the absence of pollen limitation in a population, pollen discounting makes it more likely that a mixed mating system can be maintained in a population (Holsinger 1991). However, this conclusion does not hold when inbreeding depression changes with selfing rate. With a dynamic model of inbreeding depression, complete outcrossing is the most likely outcome when pollen discounting is present and there is no pollen limitation in the population (Porcher and Lande 2005). With pollen limitation and pollen discounting, high selfing rates (close to 1) represent the stable conditions (Porcher and Lande 2005). Low to moderate levels of selfing can only occur in the absence of pollen limitation and under restricted conditions. Thus, Porcher and Lande (2005) expected that many of the observed low to moderate selfing rates observed in plant populations to result from geitonogamous selfing in populations, where selection would favor complete outcrossing. An important question in evolutionary biology is to determine whether selfing is maintained for reproductive assurance, and thus selected for, or whether it is merely a consequence of geitonogamous selfing in a plant with many flowers opened simultaneously and thus requires no adaptive explanation.

While the influence of pollination ecology on the evolution of selfing has concentrated on the role of pollen limitation, pollen discounting and modes of selfing, little work has examined how distinct pollinator types influence selfing rate (Lloyd and Schoen 1992). Pollinator behavior can have a significant impact on the level of selfing, via their impact on geitonogamy. First, pollinators are often attracted to large floral

displays and have been found to visit more flowers sequentially on large floral displays, thus increasing geitonogamy as the pollinator accumulates and then deposits pollen as it moves among flowers on the same plant (Lloyd 1992; de Jong et al. 1993; Robertson and Macnair 1995; Harder and Barrett 1995, 1996; Snow et al. 1996; Eckert 2000; Galloway et al. 2002; Karron et al. 2004; Mitchell et al. 2004; Makino et al. 2007). Consequently, flowers visited later in succession will have increased levels of geitonogamy compared to the first flowers visited on a plant (Barrett et al. 1994; Harder and Barrett 1995). Second, pollinators may show a preference for either male- or female-phase flowers on dichogamous plants, which express male- and female-phases at different times within a flower. For example, a pollinator that collects pollen may show a preference for male-phase flowers when pollen is available (Bell et al. 1984; Johnson et al. 1995; Lau and Galloway 2004). The level of geitonogamy may increase if a male-phase flower is preferentially visited first rather than a female-phase flower. By visiting a male flower first on a plant, a pollinator increases the chance that pollen will be transferred from the first flower to other flowers visited consecutively on the plant. Third, pollinator movement on an inflorescence can influence levels of geitonogamy. For example, geitonogamous selfing could increase if a pollinator tends to move from the bottom to the top of an inflorescence (as has been observed in some species of bees) of a protandrous plant species whose flowers open from the top to bottom. Because more male-phase flowers would be open on the bottom of the inflorescence while more female-phase flowers would be open on the top of the inflorescence, there is an increased probability that pollen will be transferred from the first male-phase flowers visited to female-phase flowers as the pollinator moves up the inflorescence (Proctor et al. 1996).

In contrast, pollinators can affect the level of selfing via their impact on autogamy. Non-facilitated autogamy can ensure seed set during times of low pollinator abundance. Studies have shown that selfing is common in populations or habitats where

pollinators are rare or in species that flower at times of low pollinator abundance (Motten 1982; Piper et al. 1986; Barrett 1996; Fausto et al 2001). In addition, some plant species have demonstrated an increased capacity for non-facilitated autogamy during times of reduced pollinator visits (Wyatt 1986; Sih and Baltus 1987; Jennersten 1988; Fausto et al. 2001). Pollinator species differ in how effectively they deposit and remove pollen from individual flowers and in the extent to which they can transfer pollen among flowers (Schemske and Horvitz 1984; Herrera 1987; Wilson and Thomson 1991; Fishbein and Venable 1996; Lau and Galloway 2004). A pollinator with poor pollen transfer efficiency decreases the amount of outcross pollen plants receive (pollen limitation), which may increase selection for non-facilitated autogamy to ensure seed set within a flower (Kalisz and Volger 2003; Kalisz et al. 2004). Similarly, pollinators that visit multiple plant species during a foraging bout will increase pollen limitation due to transfer of interspecific pollen among plant species, thus potentially increasing the selection for non-facilitated autogamy to ensure seed set (Waser 1978; Waser 1983; Campbell 1985; Fisherman and Wyatt 1999; Brown and Mitchell 2001; Bell et al. 2005). Poor pollen transfer among flowers creates conditions of pollen limitation, which in turn may select for autogamous selfing as a mechanism to provide reproductive assurance (Kalisz and Vogler 2003; Kalisz et al. 2004). Different pollinators could distinctly affect pollen limitation and consequently levels of autogamous selfing.

Nearly all plant species are pollinated by more than one species of pollinator (Waser et al. 1996; Proctor et al. 1996), often from different taxonomic groups that vary significantly in their foraging behavior (Handel 1983; Schemske and Horvitz 1984; Herrera 1987; Ramsey 1988; Young and Stanton 1990). However, few studies have attempted to determine whether different pollinator types affect levels of selfing or outcrossing. Two different studies found no difference in outcrossing rate among populations with different pollinator types. Schmidt-Adam et al. (2000) found no difference in outcrossing rates between mainland and island populations of the New

Zealand endemic *Metrosideros excelsa*. Introduced birds and bees visited the island populations, while native birds and bees visited the mainland populations. In another study, Eckert (2002) hypothesized that differences in foraging behavior between bees and butterflies would influence levels of outcrossing, but found no difference in allozyme estimated outcrossing rates between populations of *Decodon verticillatus* located in Florida/Georgia that have a greater abundance of butterflies and Michigan/Ontario which have more bees.

In this study we examined whether selfing in a population of *Aquilegia coerulea* is selected for or is merely a nonadaptive consequence of adaptations for cross-pollination in a plant with large floral displays. We also determined whether distinct pollinator types differentially affect selfing rate in this plant species. Previous mating system studies done in the blue columbine, *Aquilegia coerulea*, found that most populations maintain a mixed mating system, with selfing rates between 0.07 and 0.59 (Brunet and Sweet 2006). Bumblebees and hawkmoths are the two major pollinators of this plant species (Miller 1978, 1981). While bumblebees collect pollen during the day, hawkmoths hover above flowers to obtain nectar at dusk. In an experiment in which all the flowers on a plant were either unmanipulated (control) or emasculated, Miller (1981) found greater seed set in control plants during a year of high bumblebee abundance, but no difference in seed set in control versus emasculated plants in a year of high hawkmoth abundance. Because selfing cannot occur in emasculated plants, these data suggest that hawkmoths increase outcrossing rate (Miller 1981). In addition, when comparing different populations, Brunet and Sweet (2006) showed that outcrossing rate increased with hawkmoth abundance, but not with the abundance of the other floral visitors (bumblebees, solitary bees and syrphid flies). Outcrossing rate was also influenced by floral display size, but not by population size, plant density or herkogamy (Brunet and Sweet 2006). While evidence among populations supports the hypothesis that pollinator types can differentially influence selfing and outcrossing rates, no such data exist within a single population. Furthermore, more data are needed

to pinpoint the pollinator behaviors responsible for the decrease in selfing rate associated with hawkmoths compared to bumblebees and other floral visitors.

Research objectives

In this study, we examined how autogamy and geitonogamy contribute to the maintenance of selfing in a mixed mating system found in a population of the blue columbine, *Aquilegia coerulea* during the summer of 2002. Within the same population we examine how the behavior and abundance of hawkmoths, bumblebees, and other floral visitors influence levels of selfing over three years of study, 2001-2003. The objectives of this study are to:

- 1) Determine whether autogamy provides reproductive assurance in the population.
- 2) Quantify the genetic contribution of autogamy and geitonogamy to the selfing rate in the *A. coerulea* population using allozyme data.
- 3) Measure the potential levels of autogamy and geitonogamy in the population and contrast these estimates to the realized levels measured using the allozyme data.
- 4) Document the number of different pollinators, pollinator behavior, and yearly variation in pollinator types and abundance within the population of *A. coerulea*.
- 5) Determine if there is yearly variation in selfing rate in the population of *A. coerulea* and determine if yearly variation in selfing rate is correlated with variation in pollinator type and abundance.
- 6) Pinpoint aspects of pollinator behavior that can explain potential differences in selfing rate among pollinator types.

Chapter 2

METHODS

The species, *Aquilegia coerulea*

The blue columbine, *Aquilegia coerulea* James, (Ranunculaceae) is a perennial herbaceous plant found throughout the central and southern Rocky Mountains of North America in montane and subalpine habitats at elevations of 2100-3700m. The plant is self-compatible and does not reproduce clonally. The radially symmetrical flowers have five petals and five sepals. The white petals consist of an upper flattened lamina and an elongated spur containing nectar. The sepals extend past the petals and can range in color from white to deep blue or purple. The flowers open sequentially on inflorescences with distal flowers opening first. Flower position refers to the order in which a flower opens on the inflorescence. A first-position flower is the first flower to open on an inflorescence followed by second-, third-, and later-position flowers. Flowers are hermaphroditic and protandrous, thus pollen dehiscence before stigmas become receptive within each flower. The male reproductive organ includes 50-130 stamens and surrounds the female reproductive organ which consists of five to ten unfused carpels. Each carpel can independently develop into a mature follicle. The male-phase of a flower lasts on average three days. It begins when pollen first dehiscence from the anthers and ends when no pollen grains remain on the anthers. The female-phase of a flower lasts an average four to five days and begins when stigmas become receptive. Stigma receptivity can be determined by the swelling of papillae on the stigmatic surface. There is typically little to no pollen available when stigmas become receptive within a flower. The major pollinators of *A. coerulea* are hawkmoths and bumblebees (Miller 1978, 1981). Hawkmoths forage at dusk using their long tongues to

feed on nectar located at the base of the spur. Bumblebees collect pollen from the anthers throughout the day.

The population

The Falls Creek population of *A. coerulea* is located on Black Mountain in Routt National Forest approximately 20 miles north of Craig in northwest Colorado, USA at an elevation of 2745 m. The population consists of approximately 300 flowering plants growing among large rock outcrops surrounding Falls Creek. Plants in this population bloom mid-June through early August and have an average of 4.0 inflorescences per plant (range 1 – 20), 4.5 flowers per inflorescence (range 1 – 9.6) and 18.2 flowers per plant (range 1 – 94). Experiments designed to estimate the contribution of autogamy to seed set and to measure the levels of autogamous and geitonogamous selfing in the population were conducted in the summer of 2002. Pollinator observations and estimates of population selfing rate were obtained over three years, 2001-2003.

Contribution of autogamy to seed set

In this experiment, we selected two flowers at a similar developmental stage (sexual stage) and position on the inflorescence on each of 50 plants in the population. On each plant, we emasculated one flower before any anthers dehisced while the other flower was left unmanipulated (control). Only first- and second-position flowers were used in the experiment because seed set decreases with increasing flower position in *A. coerulea* (Brunet 1996). Because the follicles can split open before the seeds are fully mature, we placed a clear polyethylene mesh bag over the fruits just before the follicles

opened to ensure collection of all seeds. We collected mature fruits and counted the number of developed follicles and total number of mature seeds per flower. Fourteen plants lost one or both experimental flowers to herbivory leaving 36 plants with a pair of control and emasculated flowers.

While outcrossing and geitonogamy (selfing among flowers) can happen in flowers that do not contain stamen (emasculated flowers), autogamy (selfing within a flower) cannot. Therefore difference in seed set between emasculated and control flowers should determine the contribution of autogamy to seed set in the population. In addition, if autogamy occurs in the absence of pollinator visits (autonomous autogamy) then an increase in seed set in the control compared to emasculated flowers represents the level of reproductive assurance in the population. Thus, we compared total seed set per flower and average seed set per follicle between emasculated and control flowers using a paired t-test to determine the contribution of autogamy to seed set in the population (SAS Version 8.1).

Contribution of autogamy and geitonogamy to selfing rate

For each of the 36 pairs of emasculated and control flowers, we assayed 10-18 seeds per flower via horizontal starch gel electrophoresis using a histidine citrate buffer system (pH = 6) and standard staining protocols (Wendel and Weeden 1998). We assayed the seeds at five polymorphic allozyme loci: MPI (mannose phosphate isomerase), ADH (alcohol dehydrogenase), 6PGD (6-phosphogluconate dehydrogenase), MDH (malate dehydrogenase), and PGM (phosphoglucomutase). We estimated selfing rates for the group of emasculated and control flowers separately using the computer program MLTR (Ritland 2002). This program allows the estimation of mating parameters for different groups of plants with similar pollen allele frequencies. We obtained estimates of the selfing rate using Newton-Raphson iterations

and the most likely parent method in the computer program MLTR for Windows (Ritland 2002). Selfing rate estimates converged to the same value from different starting points. We calculated standard error of the selfing rate estimate for the group of control or emasculated flowers as the standard deviation of 1000 bootstrap values generated with the progeny array as the unit of resampling.

We used pairwise comparisons of bootstrap estimates to compare selfing rate estimates between emasculated and control flowers (Eckert and Barrett 1994). Selfing rate in the control flowers is considered significantly greater than in emasculated flowers if 95 % or more of the differences between randomly paired bootstrap estimates of control versus emasculated flowers lay above zero (Eckert and Barrett 1994; Eckert 2000). The test was one-tailed as we expected autogamy to increase selfing rate of control flowers.

The selfing rate estimates calculated using genetic markers and progeny arrays do not distinguish between true selfing and mating with close relatives. To determine the contribution of mating between close relatives to the selfing rate estimate, we calculated the level of biparental inbreeding in both the control and emasculated flowers. Biparental inbreeding is calculated as the difference between the multilocus selfing rate estimate and the mean of the single locus selfing rate estimates (Ritland 1990).

While autogamous and geitonogamous selfing and outcrossing occur in the control flowers, only geitonogamous selfing and outcrossing can take place in the emasculated flowers. Thus, the level of autogamous selfing can be directly calculated as the difference in selfing rate between control and emasculated flowers. However, in the absence of competition from autogamous pollen, the level of geitonogamous selfing and outcrossing may increase in the emasculated flowers. To correct for this effect, we assumed that the relative proportions of outcrossed and geitonogamous selfs were the

same in control and emasculated flowers (Schoen and Lloyd 1992). With this assumption, if s is the selfing rate, g is geitonogamous selfing, and a is autogamous selfing, and if we apply the subscripts c for control and e for emasculated flowers, then:

$$g_c / (1-s_c) = g_e / (1-s_e). \quad (1)$$

Because all selfing is from geitonogamy in emasculated flowers,

$$g_c / (1-s_c) = s_e / (1-s_e). \quad (2)$$

Therefore,

$$g_c = s_e (1 - s_c) / (1-s_e) \quad (3)$$

and because selfing consists of both autogamous and geitonogamous selfing,

$$a_c = s_c - g_c. \quad (4)$$

(Schoen and Lloyd 1992; Eckert 2000). To obtain the standard errors of the geitonogamous and autogamous selfing rate estimates, first, for each of 1000 random pairs of bootstrap values generated for the control and emasculated flowers, we calculated geitonogamous and autogamous selfing using equations (3) and (4) respectively. We then used the standard deviation of the 1000 geitonogamous and autogamous selfing rate estimates calculated from the bootstrap values as standard errors of the mean geitonogamous and autogamous selfing rate respectively.

Bumblebee preference for control or emasculated flowers

The experiment designed to estimate the contribution of autogamy to seed set assumes that pollinators will visit both control and emasculated flowers with equal frequency. Of the two major pollinators, hawkmoths and bumblebees, only the bumblebees forage for pollen. Thus, the bumblebees may have a preference for control flowers. An increase in the number of visits to the control flowers could increase seed set in the control versus emasculated flowers irrespective of autogamy. To test whether bumblebees preferred control flowers we simultaneously presented one control and one

emasculated flower, each held in a separate florist tube, to a bumblebee. Both flowers were similar in color, size, and floral stage. Furthermore, all selected flowers had dehiscent pollen. For each of 22 independent bumblebee visits, we recorded the flower type (control or emasculated) that the bumblebee visited first.

Potential for autogamy

The potential for autogamy can be determined by the number of seeds set in the absence of all pollinators. To quantify the potential for autogamy, we paired 24 plants according to size (total number of flowers per plant) and floral stage. Before any flower opened, we caged and bagged with bridal veil one plant per pair to exclude all pollinators. The other plant of the pair was left untouched. We removed the cage and veil when the stigmas of the last flower to open on the plant were no longer receptive. We recorded the number of flowers per plant, and the number of carpels and mature follicles per flower. To prevent loss of seeds from follicles, we placed clear polyethylene mesh bags over maturing fruits. We collected mature fruits and determined fruit set as the percentage of flowers that gave mature fruits. We calculated seed set both as the total number of seeds per flower (all follicles combined) and the average number of seeds per follicle. The potential for autogamy was quantified as fruit and seed set of caged plants. We compared fruit and seed set of caged and open-pollinated plants using paired t-tests (SAS Version 8.1).

Potential for geitonogamy

In a dichogamous plant the potential for geitonogamy estimates the probability that flowers can receive pollen from another flower on the same plant based solely on the flowering phenology of that plant (number of male- and female-phase

flowers open on the plant each day) (Brunet 2005). The calculations assume random pollinator movement on inflorescences (each flower has the same probability of being visited) and equal fertilization of ovules by self- and outcross-pollen. Hence, the potential for geitonogamy in a dichogamous plant predicts the impact of the flowering phenology on the level of geitonogamous selfing. Differences between the realized level of geitonogamy measured using allozymes and the potential level of geitonogamy based solely on flowering phenology would indicate non-random pollinator movement on inflorescences and/or differential ovule fertilization by self- versus outcross-pollen.

To calculate the potential for geitonogamy, we recorded the first day each flower opened on 32 plants in the experiment. We reconstructed the flowering phenology of each plant using the first day each flower opened combined with the average number of days a flower spends in the male- and female-phases in the population. We obtained these averages by recording the sexual phase for each day a flower was open for 13 flowers on 11 plants in the population. We then used the flowering phenology data (number of male- and female-phase flowers open on the plant each day) to calculate the probability that flowers on a plant would receive geitonogamous pollen. For each day a flower is open on a plant, we calculated the probability that the flower received self-pollen based on the ratio of male- to female-phase flowers open that day (see Brunet 2005, protocol 2.26 for details). If a flower was in male-phase or if only male- or female-phase flowers were open on a given day, the probability of geitonogamy was zero. We first calculated the potential for geitonogamy using all flowers on a plant, and then used only the emasculated and control flowers (first or second flowers to open on an inflorescence in this experiment). We performed these two sets of calculations because geitonogamy tends to decrease in later-opening flowers (Brunet 2005). To determine whether pollinator movement influenced the level of geitonogamous selfing, we compared the potential for geitonogamy calculated from the flowering phenology data to the realized levels of geitonogamous selfing obtained using allozyme markers.

Pollinator abundance and behavior

Pollinator observations were conducted between July 16-21 in 2001; June 21-July 12 in 2002; and July 21-24 in 2003. In the summer of 2001 pollinator observations included two one-hour periods: 1:30- 2:30 p.m., and dusk 8:30 – 9:30 p.m. In the summers of 2002 and 2003, we observed pollinators during four one-hour periods: 9:30 – 10:30 am; 12:30 – 1:30 pm; 3:30 – 4:30 pm; and 8:30 – 9:30 pm. The time periods accommodated the distinctive diurnal activity patterns exhibited by the different types of pollinators including hawkmoths, which tend to come out at dusk. In 2001 pollinator observations totaled seven hours, two hours during the day and five hours at dusk. In 2002 we observed pollinators for a total of 26 hours, 6 hours for each of the three day time periods and 8 hours at dusk. In 2003 we collected data for a total of 32 hours, 8 hours per time period.

Before each period of pollinator observations, we noted the number of plants per observational patch, the number of open flowers per plant, and the sexual stage of each flower in the patch. During pollinator observations, we recorded the type of pollinator entering the patch, the time spent by each pollinator type in a patch, the number of flowers visited per patch, and the number of flowers visited in succession on a plant. We also recorded the sexual stage of each flower and the order in which it was visited on a plant. At least one specimen of each floral visitor was collected in the field for proper identification. Once identified, floral visitors were grouped into five categories; hawkmoths, bumblebees, solitary bees and wasps, pollinating flies and Muscidae flies. Hawkmoths and bumblebees were identified to species. Solitary bees and wasps and pollinating flies were identified to genus, while house flies were identified to family (Muscidae). Each type of floral visitor was visually checked for the presence of pollen on its body immediately after collection in the field.

We calculated pollinator abundance for each pollinator type as the number of visits per flower per hour. This measure combined information on the number of pollinator visits per hour with the number of flowers visited per foraging bout. We compared pollinator abundance among years for each pollinator type using analysis of variance (SAS Version 8.1). We examined whether different groups of pollinators spent the same amount of time per flower, visited the same number of flowers in succession on a plant, and visited the same number of flowers in succession in a foraging sequence bout using analysis of variance and Tukey mean tests (SAS Version 8.1). To determine whether pollinators had a preference for a sexual phase of a flower, for each new visit to a plant, for each pollinator species, we calculated the proportion of times that a male-phase flower was visited first on the plant (as opposed to a female-phase flower). We then examined whether the number of flowers visited in succession on a plant varied when a male- or a female-phase flower was visited first on a plant. In addition, we observed pollinator movement on an inflorescence and noted any directional movement up or down the inflorescences for all pollinator types during pollinator observations. We also noted whether each pollinator type visited other plant species during foraging bouts or only visited flowers of *A. coerulea*. Finally, in the summer of 2003, we measured the distance traveled between *A. coerulea* plants within observational patches by bumblebees and hawkmoths and then compared the distance traveled between the two pollinator groups using a permutation test (Microsoft Excel 2003). Due to the difficulty of following hawkmoths at dusk, we recorded significantly fewer measurements for hawkmoths ($n = 6$) compared to bumblebees ($n = 62$). The permutation test combined all 68 measurements and then compared the data generated from 200 data sets where 6 data points were randomly assigned to hawkmoths and 62 data points to bumblebees. The P-value is based on percentage of times the generated data showed a difference in distance greater than the observed difference.

Temporal variation in population selfing rate

The population selfing rate was estimated over a three year time period, 2001-2003. We collected fruits from one to four first- and second- position flowers per plant (depending on plant size) for 41 plants in 2001 and 40 plants in 2003. The plants chosen represented the distribution of plant size in the population. In 2002, we used the 36 plants used in the experiment to determine the contribution of autogamy to seed set and obtained seeds from the control flowers and from one additional second-position flower on a separate plant. A random sample of 8-20 seeds (stratified across flower positions) per plant were assayed via horizontal starch gel electrophoresis for allozyme variability at five putative loci, MPI, ADH, 6PGD, MDH, and PGM. Population selfing rates and pollen ovule allele frequencies were jointly estimated using the maximum likelihood computer program MLTR (Ritland 2002).

Relationship between pollinator type and population selfing rate

For each pollinator type, we visually examined whether there was a relationship between the yearly pollinator abundance and selfing rate. We determined whether selfing rate varied significantly among years using pairwise comparisons of bootstrap estimates (2001 vs. 2002; 2002 vs. 2003; and 2001 vs. 2003) (Eckert and Barrett 1994; Eckert 2000). More specifically, we tested whether selfing rate was lower in years of greater hawkmoth abundance. Tests were one-tailed as we examined whether the selfing rate estimate was significantly greater in 2002 relative to 2001, 2002 vs. 2003 and finally 2001 vs. 2003. We considered the selfing rate estimate in 2003 for example to be greater than the selfing rate estimate in 2002 if 95 % of more of the differences between randomly paired bootstrap estimates from each of these two years laid above zero (Eckert and Barrett 1994; Eckert 2000). Finally, we ran a simple regression between yearly hawkmoth abundance and selfing rate (SAS Version 8.1).

Chapter 3

RESULTS

Contribution of autogamy to seed set

Control flowers set 32.3 % more seeds per flower and 24.2 % more seeds per follicle relative to emasculated flowers (seed set per flower = 128.5 ± 8.1 for control and 97.1 ± 9.1 for emasculated) (paired $t = 3.41$, $df = 35$, $P = 0.0017$); (seed set per follicle = 21.2 ± 1.1 for control and 17.1 ± 1.4 for emasculated) (paired $t = 3.15$, $df = 35$, $P = 0.0033$). Seed set of control flowers was greater than seed set of emasculated flowers in 21 pairs, equal in 9, and smaller in 6 pairs. Autogamy appears to significantly contribute to seed set in this population of *A. coerulea* in 2002.

Contribution of autogamy and geitonogamy to selfing rate

The number of alleles per locus and their frequencies for control and emasculated flowers are presented in Table 3.1. The 2002 population selfing rate estimate for the control flowers was $s = 0.59 \pm 0.06$ (mean \pm SE) based on five loci in 434 progenies from 36 families. The level of biparental inbreeding, measured as the difference between the multilocus selfing rate estimate ($s_m = 0.59 \pm 0.06$) and the mean of the single locus selfing rate estimate ($s_s = 0.61 \pm 0.06$) was low in the population (0.02 ± 0.02), indicating that mating among close relatives did not contribute significantly to the selfing rate estimate. The selfing rate estimate for emasculated flowers ($s_e = 0.55 \pm 0.05$, based on five loci from 400 progenies in 36 families) did not differ significantly from the selfing rate estimate of the control flowers ($s_c = 0.59 \pm$

0.06) ($P = 0.33$ based on pairwise comparisons of bootstrap estimates). The level of autogamous selfing estimated by directly subtracting the selfing rate of control flowers ($s_c = 0.59 \pm 0.06$) from the selfing rate of emasculated flowers ($s_e = 0.55 \pm 0.05$) was 0.04. The level of autogamous selfing estimated using the correction for reduced level of competition in emasculated flowers (see Materials and Methods section) was 0.08 ± 0.18 and did not differ significantly from zero ($P = 0.33$ based on comparisons of bootstrap estimates). The contribution of autogamous selfing to the population selfing rate was negligible. With a population selfing rate of $s = 0.59$, and a level of autogamous selfing of $a = 0.08$, the level of geitonogamous selfing in the population is expected to be $g = 0.51$. Hence, the majority of selfing in the population occurs via geitonogamous selfing ($g = 0.51 \pm 0.14$).

Table 3.1. Number of alleles and gene frequencies at five allozyme loci used to estimate selfing rate from 36 pairs of control and emasculated flowers in the Falls Creek population of *A. coerulea* in 2002. The allozyme loci are malate dehydrogenase (MDH), mannose-6-phosphate isomerase (MPI), phosphoglucomutase (PGM), mannose-6-phosphate isomerase (MPI), 6-phosphogluconate dehydrogenase (6-PGD), and alcohol dehydrogenase (ADH).

Locus-allele	Allelic frequency for control flowers	Allelic frequency for emasculated flowers
Mdh-1	0.966	0.959
Mdh-2	0.034	0.040
Mdh-3	0.000	0.001
Pgm-1	0.806	0.820
Pgm-2	0.194	0.170
Pgm-3	0.000	0.010
Mpi-1	0.715	0.700
Mpi-2	0.168	0.172
Mpi-3	0.070	0.058
Mpi-4	0.048	0.071
6pgd-1	0.978	0.967
6pgd-2	0.010	0.015
6pgd-3	0.011	0.018
Adh-1	0.858	0.832
Adh-2	0.087	0.103
Adh-3	0.041	0.038
Adh-4	0.014	0.027

Bumblebee preference for control or emasculated flowers

When presented simultaneously with a control and emasculated flower, bumblebees visited control flowers first 17 out of 22 times (77.3% of the time). Bumblebees demonstrated a strong preference for control flowers.

Potential for autogamy

We observed high fruit and moderate seed set in both caged and open-pollinated plants (fruit per flower = 0.81 ± 0.09 for caged and 0.78 ± 0.08 for open-pollinated), (seeds per flower = 46.7 ± 9.0 for caged and 45.2 ± 8.5 for open-pollinated), (seeds per follicle = 8.2 ± 1.5 for caged and 8.6 ± 1.3 for open-pollinated), with no statistically significant differences in fruit and seed set between caged and open-pollinated plants (paired $t = 0.29$, $df = 11$, $P = 0.79$ for fruit set), (paired $t = 0.14$, $df = 11$, $P = 0.90$ for seed set per flower), and (paired $t = 0.24$, $df = 11$, $P = 0.82$ for seed set per follicle). Thus, potential for autogamy is high in this population in 2002.

Potential for geitonogamy

Flowers in the population spent an average of three days in the male-phase followed by four days in the female-phase during the summer of 2002. The flowering phenology of one plant with two inflorescences and four flowers with the first two flowers opening on July 5, the third on July 7, and the fourth on July 8 is presented as an example in Table 3.2. Only male-phase flowers are open in the population on days 1-3 and thus, the potential for geitonogamy for flowers open those days is zero (Table 3.3). There are two male- and two female-phase flowers open on the plant on day 4.

The probability that self pollen will reach female-phase flowers that day is 1 (2/2). Male-phase flowers have a zero potential for geitonogamy (Table 3.3). One can calculate the probability of geitonogamous selfing for each flower on the plant and average the values to obtain the probability of geitonogamous selfing for the whole plant (average per flower per plant) (Table 3.3).

The average potential for geitonogamy for the 32 experimental plants was 0.25 ± 0.02 when averaged over all flowers, and 0.38 ± 0.04 when averaged only over the control and emasculated flowers used in the experiment. We expected this difference between the two methods of calculation because early flowers were selected for the control and emasculated flowers and the potential for geitonogamy is typically larger in earlier- relative to later-opening flowers in protandrous plants (Table 3.3) (Brunet 2005; Brunet and Charlesworth 1995) because in protandrous plants earlier-opening flowers are in the female phase when later-opening flowers are in the male phase, but this is not true of later-opening flowers (Brunet and Charlesworth 1995).

The realized level of geitonogamy measured using allozyme markers was $g = 0.51 \pm 0.14$ compared to a value of 0.38 ± 0.04 for the potential for geitonogamy calculated for control and emasculated flowers for 32 plants in the experiment. Because the potential for geitonogamy represents the level of geitonogamy based solely on the flowering phenology, the discrepancy between the realized and potential for geitonogamy suggests either that pollinator movement on inflorescences increases geitonogamous selfing or that self pollen is more likely to fertilize ovules when competing with outcross pollen. Because we know that outcross pollen outcompete self pollen in *A. coerulea* (Montalvo 1992), these data suggest that pollinator movement on inflorescences promotes geitonogamous selfing in this plant species.

Table 3.2. The flowering phenology of a plant with two inflorescences (I) and two flowers (Fl) per inflorescence. The first day each flower opened was recorded (day 1= June 5) and we estimated that plants in the population spend an average of three days in the male phase (M) followed by four days in the female phase (F).

I #	Fl #	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
1	1	M	M	M	F	F	F	F			
	2			M	M	M	F	F	F	F	
2	1	M	M	M	F	F	F	F			
	2				M	M	M	F	F	F	F

Table 3.3. The potential for geitonogamy for the plant with the flowering phenology presented in Table 3.2. For flowers in the female-phase, the probability of geitonogamy is based on the ratio of male to female flowers open on the plant that day. If only male- or female-phase flowers are open on a given day, the probability of geitonogamy is zero that day. For each flower, the daily potential for geitonogamy is averaged over the seven days the flower is open. The potential for geitonogamy for the whole plant is obtained by averaging over all flowers.

I #	Fl #	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Potential for geitonogamy
1	1	0	0	0	2/2	2/2	1/3	0				0.33
	2			0	0	0	1/3	0	0	0		0.05
2	1	0	0	0	2/2	2/2	1/3	0				0.33
	2				0	0	0	0	0	0	0	0.00
Plant: 0.18												

Pollinator abundance

Pollinators seen visiting *A. coerulea* flowers in this population between the years 2001-2003 included one species of hawkmoth (*Hyles lineata*) and three species of bumblebees (*Bombus flavifrons*, *Bombus occidentalis*, and *Bombus rufocinctus*) (Table 3.4). Other floral visitors included solitary bees from four different families (Halictidae, Megachilidae, Anthophoridae and Andrenidae) and solitary wasps from one family (Vespidae) (Table 3.4). We observed pollen on the bodies of hawkmoths, bumblebees, solitary bees and wasps. Pollen was also found on the bodies of individuals from two families of flies, Syrphidae and Bombyliidae, but not from members of the house fly family Muscidae. Muscidae spent long periods of time within each flower, frequently landed on non-sexual plant structures to groom, did not commonly visit more than one flower in a patch, and often visited other plant species, and thus they do not appear to be pollinators of *A. coerulea* (Table 3.5).

Pollinator abundance, measured as number of visits per flower per hour, varied significantly among years (df 2, $F= 5.68$, $P < 0.0042$) and among pollinator groups (df 3, $F= 3.02$, $P= 0.0317$). There was a significant interaction between pollinator group and year on pollinator abundance (df 6, $F= 5.7$, $P < 0.001$) indicating that the relative abundance of the different pollinator groups varied among years (Table 3.4; Figures 3.1 and 3.2). Each pollinator group except the solitary wasps and bees varied significantly among years (Figure 3.1) Bumblebees were present each year and most abundant in 2003 with the species *Bombus flavifrons* making up the majority of bumblebee visits each year (Table 3.4; Figures 3.1 and 3.2). Hawkmoths were common in 2001, rare in 2002, and most common in 2003 (Table 3.4; Figures 3.1 and 3.2). Solitary bees and wasps were most common the year that hawkmoths were rare (Table 3.4, Figures 3.2 and 3.2). Pollinating flies were rare in 2003, but more common in 2001 and 2002 (Table 3.4; Figures 3.1 and 3.2). While bumblebees, solitary bees and wasps, and pollinating flies occurred throughout the day, hawkmoths visited *A. coerulea* flowers only at dusk (Table 3.4).

Table 3.4. Pollinator abundance and visitation rate in a population of *A. coerulea* over three years (2001-2003). Pollinator abundance represents the average number of visits per flower per hour; visitation rate is the average number of pollinator visits per hour per patch. In 2001 day observations were obtained over one time period (1:30-2:30); in 2002 and 2003 day observations were made over three time periods (9:30 – 10:30 a.m.; 12:30- 1:30 p.m.; and 3:30 – 4:30 p.m.). Evening observations were obtained over one time period each year (8:30- 9:30 p.m.). Standard errors are in parentheses.

	2001		2002		2003	
	Abundance	Visit rate	Abundance	Visit rate	Abundance	Visit rate
DAY VISITORS						
Bumblebees:	0.47 (0.30)	3.0	0.43 (0.08)	2.9	1.08 (0.21)	6.7
Apidae <i>Bombus flavifrons</i>	0.43 (0.26)	2.5	0.43 (0.08)	2.9	1.03 (0.21)	6.4
Apidae <i>Bombus occidentalis</i>	0.04 (0.04)	0.5	0.00 (0.00)	0.0	0.00 (0.00)	0.0
Apidae <i>Bombus rufocinctus</i>	0.00 (0.00)	0.0	0.00 (0.00)	0.0	0.05 (0.04)	0.29
Solitary Bees and Wasps:	0.00 (0.00)	0.0	0.40 (0.13)	3.7	0.10 (0.07)	0.79
Halictidae <i>Lasioglossum</i> sp.	0.00 (0.00)	0.0	0.08 (0.03)	1.1	0.00 (0.00)	0.0
Megachilidae <i>Megachile latimanus</i>	0.00 (0.00)	0.0	0.11 (0.05)	0.61	0.01 (0.01)	0.08
Anthophoridae <i>Anthophora</i> sp.	0.00 (0.00)	0.0	0.07 (0.03)	0.5	0.00 (0.00)	0.0
Andrenidae <i>Andrena</i> sp.	0.00 (0.00)	0.0	0.07 (0.03)	0.83	0.00 (0.00)	0.0
Vespidae <i>Ancistrocerus</i> sp. and <i>Stenodynerus</i> sp.	0.00 (0.00)	0.0	0.06 (0.03)	0.67	0.09 (0.07)	0.71
Pollinating Flies:	0.38 (0.38)	3.5	0.28 (0.05)	2.1	0.07 (0.04)	0.67
Syrphidae <i>Chrysotoxum</i> sp. and <i>Didea</i> sp.	0.38 (0.38)	3.5	0.13 (0.06)	1.0	0.00 (0.00)	0.0
Bombyliidae <i>Villa</i> sp.	0.00 (0.00)	0.0	0.11 (0.04)	1.1	0.07 (0.04)	0.67
Non-Pollinating Flies:	2.20 (0.11)	24	0.68 (0.14)	8.05	0.16 (0.06)	2.7
Muscidae unknown genus	2.20 (0.11)	24	0.68 (0.14)	8.05	0.16 (0.06)	2.7
EVENING VISITORS						
Hawkmoths:	0.25 (0.09)	3.4	0.04 (0.03)	0.25	1.03 (0.16)	2.6
Sphingidae <i>Hyles lineata</i>	0.25 (0.09)	3.4	0.04 (0.03)	0.25	1.03 (0.16)	2.6
Bumblebees:	0.10 (0.10)	0.4	0.30 (0.12)	1.0	0.20 (0.11)	1.25
Apidae <i>Bombus flavifrons</i>	0.10 (0.10)	0.4	0.30 (0.12)	1.0	0.20 (0.11)	1.25
Non-Pollinating Flies:	0.05 (0.05)	0.8	0.12 (0.07)	1.5	0.00 (0.00)	0.0
Muscidae unknown genus	0.05 (0.05)	0.8	0.12 (0.07)	1.5	0.00 (0.00)	0.0

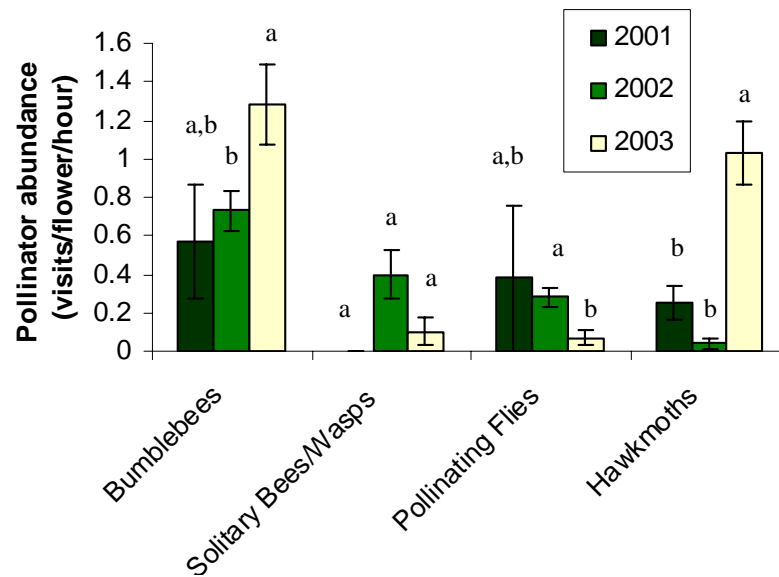


Figure 3.1. Yearly variation in pollinator abundance (visits per flower per hour) for each pollinator type over three years (2001-2003). Letters (a,b,c) above each bar represent statistical differences in abundance between years for each pollinator group.

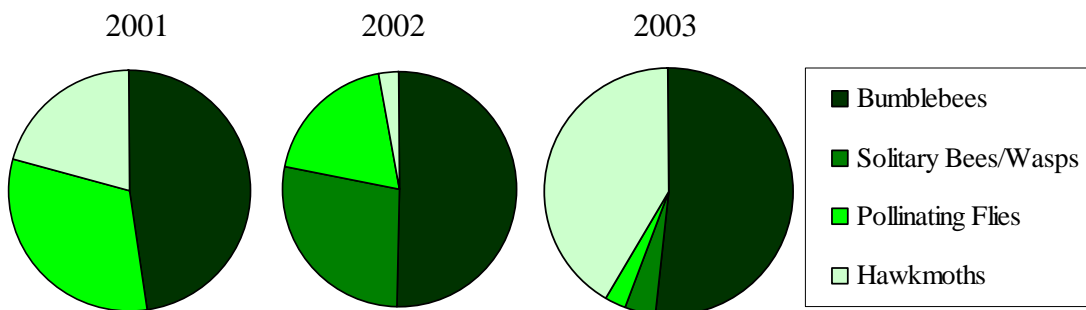


Figure 3.2. Relative abundance of the different pollinator types each year (2001-2003). Relative abundance is calculated as pollinator abundance (visits per flower per hour) for each pollinator type divided by total pollinator abundance for each year.

Pollinator behavior

Bumblebees, solitary bees and wasps, and pollinating flies collect pollen from the flowers of *A. coerulea* while hawkmoths collect nectar. On rare occasions in 2002, Vespidae wasps were seen robbing nectar from the nectary at the base of the spur. The time spent per flower varied significantly among the pollinator groups (df (3,432); $F=5.37$; $P=0.0012$), with solitary bees and wasps spending significantly more time per flower relative to bumblebees and hawkmoths, but not pollinating flies (Table 3.5; Figure 3.3A). Pollinator groups visited significantly different number of flowers in succession on a plant (df (3, 574); $F=10.84$; $P < 0.001$) and hawkmoths visited significantly more flowers relative to all other pollinator groups (Figure. 3.3B). Bumblebees visited significantly more flowers per plant than pollinating flies, but not solitary bees and wasps (Figure 3.3B). Hawkmoths also visited more flowers during a foraging bout than the other three groups of pollinators (Figure 3.3C). Bumblebees visited significantly more flowers per foraging bout than pollinating flies, but not compared to solitary bees and wasps (Figure 3.3C).

Table 3.5. The average time spent per flower, average number of flowers visited per foraging sequence bout, and the average number of flowers visited per plant by different visitors of *A. coerulea* flowers. Sample sizes (n) are combined for 2001, 2002, and 2003 day and evening observations. Standard errors are in parentheses. Values are presented for each pollinator species, and for groups of pollinators such as bumblebees.

	Time (sec)	n	# fl visited /foraging sequence bout	n	# fl visited / plant	n
Hawkmoths:	3.61 (0.49)	38	5.39 (0.70)	38	3.16 (0.31)	46
Sphingidae <i>Hyles lineata</i>	3.61 (0.49)	38	5.39 (0.70)	38	3.16 (0.31)	46
Bumblebees:	3.83 (0.19)	240	3.38 (0.16)	240	2.13 (0.08)	334
Apidae <i>Bombus flavifrons</i>	3.82 (0.20)	232	3.42 (0.18)	232	2.16 (0.08)	321
Apidae <i>Bombus occidentalis</i>	2.00	1	1.00	1	1.00	1
Apidae <i>Bombus rufocinctus</i>	4.79 (1.19)	7	2.29 (0.56)	7	1.33 (0.14)	12
Solitary Bees and Wasps:	6.97 (1.18)	85	2.61 (0.22)	85	1.86 (0.10)	116
Halictidae <i>Lasioglossum</i> sp.	10.32 (3.62)	20	1.36 (0.12)	20	1.43 (0.15)	22
Megachilidae <i>Megachile latimanus</i>	3.51 (0.96)	12	5.00 (1.06)	12	2.00 (0.27)	26
Anthophoridae <i>Anthophora</i> sp.	2.48 (0.54)	9	3.22 (0.60)	9	1.81 (0.25)	16
Andrenidae <i>Andrena</i> sp.	5.76 (1.38)	15	2.27 (0.28)	15	1.89 (0.21)	18
Vespidae <i>Ancistrocerus</i> sp. and <i>Stenodynerus</i> sp.	7.79 (2.08)	29	2.41 (0.27)	29	2.06 (0.17)	34
Flies:	4.56 (1.13)	61	2.20 (0.24)	61	1.66 (0.11)	80
Syrphidae <i>Chrysotoxum</i> sp. and <i>Didea</i> sp.	8.25 (2.59)	25	2.64 (0.53)	25	1.76 (0.18)	37
Bombyliidae <i>Villa</i> sp.	2.00 (0.23)	36	1.89 (0.15)	36	1.58 (0.13)	43
Non-Pollinating Flies:	91.77 (8.81)	274	1.36 (0.04)	274	1.29 (0.04)	284
Muscidae unknown genus	91.77 (8.81)	274	1.36 (0.04)	274	1.29 (0.04)	284

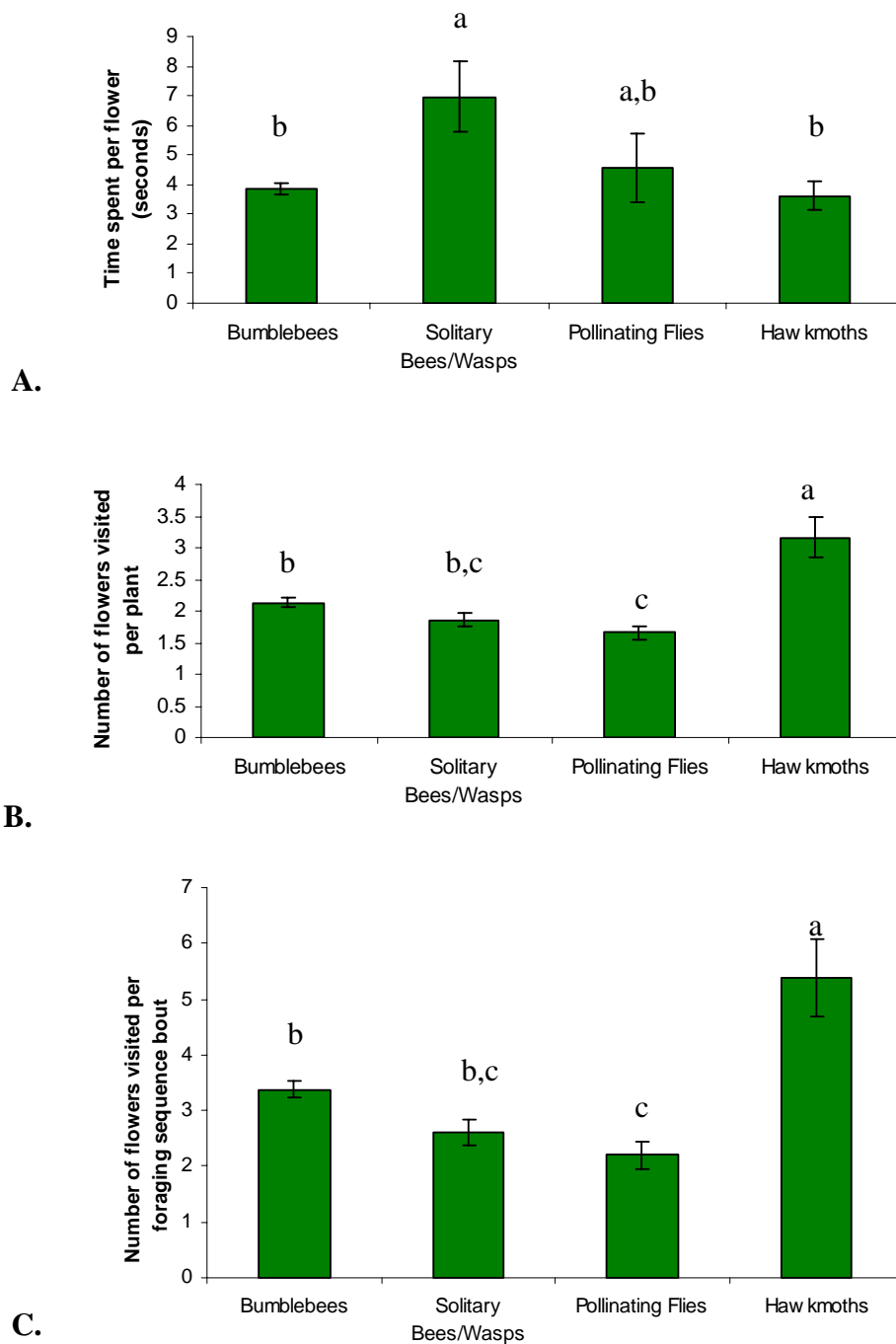


Figure 3.3. Comparison of pollinator behavior including A) average time in seconds spent per flower, B) average number of flowers visited in succession on a plant, and C) average number of flowers visited per foraging sequence bout. All values are averaged from three years of pollinator observations (2001-2003). Letters (a, b, c) above each bar represent statistical differences between pollinator types.

Bumblebees, solitary bees and wasps, and flies all preferred male-phase flowers as the first flower to visit on a plant (Table 3.6). Hawkmoths on the other hand preferred going to female-phase flowers first (Table 3.6). However, there was no difference in the number of flowers visited in succession on a plant when a male- as opposed to a female-phase flower was visited first on a plant (male first $n=2.28$; female first $n=2.05$) ($df=1$, $F= 2.03$, $P= 0.15$) and this hold true for all pollinator groups. There was no significant interaction between the effects of pollinator group and sexual phase of first-visited flower on the number of flowers visited in succession on a plant ($df = 3$; $F = 0.91$; $P = 0.44$).

Table 3.6. Pollinator preference for male- and female-phase flowers of *A. coerulea*. The variable % M1 represents the proportion of times that the first flower visited on a plant was a male-phase flower, % F1, a female-phase flower. The next variable is the number of flowers visited in succession on a plant if the first flower visited was a male-phase (# fl M1) or a female-phase (#fl F1) flower respectively. Values are calculated over all three years (2001-2003) for each species and for groups of pollinators.

	% M 1	% F 1	n	# fl M1	n	# fl F1	n
Hawkmoths:	39.13	60.87	46	3.33 (0.47)	18	2.86 (0.38)	28
Sphingidae <i>Hyles lineata</i>	39.13	60.87	46	3.33 (0.47)	18	2.86 (0.38)	28
Bumblebees:	79.64	20.36	334	2.21 (0.08)	266	1.81 (0.17)	68
Apidae <i>Bombus flavifrons</i>	80.06	19.94	321	2.24 (0.08)	257	1.87 (0.18)	64
Apidae <i>Bombus occidentalis</i>	0.00	100.00	1	0.00	0	1.00	1
Apidae <i>Bombus rufocinctus</i>	75.00	25.00	12	1.44 (0.18)	9	1.00 (0.00)	3
Solitary Bees and Wasps:	69.83	30.17	116	1.83 (0.11)	81	1.91 (0.21)	35
Halictidae <i>Lasioglossum sp.</i>	68.18	31.82	22	1.40 (0.16)	15	1.29 (0.18)	7
Megachilidae <i>Megachile Latimanus</i>	84.62	15.38	26	2.09 (0.21)	22	1.50 (0.50)	4
Anthophoridae <i>Anthophora sp.</i>	81.25	18.75	16	1.85 (0.30)	13	1.67 (0.33)	3
Andrenidae <i>Andrena sp.</i>	55.56	44.44	18	1.80 (0.29)	10	2.00 (0.19)	8
Vespidae <i>Ancistrocerus sp.</i> and <i>Stenodynerus sp.</i>	61.76	38.24	34	1.86 (0.27)	21	2.38 (0.51)	13
Flies:	63.75	36.25	80	1.73 (0.14)	51	1.55 (0.13)	29
Syrphidae <i>Chrysotoxum sp.</i> and <i>Didea sp.</i>	81.08	18.92	37	1.80 (0.19)	30	1.57 (0.20)	7
Bombyliidae <i>Villa sp.</i>	48.84	51.16	43	1.62 (0.21)	21	1.55 (0.16)	22
Non-Pollinating Flies:	58.10	41.90	284	1.28 (0.06)	165	1.33 (0.05)	119
Muscidae unknown genus	58.10	41.90	284	1.28 (0.06)	165	1.33 (0.05)	119

Observations indicate no regular or directional movement up or down the inflorescence for any pollinator type. All pollinator types were observed visiting only *A. coerulea* flowers throughout the flowering season, except for the bumblebee, *Bombus flavifrons*, which occasionally visited flowers of two other plants; thimbleberry (*Rubus parviflorus*) and mountain blue bell (*Mertensia sp.*), while also visiting *A. coerulea* flowers in early- to mid-July 2002 and 2003. Finally, hawkmoths traveled slightly longer distances ($x = 56.67 \pm 9.19\text{cm}$, $n = 6$) between plants within observational patches compared to bumblebees ($x = 45.24 \pm 3.77\text{cm}$, $n = 62$), although the difference was not statistically significant ($P = 0.17$).

Pollinator types and yearly variation in population selfing rate

The gene frequencies at five allozyme loci in the Falls Creek population were consistent among years (Table 3.7). The population selfing rate varied among years (Table 3.8). Based on pairwise comparisons of bootstrap estimates the population selfing rate was greater in 2002 compared to 2001 and 2003 ($P < 0.001$ for 2001-2002 and for 2002-2003). The population selfing rate was not greater in 2001 than in 2003 ($P = 0.115$ from pairwise comparisons of bootstrap estimates 2001-2003). Biparental inbreeding, calculated as the difference between the multilocus and the mean of single locus selfing rate estimates, was low each year suggesting that mating among close relatives did not significantly affect the selfing rate (Table 3.8).

Yearly variation in bumblebees, solitary bees and wasps, or pollinating flies abundance could not account for the variation in selfing rate observed among years (Figure 3.1 and Table 3.8). For example, bumblebees were most abundant in 2003 and of similar abundance in 2001 and 2002 while selfing rate was greatest in 2002 and lowest and similar in 2001 and 2003. However the pattern of hawkmoth abundance corresponded to the changes in selfing rate (Figure 3.1; Table 3.8), which suggests that

greater hawkmoth abundance may decrease selfing rate. However, the simple linear regression between hawkmoth abundance and selfing rate was not statistically significant ($Y = 0.29X - 0.52$, $P = 0.36$, $R^2 = 0.71$), thus larger sample sizes will be needed before we can confirm such a trend (Figure 3.4).

Table 3.7. The number of alleles and gene frequencies at five allozyme loci from plants used to estimate population level selfing rate in the Falls Creek population of *A. coerulea* for 2001, 2002 and 2003. The allozyme loci are malate dehydrogenase (MDH), mannose-6-phosphate isomerase (MPI), phosphoglucomutase (PGM), mannose-6-phosphate isomerase (MPI), 6-phosphogluconate dehydrogenase (6-PGD), and alcohol dehydrogenase (ADH).

Treatment /locus	2001	2002	2003
MDH-1	0.949	0.962	0.954
MDH-2	0.051	0.038	0.046
MDH-3	0.000	0.001	0.000
PGM-1	0.850	0.817	0.833
PGM-2	0.145	0.179	0.167
PGM-3	0.005	0.005	0.000
MPI-1	0.773	0.710	0.843
MPI-2	0.130	0.164	0.086
MPI-3	0.075	0.065	0.028
MPI-4	0.022	0.060	0.043
6PGD-1	0.908	0.972	0.947
6PGD-2	0.074	0.013	0.013
6PGD-3	0.018	0.015	0.040
ADH-1	0.821	0.843	0.885
ADH-2	0.127	0.097	0.086
ADH-3	0.037	0.040	0.017
ADH-4	0.015	0.020	0.013

Table 3.8. Estimates of multilocus selfing rate (s_m), single locus selfing rate (s_s), and biparental inbreeding (BI) in a population of *A. coerulea* over three years (2001- 2003). The total number of different plants (families) and seeds (progeny) used for selfing rate estimates are included in the Table. Standard errors are in parentheses.

Year	s_m	s_s	BI	# families	# progeny
2001	0.33 (0.06)	0.35 (0.06)	0.02 (0.02)	41	405
2002	0.59 (0.06)	0.61 (0.06)	0.02 (0.02)	37	565
2003	0.23 (0.05)	0.27 (0.05)	0.04 (0.02)	40	563

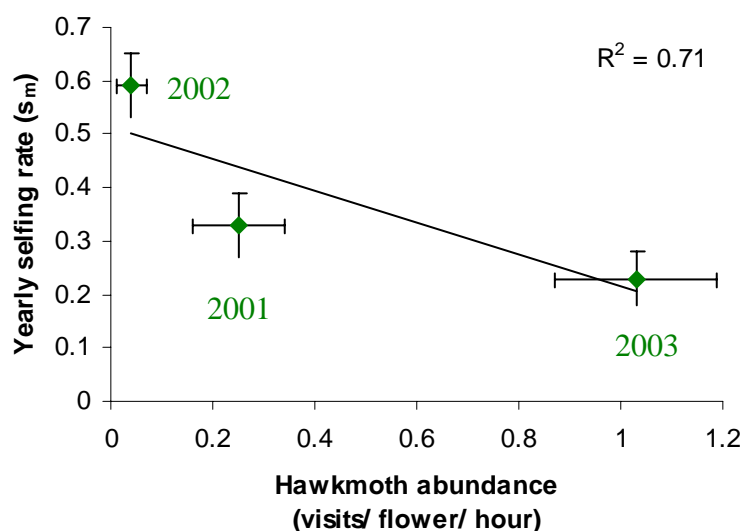


Figure 3.4. Simple linear regression between yearly variation in hawkmoth abundance (visits per flower per hour) and variation in population level selfing rate between 2001 and 2003.

Chapter 4

DISCUSSION

Geitonogamy contributed to the majority of the selfing rate in the population. Geitonogamy is almost never advantageous because it relies on the same pollen transfer mode as outcrossing, provides little or no reproductive assurance, and can result in severe pollen and seed discounting (Harder and Barrett 1995). Hence, the maintenance of a mixed mating system via geitonogamy can be viewed as an incidental cost of outcrossing combined with large floral displays in a self-compatible plant. Geitonogamy has been found to comprise a significant proportion of the total selfing rate in many plant species with large floral displays and some with clonal habits (Schoen and Lloyd 1992; Leclerc-Potvin and Ritland 1994; Eckert 2000; Levri 2000; Montaner et al. 2000), supporting the hypothesis that mixed mating systems are often a non-adaptive by-product of adaptations for outcrossing in plants with large floral displays.

The high level of geitonogamy in the population estimated in 2002 was influenced not only by the flowering phenology of a plant with large floral displays, but also by the behavior of the pollinators visiting the inflorescences. The increase in realized level of geitonogamy measured using allozymes compared to the potential for geitonogamy calculated from flowering phenology indicates either that pollinators do not have an equal probability of visiting flowers on inflorescences or that self pollen is more likely to fertilize ovules compared to outcross pollen. Experiments conducted by Montalvo (1992) showed that outcross pollen is more successful at fertilizing ovules compared to self pollen in *A. coerulea*. However, pollinator observations conducted between 2001 and 2003 indicate that pollinator behavior can favor geitonogamous

selfing. We have observed that the major pollinators of 2002, bumblebees, solitary bees, wasps, and flies, all prefer male-phase relative to female-phase flowers as the first flower visited on a plant. Visiting male-phase flowers first on a plant would increase the level of geitonogamous selfing relative to equal visits to male- and female-phase flowers. Thus, the behavior of pollinators visiting the inflorescences increased the realized level of geitonogamy above the level expected solely based on the flowering phenology of the plant.

In contrast, the realized level of autogamy estimated using allozyme data was negligible and not significantly different from zero. These data suggest that autogamous selfing is not being selected for as a mechanism for reproductive assurance in this *A. coerulea* population. However, control flowers set significantly more seeds than emasculated flowers in our experiment indicating that autogamy contributed to seed set, and potentially to reproductive assurance. We need to reconcile these two sets of contradictory data. The experimental design used to measure the contribution of autogamy to seed set assumes that pollinator visitation rate to emasculated and control flowers is equal. However, in the flower choice experiment, bumblebees visited control flowers first 77.3 % of the time compared to emasculated flowers. Furthermore, bumblebees, solitary bees, wasps and flies, which were the major pollinators in 2002, all forage for pollen rather than nectar and showed a preference for male-phase flowers. Hawkmoths were rare in the population in 2002. These data indicate that control flowers were more likely to be visited than emasculated flowers in our experiment. Miller (1981) also observed increased visitation rate by bumblebees to control versus emasculated flowers. Therefore, the increased seed set in the control compared to emasculated flowers does not result from autogamy in control flowers, but rather reflects greater pollinator visitation rate and increase in pollen load in control flowers. Furthermore, pollen must be limiting in the population in at least some years for reproductive assurance to be selected for. Although pollen limitation was not measured in this population, previous estimates of pollen limitation in two different populations

of *A. coerulea* in Colorado and Utah showed no difference in seed set between flowers that received outcrossed pollen in addition to natural pollination (pollen augmentation) and control flowers (unmanipulated) (Brunet 1996; Brunet unpublished data). These data provide no evidence that pollen is limiting in populations of *A. coerulea*. Although other studies have found that autogamous selfing may provide reproductive assurance and be adaptive in some plant species (Kalisz and Vogler 2003; Karron et al. 2004), the maintenance of mixed mating in our population does not appear to be due to autogamy providing reproductive assurance.

The maintenance of mixed mating in a plant population is influenced by genetic factors such as inbreeding depression and gene transmission and by ecological factors including reproductive assurance, pollen limitation, and pollen and seed discounting (Holsinger 1991; Johnston 1998; Porcher and Lande 2005). Gene transmission, reproductive assurance and pollen limitation will select for selfing. In contrast, high levels of inbreeding depression, seed discounting and pollen discounting favor outcrossing. We found no evidence for autogamy and reproductive assurance in this population. Furthermore, pollen limitation has not been observed in the two *A. coerulea* populations where it has been measured (Brunet 1996; Brunet unpublished data). A previous estimate of inbreeding depression from an *A. coerulea* population in Colorado indicates a moderate level of inbreeding depression (~ 0.5) (Montalvo 1994). Finally, although pollen discounting has not been measured in *A. coerulea*, high levels of pollen discounting are common in populations with high levels of geitonogamy (Lloyd 1992; Harder and Barrett 1995). A theoretical model developed by Porcher and Lande (2005) indicates that predominant selfing or predominant outcrossing are typically selected for even when ecological factors such as pollen limitation and pollen and seed discounting are included in the model. The authors concluded that low to moderate levels of selfing maintained in a population are likely due to unavoidable geitonogamous selfing in plants with large floral displays. Our observation that moderate selfing rate is maintained via geitonogamy in the *A. coerulea* population with

potentially no pollen limitation and high pollen discounting supports Porcher's and Lande's (2005) hypothesis that moderate selfing rates often result from unavoidable geitonogamous selfing in populations where selection would otherwise favor outcrossing.

Although selfing may not be selected for in this population, the potential for autogamy, measured in 2002 as fruit and seed set in the absence of pollinators, remains high in the population. A potential for autogamy has been measured in four different *A. coerulea* populations and was high in all populations, irrespective of the population selfing rate (Brunet and Eckert 1998; Brunet and Sweet 2006). It is difficult to explain why the potential for autogamy remains high in all these populations if selfing is solely maintained via geitonogamy. Because pollen limitation need only occur some years for autogamy to be selected for as a mechanism of reproductive assurance, it is possible that autogamy is selected for during those years of very low pollinator abundance. In a related species, *Aquilegia canadensis*, Herlihy and Eckert (2002) found that, although autogamy contributed significantly to seed set and selfing rate, autogamous selfing was not maintained as a reproductive assurance mechanism because high seed discounting and inbreeding depression negated the advantages of the extra seeds produced via autogamous selfing. Thus, the maintenance of autogamous selfing remains unclear in the two *Aquilegia* species where it has been examined.

Selfing rates varied significantly among years in the *A. coerulea* population. While population size, plant density, floral display size, and levels of herkogamy are known to affect selfing rate (Karron et al. 1995; Harder and Barrett 1996; Snow et al. 1996; Karron et al. 1997; Brunet and Eckert 1998; Franceschinelle and Bawa 2000; Karron et al. 2004; Mitchell et al. 2004; Brunet and Sweet 2006; Makino et al. 2007), these factors did not vary from year to year within our population (personal observation). In contrast, all pollinator types except solitary bees and wasps varied significantly among years. We did not observe a relationship between yearly selfing

rates and the abundance of all pollinators combined. Furthermore, yearly change in abundance of bumblebees, solitary bees and wasps, and flies did not correspond to the yearly variation in selfing rate. However, lower selfing rates were observed during years when hawkmoths were present and selfing rate decreased as hawkmoth abundance increased. Miller (1981) found greater seed set in control versus emasculated plants during a year of high bumblebee abundance, but no difference in seed set between the two groups in a year of high hawkmoth abundance, also suggesting that hawkmoths increased outcrossing (or decreased selfing). In addition, data collected among populations of *A. coerulea* indicated that population selfing rate decreased significantly when hawkmoth abundance increased (Brunet and Sweet 2006). The evidence suggests that different pollinator types can differentially influence levels of selfing and that increased hawkmoth abundance is associated with a decrease in selfing rate in *A. coerulea*.

Why would greater hawkmoth abundance decrease selfing rate? Pollinator behavior could influence levels of geitonogamy, which is responsible for the majority of the selfing in this population. Hawkmoths visited more consecutive flowers per plant relative to other pollinator types, a behavior known to increase selfing rate (Harder and Barrett 1995; Snow et al. 1996; Karron et al. 2004) and thus, can not explain the decrease in selfing rate associated with hawkmoth abundance. Second, all pollinators indicated non-directional movement up or down the inflorescence while foraging, thus vertical pollinator movement on an inflorescence is not influencing levels of geitonogamy in this population. Third, hawkmoths traveled slightly longer distances between plants within an observational patch compared to bumblebees, which could decrease the level of biparental inbreeding in the population (mating between close relatives). However, we found low levels of biparental inbreeding for all three years of the study and found no increase in biparental inbreeding in 2002 when hawkmoths were rare. Thus, differences in the selfing rate were not due to differences in mating between close relatives or shorter distances traveled between plants by bumblebees, solitary

bees, wasps and flies relative to hawkmoths. Finally, geitonogamous selfing can be influenced by pollinator preference for sexual stage of flowers visited. Hawkmoths preferred to visit female-phase flowers first on a plant while bumblebees, solitary bees, wasps and flies all visited male-phase flowers first. By visiting a female-phase flower first, hawkmoths ensure outcrossing. Given that the number of flowers visited per plant is the same whether a male-phase or female-phase flower is visited first, the chance of transferring pollen to other flowers visited consecutively on the same plant is less for the hawkmoth that visits a female-stage flower first. In contrast, bumblebees, solitary bees, wasps and flies may increase levels of geitonogamous selfing relative to hawkmoths because of a preference for male flowers. Therefore, the observed variation in yearly selfing rate and its relationship to hawkmoth abundance is most likely explained by hawkmoths preference for female-phase flowers.

Yearly variation in abundance of the different *A. coerulea* pollinator types, each with different foraging behaviors and differential influence on selfing rates, can have important implications on the evolution of floral morphology in *A. coerulea*. For example, because only hawkmoths forage for nectar at the base of the spur, it is the only pollinator likely to select for spur length. Miller (1981) suggested that different species of hawkmoths are associated with different spur lengths and that the spur length will be positively correlated with hawkmoth tongue length in *A. coerulea*. Hodges (1997) and Hodges and Arnold (1994) suggest that the floral nectar spurs in *Aquilegia* are a key innovation that has triggered rapid speciation in this genus. Variation in floral nectar spurs may adapt columbine species to pollination by different pollinators hence floral spurs may affect processes important in species diversification (Hodges and Arnold 1994; Hodges 1997). Only one species of hawkmoth, *Hyles lineata*, was observed in the Falls Creek population; therefore, spur length is likely correlated with the tongue length of this species. Greater variance in spur length may occur in populations where bumblebees are common and hawkmoths are rare or absent.

While floral spur length may be primarily selected for by hawkmoths, floral color may be selected for by both hawkmoths and bumblebees. Four taxonomic varieties are recognized within *A. coerulea* (Munz 1946; Whittemore 1997) and are largely distinguished by floral color, which range from white to deep blue. A range of flower colors is also observed within individual populations (Miller 1981). In a Colorado population containing both white and blue flowers, Miller (1981) found greater seed set in blue flowers during a year of high bumblebee abundance, and higher seed set in white flowers in a year of high hawkmoth abundance. These data suggest a preference of bumblebees for blue flowers and hawkmoths for white flowers. Miller (1981) also observed that *A. coerulea* populations containing mostly white flowers were potentially associated with higher hawkmoth abundance. The evidence suggests that opposing selective pressure on flower color by bumblebees and hawkmoths may be occurring. When both pollinators are present in a population, such as the Falls Creek population, pollinators could maintain polymorphisms for flower color. A range of white to blue flowers is in fact observed in this population. However, the selective pressure of bumblebees and hawkmoths on floral color may not be equivalent. Recent phylogenetic reconstruction of the *Aquilegia* genus suggests that the presence of anthocyanins (floral pigment) is a primitive trait in this genus, while loss of color has evolved more recently (Whittall et al. 2006). Interestingly, *A. coerulea* is the only species in the genus where a strong polymorphism for anthocyanins is maintained (Whittall et al. 2006). This pattern may reflect the differential selection pressure exerted on floral color by the two major pollinators of this plant species hawkmoths and bumblebees.

Many plant species are pollinated by more than one species of pollinator (Waser et al. 1996; Proctor et al. 1996), which can vary significantly in their foraging behavior (Handel 1983; Schemske and Horvitz 1984; Herrera 1987; Ramsey 1988; Young and Stanton 1990). Thus, we expect that the mating systems and selection for floral characteristics are differentially influenced by different pollinator types in many

plant populations besides *A. coerulea*. In addition to other ecological and genetic factors, the types of pollinator, their abundance, and their behavior play an important role in the maintenance of mixed mating and evolution of plant populations.

Conclusions

In conclusion, selfing observed in this population of *A. coerulea* is primarily due to geitonogamy rather than autogamy. The minimal levels of autogamy found suggest that self-fertilization is not being selected for and that reproductive assurance does not play an important role in the maintenance of selfing in the Falls Creek population. Instead, selfing appears to be a negative consequence of a mechanism for pollinator mediated outcrossing in a population with large floral displays. Our results suggest that geitonogamy rather than autogamy may be more influential in the maintenance of mixed mating systems found in many plant species.

Pollinators play an important role in the maintenance of mixed mating in plant populations. Our *A. coerulea* population was visited by four different pollinator types; hawkmoths, bumblebees, solitary bees and wasps, and flies. Pollinators varied in abundance among years. Furthermore, our study indicates that differences in pollinator behavior among pollinator types could influence levels of selfing. An increase in hawkmoth abundance is associated with a decrease in selfing compared to bumblebees and other floral visitors of *A. coerulea*, which supports data found among populations (Brunet and Sweet 2006). Our data suggest that hawkmoths decrease levels of geitonogamy, compared to other pollinator types, by preferentially visiting female- rather than male-phase flowers first on a plant. Selfing rate and mixed mating in this population of *A. coerulea* is not likely maintained by selection, but results from geitonogamous selfing whose rate appears to be differentially influenced by pollinator type. Other plant species pollinated by more than one type of pollinator may experience

similar variations in their mating system as a result of differences in pollinators' abundance and behavior.

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